Beetroot Supplementation in Women Enjoying Exercise Together

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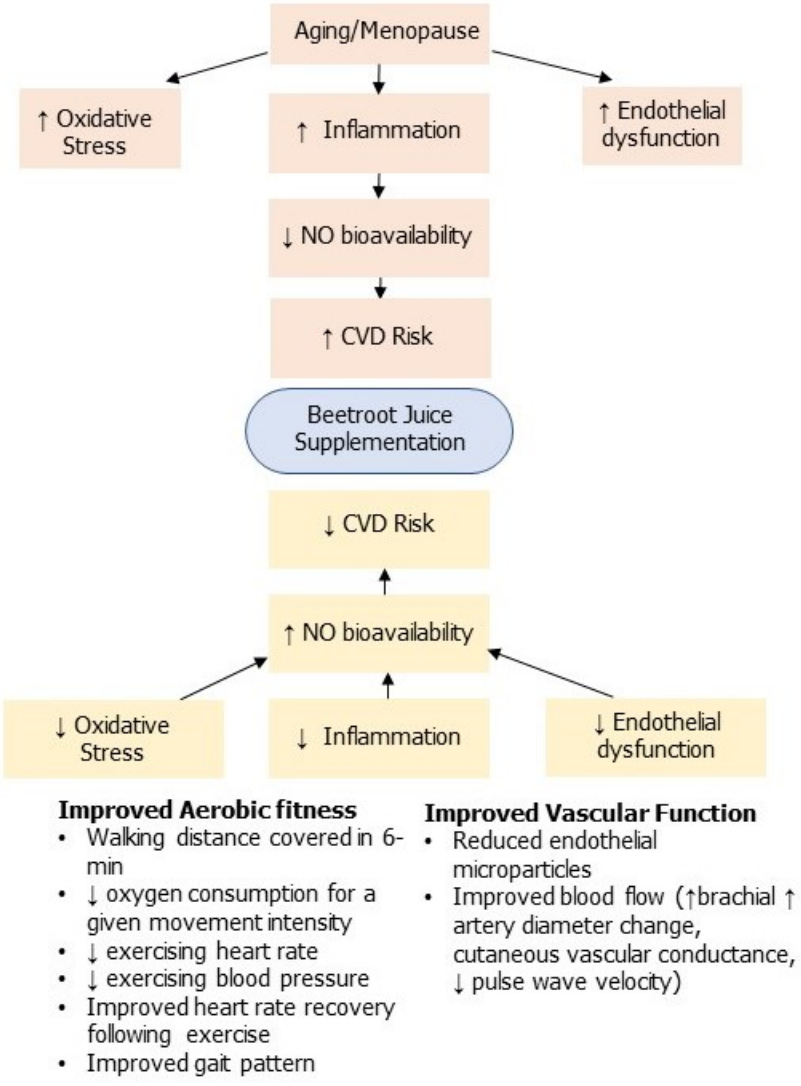
Table of Contents:

1.0 Background & Rationale

Advancing age is a primary risk factor for cardiovascular disease (CVD), which remains a pervasive source of death and disability in modern society. Postmenopausal women, in particular, represent a segment of the population that exhibit greater rates of disability and CVD with advancing age compared to men (1). Based on data from the Women's Health Initiative, wherein estrogen replacement therapy did not exhibit cardioprotective benefits (2), it is essential to consider alternative approaches that may possess therapeutic potential for attenuating CVD risk among postmenopausal women. Indeed, it has become increasingly clear that exercise training is vital to preservation and restoration of physiologic function to achieve optimal health/longevity in many populations (3). However, given that postmenopausal women do not characteristically possess the same responsiveness to exercise training compared to *pre*menopausal women (4) or men (5) – targeted efforts are needed to overcome the limitations of current exercise prescription. Such efforts may attenuate age-related shifts in arterial aging and cardiovascular disease risk by proving to be more effective at reducing vascular oxidative stress/inflammation, as well as, increasing gait/mobility and endothelial function in postmenopausal women.

CVD risk steepens during the menopausal transition (6), likely due to the combined effects of age-related oxidative stress (7), low-grade inflammation (8), and diminishing estrogen (9). Since nitric oxide is implicated in numerous physiological functions, including vasodilation and inhabitation of platelet aggregation, boosting nitric oxide bioavailability represents an ideal target to restore physiologic function. From a mechanistic standpoint, age-related oxidative stress reduces nitric oxide bioavailability via the uncoupling of eNOS following the formation of peroxynitrate (10). Historically, the L-arginine-nitric oxide pathway was thought to be the primary source of endogenous nitric oxide production. More recently, though, the nitrate-nitrite-nitric oxide pathway has been identified wherein commensal bacteria on the dorsal surface of the tongue reduce ingested nitrate (e.g. beetroot juice) to nitrite (11). Swallowed nitrite can then be further reduced to stimulate nitric oxide-dependent signaling within the systemic circulation. Functionality of the nitrite-



nitric oxide circuit is best represented by the compelling data in humans showing reduced blood pressure (12) and augmented exercise performance (13).

Since peak plasma concentrations of nitrate and nitrite occur within 1-2 hours and 3-4 hours (following ingestion) (14), respectively, there is a reasonable window of time to perform exercise. By harnessing the reasonable time (2-3 hours), during which, plasma nitrate and nitrite levels are at a maximum, beetroot juice consumption can be planned to coincide to the start of exercise to acutely elicit the following effects in postmenopausal women: 1) greater distance covered during a 6-minute walk; 2) increased endothelial-dependent vasodilation as determined by flow-mediated dilation and; 3) reduced endothelial microparticles (e.g. sVCAM-1, sICAM-1, sE-selectin). Potential findings could inform a larger clinical trial to determine if pre-exercise beetroot supplementation is an efficacious strategy to promote health related effects of exercise training including improved gait/mobility and reduced CVD risk. *To test our hypotheses, we propose a 2-arm randomized controlled pilot to examine the following aims:*

2.0 Objective(s)

2.1 Primary Objective

Aim 1. Determine the feasibility of an 8-week (3x/week) exercise training progression (EX) coupled with the consumption of beetroot juice (BR+EX) prior to exercise in postmenopausal women. Prior work supports daily, short-term (21 days) consumption of beetroot juice in men and women aged 62 +/- 1 years, yet this was performed in the absence of exercise training. Research unequivocally supports the utility of exercise training, still in the context of this proposal, feasibility is unknown.

2.2 Secondary Objective

Aim 2a. Determine preliminary effects sizes for pre-exercise beetroot juice consumption + exercise training (BR+EX) compared + exercise training only (EX) on distance (feet) covered during a 6-minute walk test. Performance during the 6-minute walk has been shown to accurately estimate peak oxygen uptake (i.e., aerobic fitness) and functional mobility. Consistent with others showing performance benefits of pre-exercise beetroot juice consumption and acute exercise, we anticipate a small to medium effects size in a beneficial direction (i.e., greater positive change) for BR+EX compared to EX.

Aim 2b. Determine preliminary effect sizes for BR+EX compared to EX on improvement of endothelial-dependent vasodilation as evidenced by percent increase in flow-mediated dilation. Based on our working premise that beetroot juice may offset the burdens of endothelial dysfunction/activation by increasing nitric oxide bioavailability, coupled with the effects of exercise training, we anticipate a small to medium effect size in a beneficial direction for BR+EX compared to EX.

2.3 Tertiary/Exploratory/Correlative Objectives

Aim 3. Determine preliminary effect size for BR+EX compared to EX on reducing soluble endothelial microparticles including, sICAM-1, s-VCAM-1, sNCAM, MPO, IFNg, IL-1β, BDNF, TNF-α, and IL-10. Given the link between nitric oxide and regulation of NF-κB, it is reasonable that BR+EX may have synergistic properties that could contribute to

favorable effects on the vascular endothelium. As such, we anticipate a small to medium effect size in a beneficial direction for BR+EX compared to EX.

3.0 Outcome Measures/Endpoints

3.1 Primary Outcome Measures

Outcomes of interest include recruitment, retention, adherence (i.e., exercise prescription and assessments), perceived exercise difficulty, and adverse event rates. Project success will be based on 83% [20 of 24] of randomized participants completing 8 weeks of exercise training.

3.2 Secondary Outcome Measures

2a. The distance (feet) covered during a 6-minute walk test at baseline prior to the intervention and following BR+EX is the main secondary outcome of interest. To confirm the relationship of the functional 6-minute walk test to aerobic fitness, measures of oxygen consumption (ml/kg/min or L/min), heart rate (bpm), and blood pressure (mm Hg) will be taken during constant speed walking at a typical walking pace prior to the 6-minute walk test. Additionally, functional mobility will be assessed by analyzing triaxial movement patterns from an accelerometer worn on the non-dominant hip during the 6-minute walk test.

The BR+EX intervention will be considered successful in the event that there is a statistically significant increase in 6-minute walk distance (feet) along with a decrease in oxygen consumption (ml/kg/min or L/min), decrease in exercising heart rate (bpm), improvement in heart rate recovery following exercise (post exercise heart rate – heart rate 1 min, 2 min after exercise (bpm)), decrease in exercising blood pressure (mm Hg), decrease in post-exercise blood pressure (mm Hg), and/or improvement in gait function (i.e. preferred walking speed, center of mass characteristics) from baseline to post-intervention above any improvement seen in EX.

2b. Percent changes in brachial artery diameter (%), cutaneous vascular conductance (%), and pulse wave velocity (m/s) at baseline prior to the intervention and following BR+EX are secondary outcomes of interest, representing changes in endothelial dependent vasodilation. A non-invasive technique of flow mediated dilation of the brachial artery using ultrasound imaging will be used to assess the percent change in vessel diameter following occlusion. A local heating protocol using non-invasive Laser Doppler flowmetry in conjunction with blood pressure measurements will be used to obtain measures of red blood cell (RBC) flux and mean arterial pressure (MAP) to calculate cutaneous vascular conductance (RBC flux/ MAP). The velocity of the pulse wave traveling from the carotid to femoral artery will be assessed non-invasively using tonometry.

A statistically significant increase in the percent change in brachial artery diameter (%), cutaneous vascular conductance (%), and/or a decrease in pulse wave velocity (m/s) in BR+EX above any improvement observed in EX from baseline to post-intervention will be considered a successful outcome.

3.3 Tertiary/Exploratory/Correlative Outcome Measures

Tertiary outcome measures of interest include plasma concentrations of sICAM-1, s-VCAM-1, sNCAM, MPO, IFNg, IL-1β, BDNF, TNF-α, and IL-10 obtained from a venous blood sample taken at baseline and post-intervention.

Statistically significant decreases in endothelial microparticle concentrations from baseline to post-intervention in BR+EX that are above any decrease observed in EX will be considered a successful outcome.

4.0 Eligibility Criteria

4.1 Inclusion Criteria

- Post-menopausal women (self-report)
- Ages 75 and younger (confirmed by birth date listed on participant's driver license at screening visit)
- Physician's clearance for study participation (required prior to baseline testing)
- English-speaking
- Body mass index between 25.0 to 39.9 kg/m^2 (measured on-site at screening visit)
- Able to ambulate without assistance

4.2 Exclusion Criteria

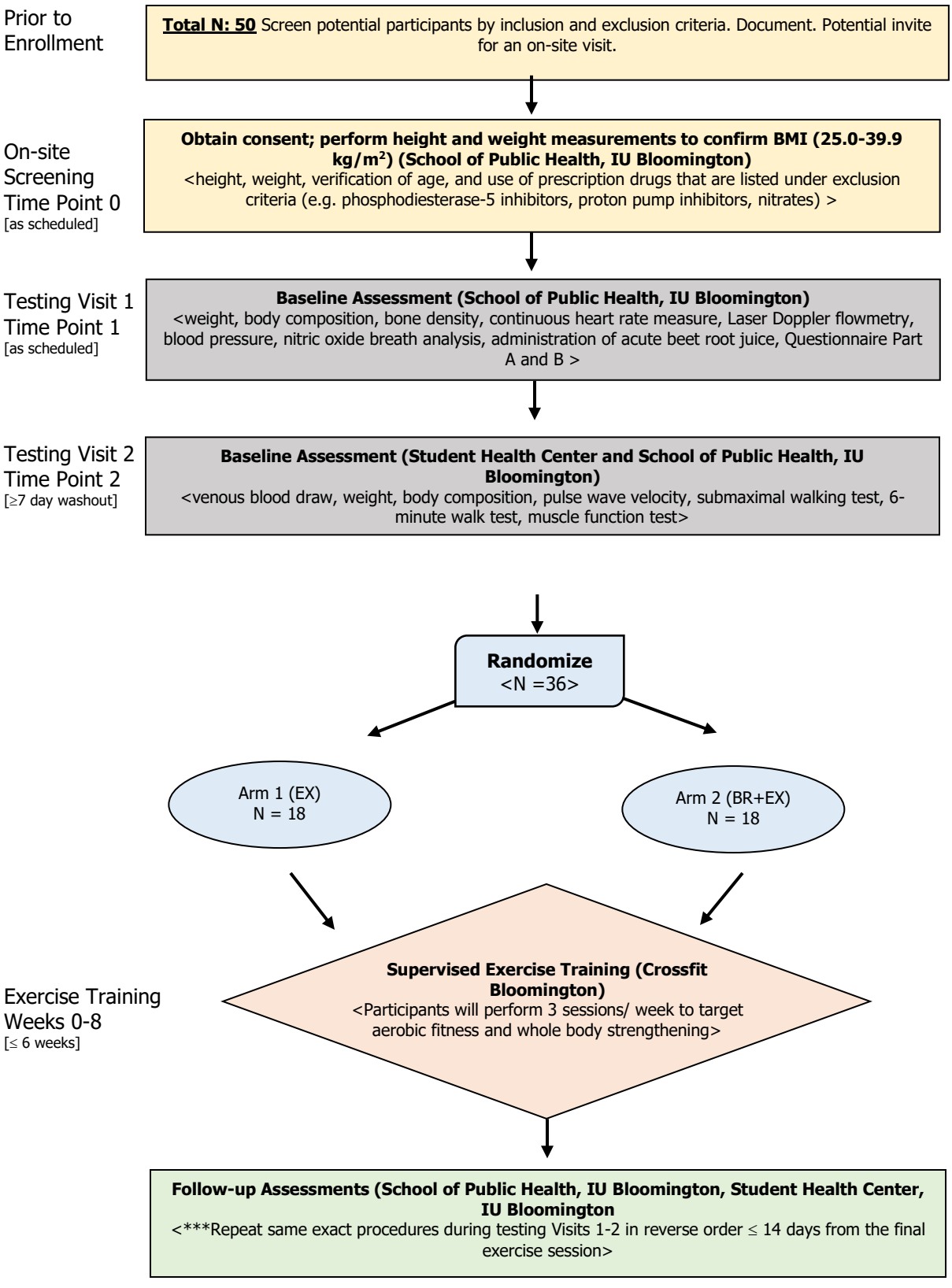
- Unable to provide informed consent
- < 18 years or > 75 years of age (confirmed by birth date listed on participant's driver license at screening visit)
- Body mass index < 25.0 or >39.9 kg/m^2 (measured on-site at screening visit)
- Greater than stage II hypertension (i.e. >159/99 mm Hg)
- Current smoker (self-report)
- Currently pregnant, lactating, or trying to become pregnant (self-report)
- Habitually exercise training ≥ 3 times per week (self-report)
- Significant orthopedic limitations or other contraindications to strenuous exercise
- Live or work >50 miles from Bloomington study site or do not have transportation to the study site
- Anticipate elective surgery during the study period
- Plan to move residence or travel out of the local area during the study period
- Current use of anti-coagulants (e.g. Coumadin or Warfarin)
- Current use of prescription medications that affect heart rate or blood vessel dilation (e.g. phosphodiesterase-5 inhibitors, proton pump inhibitors, systemic β-adrenergic blockers, nitrates, calcium channel blockers, hormone replacement therapy) (self-report and confirmed on-site at screening visit)
- Psychological or social characteristic that would interfere with their ability to fully participate in the study (i.e. taking longer than allowed time to complete cognitive assessments)

5.0 Study Design

To fulfill the purposes outlined above, a 2-arm randomized controlled study design will be employed. After completing initial screening procedures (**screening visit**), subjects will return

to the School of Public Health at Indiana University Bloomington to have baseline measures of weight, body composition, bone density, heart rate, cutaneous vascular conductance, blood pressure, assessed prior to and 3-hours following ingestion of beetroot juice (**Visit 1**). During this visit, two questionnaires will be administered to obtain information related to the health history, physical activity habits, mood state, and cognitive abilities of the subjects. Following a minimum 7-day washout period, to mitigate the risk of any carry-over effect from the acute dose of beetroot juice administered during the initial visit, subjects will have their blood drawn at the Indiana University Student Health Center followed by measures of weight, body composition, and pulse wave velocity performed at the School of Public Health at Indiana University Bloomington (**Visit 2**). During this visit they will also perform two 6-minute walking tests, and muscle function test. After baseline assessments **Visits 1-2**, participants will be randomly assigned to one of twostudy arms. All study arms will complete an 8-week exercise training protocol. The first arm will serve as a control group and will require participants to only complete the 8-week exercise training protocol (EX). The second arm will complete exercise training with ingestion of a beetroot (BR+EX). Exercise training will occur no more than 6 weeks following baseline Visit 2 to minimize the effects of potential changes in physical conditioning/ deconditioning on baseline measures. Subjects will be asked to repeat baseline Visit 2 before initiating exercise training in the event >6 weeks lapses from the completion of the final baseline assessment visit.

The same procedures as performed in **Visits 1-2** will be repeated following the treatment intervention for **Visits 3-4** in reverse order. The procedures outlined for **Visit 2** will be performed during **Visit 3**, and procedures for **Visit 1** for **Visit 4**.


Prior to Enrollment
Total N: 50 Screen potential participants by inclusion and exclusion criteria. Document. Potential invite for an on-site visit.
On-site Screening Time Point 0
[as scheduled]
Obtain consent; perform height and weight measurements to confirm BMI (25.0-39.9 kg/m²) (School of Public Health, IU Bloomington)
<height, weight, verification of age, and use of prescription drugs that are listed under exclusion criteria (e.g. phosphodiesterase-5 inhibitors, proton pump inhibitors, nitrates) >
Testing Visit 1 Time Point 1
[as scheduled]
Baseline Assessment (School of Public Health, IU Bloomington)
<weight, body composition, bone density, continuous heart rate measure, Laser Doppler flowmetry, blood pressure, nitric oxide breath analysis, administration of acute beet root juice, Questionnaire Part A and B >
Testing Visit 2 Time Point 2
[≥7 day washout]
Baseline Assessment (Student Health Center and School of Public Health, IU Bloomington)
<venous blood draw, weight, body composition, pulse wave velocity, submaximal walking test, 6-minute walk test, muscle function test>
Randomize
<N =36>
Arm 1 (EX)
N = 18
Arm 2 (BR+EX)
N = 18
Exercise Training Weeks 0-8
[≤ 6 weeks]
Supervised Exercise Training (Crossfit Bloomington)
<Participants will perform 3 sessions/ week to target aerobic fitness and whole body strengthening>
Follow-up Assessments (School of Public Health, IU Bloomington, Student Health Center, IU Bloomington
<***Repeat same exact procedures during testing Visits 1-2 in reverse order ≤ 14 days from the final exercise session>

6.0 Enrollment/Randomization

The present study will rely on participant self-referral in response to recruitment materials. Participants will be recruited from the local community through approved flyers and/or approved written scripts (See Attachments) via:

- social media
- newspaper ads
- physical postings with permission from local establishments
- or sent to the email address of local organizations with the intent that the approved recruitment flyer will be forwarded to a member of their organization Additionally, study details will be registered through the Indiana CTSI All IN for Health website. Open recruitment will occur for fixed periods (4-6 weeks).

Additional recruitment efforts include referral from the Indiana CTSI All IN for Health Volunteer Registry.

Subjects will be identified from the Indiana CTSI volunteer registry Protocol Number 1105005444. This website is created for interested individuals to contact us and sign up to receive information about ongoing studies and is supported by the Indiana CTSI. Persons in this registry have provided their health information for the purposes of being matched to appropriate research studies. The registry volunteers have signed an electronic consent/authorization so that their information can be used and they can be contacted for this reason.

The All IN manager will send an email message (see provided email text) to participants describing the study and asking them to reach out to the study team directly if they are interested in hearing more about the study.

Individuals expressing interest in the study will contact research personnel by telephone and /or email. If, by email, an agreed upon time to speak over the phone will be determined. During the phone call, research personnel will adhere to a scripted telephone screen wherein additional information detailing the inclusion/exclusion criteria, study procedures, requirements, and risks will be covered.

Based on participant responses to the questions, research personnel will determine preliminary eligibility for an on-site visit. If it appears the participant meets the preliminary eligibility, an on-site visit will be scheduled, and a consent form will be mailed / emailed to the participant for their consideration and review. After the study participant has had several days to review the consent form, research personnel will call the participant to answer any further questions regarding the consent form. Participants will be enrolled in small (n = 9) cohorts or "waves" to facilitate social support and adherence to the supervised exercise training sessions. We anticipate having 9 waves to successfully reach our target n. This assumes that each wave will have 4 participants, hence, 4 participants x 9 waves = 36 total participants.

At the on-site screening visit, research personnel will once again cover inclusion/exclusion criteria, study procedures, requirements, and risks. Following written informed consent, participants will have their body weight and standing height measured to determine their body mass index (i.e., 25.0-39.9 kg/m^2). Current prescription drug use will be recorded by having

participants physically bring their prescription labels or medication bottles to the testing site. Additionally, participants will complete a Medical History Questionnaire (see attachments).

Written authorization will be obtained from consented participants to allow research personnel to contact the participant's primary care physician to receive physician's clearance prior to study testing visits. The physician will be asked to provide their assent or dissent for their patient to participate in the exercise testing protocols and supervised group exercise sessions involved with this study. They will have the option to indicate any modifications that may be necessary for the patient to participate safely. The Physician Clearance Form (see attachments) will be faxed to the patient's physician following the screening visit.

Participants will perform baseline assessments (**Visits 1-2**) prior to randomization. Subsequently, participants will be randomly selected to: 1) control (EX only) or 2) BR+EX. Group allocation will be determined at random using a computer-based number generator in blocks of 2 to ensure equal between-group distribution (i.e. n = 2 Ex only; n = 2 BR+EX). Each number will be kept in a sealed envelope until completion of **Visits 1-2**.

7.0 Study Procedures

All study procedures are being conducted for research purposes only.

Height – A stadiometer will be used to collect standing height. Participants will be asked to remove their shoes prior to the assessment. Height will be obtained to the nearest cm.

Bioelectrical Impedance (BIA)- Participants will remove their shoes and socks to complete this assessment. They will be asked to stand on a BIA scale (Tanita) and hold two electrodes in either hand. The duration of this test is anticipated to take approximately 10 seconds per reading. Two readings will be taken and then averaged for measures of body weight (kg or lbs.), body fat percentage (%), and total body water (%). Subjects will be provided with a standard set of hospital scrubs to wear for body weight and composition measures.

Dual-Energy X-Ray Absorptiometry (DEXA)- A whole-body DEXA procedure will be used to assess bone mineral density, lean muscle weight (kg or lbs.), fat weight (kg or lbs.), and body fat percentage (%) at various body segments (e.g. total body, trunk, hip, leg). The subject will remove any metal or jewelry prior to the scan. They will be instructed to lie supine with their arms by their sides and asked to remain still while a scanning arm moves above them across the entire length of their body. One scan will be performed during **Visits 1 and 4**.

Actiheart Continuous Heart Rate- Continuous measurements of heart rate will be obtained using an Actiheart chest-strap monitor during **Visits 1 and 4** for all participants and for 3 randomly selected participants (1 per treatment group) in each cohort to monitor the intensity of exercise training sessions**.** The Actiheart is a wireless electronic measuring device that clips onto two electrodes placed approximately 1 to 2 inches below the left collarbone spaced apart at typical heart rate monitoring measurement sites.. An approximate 2 inch diameter circular area around each electrode site will be prepped using an alcohol swab, for measurement. The device electronically transmits measurements of heart rate to a software system. Subjects will

wear this device for the duration of **Visits 1 and 4**. Actiheart heart rate measurements will be taken once during week 2, week 4, and week 6 of exercise training for each of the 3 randomly selected participants (3 times total).

Pulse Wave Velocity (PWV)- Measurements of PWV (m/s) will be obtained from standard blood pressure and tonometry assessment using the ATCOR SphygmoCor device. This device is a non-invasive way to perform central blood pressure waveform assessment. During **Visits 2 and 2**, subjects will be asked to lie still in a supine position on a padded table surface for a duration of 10 minutes. A leg blood pressure cuff will be placed on the upper thigh. A technician will apply carotid tonometry simultaneously to inflating the blood pressure cuff to obtain blood pressure waveforms at the carotid and femoral artery sites. SphygmoCor software uses physical distance measurements between the sites to calculate velocity.

Laser Doppler Blood Flow Heating Protocol- Laser Doppler (Blood FlowMeter, Ad Instruments) will be used to assess cutaneous vascular conductance in response to a localized heating stimulus. Two small skin surface probes will be applied to the non-dominant forearm of the subject during **Visits 1 and 4**. Resting measurements will be obtained for 5 minutes followed by a measure of resting blood pressure. Localized skin heating will be accomplished with local heating units that will surround the skin surface probes of the laser Doppler. Both probes will be heated to a set temperature of 39°C (102°F) for 25 minutes. . The temperature of the probes will then be increased until a maximum temperature of 44°C (111°F) and held for 15 minutes . Blood pressure measurements will be obtained from the dominant arm every 10 minutes during the 40-minute heating protocol. From measures of blood pressure and the laser Doppler blood flow, cutaneous vascular conductance will be calculated as a ratio of skin blood flow to mean arterial pressure. Cutaneous vascular conductance will be expressed as a percentage of maximal conductance measured at the end of the heating protocol.

Blood Pressure- Blood pressure readings (mm Hg) of systolic and diastolic blood pressure will be taken using an automated sphygmomanometer on the dominant upper arm. During **Visits 1 and 4**, measurements will be taken during seated rest, every 10 minutes during the localized heating protocol, and every hour for 3-hours following ingestion of beetroot juice. During **Visits 2 and 3**, measurements will be taken during standing rest prior to the submaximal walking exercise, at minutes 3 and 5 during submaximal walking, and after 3 minutes of recovery following submaximal walking.

Questionnaires- A questionnaire will be given to participants in two parts (Part A and Part B) during **Visits 1 and 4**. The first part of the questionnaire consists of the Addenbrooke's Exam-III (ACE-III), which will be verbally administered by a trained member of our research team. This exam is designed to assess five cognitive domains including attention, memory, verbal fluency, language, and visuospatial abilities. Current mood states will be assessed using the Profile of Mood States (POMS) instrument. These questionnaires are also designed to obtain demographic, health, and physical activity habit information about our subjects.

Venous Blood Draw- A venous blood sample will be taken by trained personnel at the Indiana University Bloomington Student Health Center during **Visits 2 and 3**. Two 7mL, and one 4mL vials of blood will be taken at each timepoint for a total of 18mL x 2 visits = 36mL. The two 7mL vials of EDTA plasma samples will be centrifuged 1 x 10 minutes at 2500 x g at room temperature. Samples will then be aliquoted and stored at -80 degrees Celsius. Upon thawing, samples will be prepped wherein quantification of endothelial microparticles (sICAM-1, s-VCAM-1, sNCAM, MPO, IFNg, IL-1β, BDNF, TNF-α, and IL-10) will be performed. Flow cytometry will be used to obtain a complete blood count (CBC) using the 4mL vial of EDTA plasma sample.

Nitric Oxide Breath Analysis- The fraction of expired nitric oxide in exhaled breath will be assessed using a handheld digital breath analyzer (NIOX VERO). Subjects will be asked to exhale once into the analyzer prior to ingestion of beetroot juice and again 3-hours after ingestion to confirm a change in nitric oxide levels at **Visits 1 and 4**. During **weeks 3 and 6 of the supervised exercise training intervention**, random drop-ins will be conducted by a member of our research team to assess fractional exhaled nitric oxide levels to assess compliance with the beetroot supplementation by asking subjects to breathe once into the breath analyzer.

Blood Oxygen Concentrations- To monitor the concentrations of oxygen and methemoglobin in the blood both prior to and following acute beetroot juice ingestion, a fingertip pulse oximeter will be used (Masimo SpMet). Measurements of SpMet will be taken once every hour for 3-hours following ingestion of beetroot juice during **Visits 1 and 4**. A concentration of SpMet ≥ 12% indicates a serious adverse response to acute beetroot supplementation.

Beetroot Juice Administration- Participants randomized to receive exercise training and beetroot juice will be supplied with 140 mL of commercially-available beetroot juice (James White Drinks, UK) containing approximately 12 mmol of nitrate. Participants will be given a 4-week supply (24 bottles) which will then be repeated at the half-point of the exercise training progression. Participants will be instructed to consume the supplement between 2 to 3 hours prior to each exercise session to ensure training coincides with peak nitrate/nitrite bioavailability (14). Participant adherence will be determined at random, prior to exercise (on-site), during weeks 3 and 6 using a portable breath analyzer to monitor the fractional exhaled nitric oxide (NIOX MINO, NC) (17).

Dietary Recall Assessment- Participants will be asked to record all food, beverages, and supplements that they consumed within a 24hr period on six separate occasions using a paper handout supplied to them (see dietary recall handout in protocol attachments). They will be asked to record three times on one weekend and one weekday during the first week, and again during the last week of the 8Wk supervised exercise training sessions. They will be asked to complete the recalls within a 7-day period at both timepoints. Since this is not a primary outcome variable of interest, and will simply be used to describe amounts of dietary nitrate

consumed outside of the beetroot juice supplement, any subject who does not complete one or more of the recall entries will not be withdrawn from the remainder of study procedures. Subsequent analysis to obtain measures of food group equivalencies for nitrate containing food items (e.g. dark leafy greens) will be conducted using a validated Automated Self-Administered 24h (ASA24) dietary recall system. Approved study personnel will manually enter food, beverage, and supplement items into the electronic recall system to generate a report of dietary characteristics for each 24hr period.

Isokinetic Dynamometry- A Humac Norm System will be used to measure each subject's muscle contractile properties as previously described (18, 19) during **Visits 2 and 3**.. Briefly, study participant will perform maximal knee extensions with their dominant leg at angular velocities of 0, 1.57, 3.14, 4.71, and 6.28 rad/s. (Not all older individuals may be able to achieve the highest velocity – if not, the highest achieved angular velocity and associated torque will be used in all subsequent calculations.) The subject will perform 3-4 knee extensions at each velocity, with 2 minutes of rest allowed between each set of contractions. To eliminate artifacts, data will be "windowed" to isolate the isokinetic phase and smoothed using a 9 point weighted moving average filter using Microsoft excel. The highest torque generated at each velocity will be used to calculate peak power at that velocity, after which the resulting power-velocity curve will be fit with a parabolic function to determine the subject's Pmax and Vmax (i.e., Y-maximum and 2nd Y-intercept of fitted parabola, respectively) as previously described (18, 19).

Submaximal Walking Test- During **Visits 2 and 3**, subjects will be outfitted with a flexible oronasal face mask (Korr CardioCoach) and chest-strap heart rate monitor. A metabolic cart will be used to obtain continuous breath-by-breath monitoring of oxygen consumption. Subjects will be asked to stand quietly on the treadmill belt for 3 minutes while resting measurements of heart rate and blood pressure are obtained. They will then walk at 2mph at a 0% grade for 6 minutes. Heart rate and blood pressure will be obtained at minutes 3 and 5 of walking. Following exercise, they will remain standing while rested for 3 additional minutes while heart rate is recorded each minute and blood pressure after the final minute. Subjects will be asked to rate their perception of effort during exercise at minutes 3 and 5 using a discrete visual analog scale (0-10).

6-Minute Walk Test- Consistent with standard procedures (20), participants will be instructed to walk for six minutes around an indoor, closed course during **Visits 2 and 3**. A chest-strap heart rate monitor will be worn to measure changes in heart rate before and after walking, and a triaxial accelerometer will be used to examine signal profile associated with movement complexity (i.e. movement accelerations) and estimates of energy expenditure (kilocalories). The accelerometer will be worn on the non-dominant hip and fastened with an elastic belt. Subjects will additionally be asked to identify their level of perceived exertion on a continuous visual analog scale ("no exertion" to "maximal exertion") after the test.

Supervised Exercise Training (CrossFit, Bloomington)- Participants randomized to exercise training will perform combined aerobic and resistance workouts in a group setting with a cohort of approximately 4 women also enrolled in the study. There is a chance that participants will complete the exercise training sessions with other individuals who are not enrolled in the study. This will be outlined in the Informed Consent Statement provided to participants.

Full participation in the exercise training intervention is defined as attending 3 sessions per week for a total of 8 weeks (24 sessions total). The sessions will be supervised by a certified fitness professional who will make adjustments to the workouts based on individual needs/abilities. Each one-hour session will consist of a warm-up, instruction of the workout, a workout of combined aerobic and resistance training exercises, and cool-down. Intensity of the exercise sessions will be progressed gradually from weeks 1 to 8 and monitored by having participants identify their rating of perceived difficulty for each session using a discrete visual analog scale (0-10). The intensity of exercise sessions will also be monitored through continuous heart rate assessment (Actiheart) during week 2, week 4, and week 6 of training.

If participants miss (do not attend) a scheduled exercise training session, they will resume training at the next available scheduled session. The missed workout session will be recorded. Participants will be withdrawn from the study if they miss more than 3 consecutive exercise training sessions in any given week. Additionally, they must complete a minimum of 70% of the total 24 exercise training sessions (17 out of 24 sessions attended) to continue with follow-up assessments.

8.0 Study Calendar

(Screening Visit) After obtaining consent, subjects will be asked to complete a health history questionnaire, risk factor stratification document indicating their readiness to exercise, and provide information to obtain permission to contact their primary care physician. Their age will be verified by a member of our research team by having them provide a valid photo ID (e.g. driver's license), and current prescription medications will be verified by having them bring-in their medication bottles or labels. A measure of height and weight (via BIA) will be obtained to verify BMI inclusion criteria of 25.0 to 39.9 kg/m^2.

(Visit 1) All procedures during this visit will take place at the School of Public Health, Indiana University Bloomington. Measures of height, weight (BIA), body composition (BIA/DEXA), total body water (BIA), and bone density (DEXA) will be taken after having participants change into a provided standard set of hospital scrubs. The remaining procedures will be conducted with the participant resting quietly on a reclining couch. They will be outfitted with a chest-strap electrocardiograph monitor for the duration of the visit. Two laser Doppler probes will be attached to their non-dominant forearm while a localized heating protocol is initiated. A blood pressure cuff will be affixed to their dominant upper arm and blood pressure will be assessed after 5 min of seated rest, and at every 10 minutes of the 30-minute heating protocol. Following, they will perform a breath analysis to measure pre-ingestion levels of exhaled nitric oxide before ingesting 140mL of a commercial beetroot juice supplement. During a 3-hour window following ingestion, participants will be given a rest period and asked to complete a set of two questionnaires. During this time, blood pressure and methemoglobin using a fingertip pulse oximeter will be assessed once every hour. They will be asked to perform an additional breath analysis 3-hour post-ingestion to coincide with estimated maximum nitric oxide bioavailability provided by the supplement. After which, a repeat of the localized heating protocol and blood pressure measurements will be performed.

(Visit 2) A venous blood sample will be collected at the Indiana University Student Health Center. Following, all remaining procedures will take place at the School of Public Health, Indiana University Bloomington. Participants will again, be asked to change into a provided

standard set of hospital scrubs so that accurate measurements of body weight (BIA) and composition (BIA) can be obtained. They will then be asked to lie supine while resting quietly for a period of 10 minutes on a padded table while measures of pulse wave velocity are obtained. They will then be escorted to the recreational track within the School of Public Health building to complete the 6-min walking test and muscle function test.

(8-weeks Supervised Exercise Training) After being randomized into either an exercise only (EX) or beetroot and exercise (BR+EX) group, subjects in the BR+EX group will receive a 4-week supply of beetroot juice. They will additionally be asked to attend 3 sessions of supervised exercise training at CrossFit, Bloomington for a duration of 8 weeks. Subjects in BR+EX will be instructed to consume the beetroot juice 3 hours prior to each attended exercise session.

(Visits 2-3) The remaining follow-up visits will follow the exact same procedures as outlined for Visits 1-2 and will be performed in the reverse order.

	Screening	Initial Assessments		Exercise Training Intervention	Follow-up Assessments	
	Time point 0	Visit 1 (as scheduled)	Visit 2 (following ³7-day washout period)	0 to 8 weeks (following £ 6 weeks)	Visit 1 (following ³ 2 days)	Visit 2 (following ³ 2 days)
STUDY PROCEDURES						
Obtain Consent	X					
Medical History Questionnaire	X					
ACSM Risk Factor Stratification	X					
Obtain Permission to Contact Physician	X					
Verification of Age (driver's license)	X					
Prescription Medication Verification	X					
Height	X	x				X
Body Weight (BIA)	X	x	X		X	X
Body Fat Percentage (BIA)		X	X		X	X
Total Body Water (BIA)		X	X		X	X
DEXA		X				X
Heart rate (Actiheart)		X		X		X
Localized Heating Protocol using Laser Doppler		X				X
Blood Pressure		X				X
Nitric Oxide Breath Analysis		X		X		X
Acute Administration of Beetroot Juice (140 mL)		X				X
Blood Oxygen/ Methemoglobin Oximetry		X				X
Questionnaire (Part A and B)		X				X
Venous Blood Draw			X		X	
Pulse Wave Velocity			X		X	
Submaximal Walk Test			X		X	
Heart rate (chest-strap)			X		X	
Accelerometry			X		X	
6-minute Walk Test			X		X	

Rates of Exertion (continuous analog scale)			X		X	
Rates of Exertion (discrete analog scale)			X	x	X	
Isokinetic Dynamometry			X		X	
Supervised Exercise Training Sessions				X		
24hr Dietary Recall				X (3 during week 1 and 3 during week 8 of supervised exercise training)		

9.0 Reportable Events

The PI will report any adverse event or unanticipated problems involving risk to the IU IRB through the Kuali Coeus system. Collection of adverse and serious adverse events will begin to be collected at the time of consent. Events that will be promptly reported include anything that would impact subject safety, confidentiality, information security, or privacy. In the scope of this research study, the following events would be considered **promptly reportable** within 5 business days of the investigator becoming aware of the event:

1. Serious adverse events assessed by the PI as 1) unexpected, 2) related or possibly related to study participation, and 3) suggests that the research places subject(s) at greater risk or harm than previously anticipated. These may include injuries (e.g. broken bone) or medical events (e.g. chest pain) that meet the following criteria;
    a. Are life-threatening or result in death
    b. Causes disability or incapacity
    c. Requires in-patient hospitalization
    d. Results in a congenital anomaly and/or
    e. Considered an important medical event
2. Although not anticipated to occur, any major protocol deviations or noncompliance by an investigator involved with conducting research that in the opinion of the PI 1) impacted subject safety, and/or 2) affects the integrity of the data. These may include:
    a. Dosage errors with beetroot juice (nitrate) supplementation, enrolling of a subject who fails to meet inclusion criteria, and/or study visits performed outside of the outlined timeframe in the protocol document
3. Although not anticipated to occur, any incident that breaches subject privacy and/or confidentiality (i.e. loss of information containing identifiable records) would be considered a promptly reportable event. Any such incident would also be reported to the HIPPA Privacy Office.
    a. IU HIPPA Privacy Officer: 317-278-4521
4. Exceeding the study-wide enrollment number (i.e. 50 subjects).
5. Implementation of major changes (i.e. study procedures, risks and/or benefits) to the study protocol or documents without IRB approval.
6. Conduct of research procedures including interaction with subjects or review of identifiable subject information by individuals who are not listed as approved personnel and have not completed the necessary CITI training.

Additionally, events that do not meet prompt reporting criteria, but are still considered reportable will be communicated to the IU IRB at the time of the next study renewal. These events would include:

1. Local adverse events, which were not previously promptly reported and occurred at a greater frequency and/or severity than was initially anticipated. In relationship to the relevant study procedures, this may include:
    a. A greater frequency and/or severity of symptoms related to beetroot juice ingestion (e.g. gastrointestinal distress), muscle soreness during the walking and/or muscle strength tests, discomfort or bruising with needlestick blood draws.
    b. Injuries or medical conditions that do not require hospitalization and are not considered medically important (e.g. twisted ankle, sore muscles/joints, pulled muscle).
    c. Enrollment of a greater number of subjects that does not exceed the total study-wide enrollment number (i.e. 37-50 subjects enrolled).
    d. Conduct of research procedures by individuals who are not listed as approved personnel on the study protocol.
    e. Implementation of minor wording changes to study documents.

10.0 Data Safety Monitoring

Dr. Zachary Schlader (Department of Kinesiology) will work with Emily Long (Department of Kinesiology) to oversee aspects of data management and safety monitoring for the current investigation, including: data quality, subject recruitment, accrual, retention, outcome and adverse event data. Both are independent of the PI and funding source. Dr. Schlader is not directly affiliated with the study but will be responsible for performing periodic inspection of data quality and random assessments of participant recruitment, retention, adherence, and adverse events.

Dr. Schlader and Emily Long will hold in-person meetings once every 3 months to address items related to data quality. However, Dr. Schlader will perform periodic assessments at random to examine participant recruitment, retention, adherence, and adverse events.

As a safety precaution, potential side effects of acute beetroot juice ingestion will be determined for each participant during visit 1. Stopping criteria will be instituted (i.e., participant withdrawn) if a participant exhibits, during a 3-hr period, either a drop of >20 mm Hg in systolic blood pressure or a value of 12% in blood methemoglobin (determined via CO-oximeter).

Statistical analyses will be conducted on validated statistical software (e.g. SPSS, SigmaPlot) and results will be reported through traditional scientific outlets such as peer-reviewed journals and presentations. All analyses will be done without the use of any identifying features to individuals enrolled (e.g. use of ID codes, saving electronic data using ID codes rather that subject name).

11.0 Study Withdrawal/Discontinuation

A subject may elect to discontinue with study procedures by verbally notifying a member of the research team that they would like to do so either in person or by phone. Contact information for Dr. Stephen Carter and Emily Long is provided in the Informed Consent Statement. All

members associated with the research study (i.e. fitness instructors at Crossfit Bloomington) will be instructed to notify the research team in the event of a subject dropout.

Subjects may be withdrawn from the study by members of the research team if it is determined their safety is at risk (e.g. >20 mm Hg drop in systolic blood pressure with acute beetroot juice ingestions), they are unable to perform procedures as requested, or fail to follow instructions provided. Additionally, subjects may be withdrawn if they miss (do not attend) more than 3 consecutive exercise training sessions in any given week of the supervised exercise training protocol. Withdrawal is also warranted if they attend less than 70% of the scheduled exercise training sessions (e.g. less than 17 out of the 24 total sessions).

12.0 Statistical Considerations

Rather than confirm efficacy, this proposal addresses elements of feasibility such that we intend to generate preliminary effect sizes for a larger-scale investigation. Descriptive statistics and calculation of rates (e.g., recruitment, retention, adherence, and adverse event) will be used for study aim 1. Between-group differences in descriptive data along with aims 2(a,b) and 3 will be evaluated using an unpaired t-test. Effect size calculations will be performed with Cohen's d to qualify substantive differences: small (0.2), medium (0.5), and large (0.8). Potential covariates of interest include age, body mass, body fat percentage, and/or markers of inflammation. Non-normally distributed data will be log-transformed. Two-tailed parametric correlations (Pearson) will be performed and based on consequent results, multiple linear regression will be used to examine changes in inflammation, endothelial function, distance in 6-minute walk test, leg strength, and resting blood pressure/arterial stiffness.

13.0 Statistical Data Management

Primary data will be collected via paper and digital instruments and stored electronically on password protected/network secure university servers. The storage location will be backed up automatically every day. Quality assurance steps will include: 1) built in range checks using physiologically relevant values and 2) periodic means testing. The following quality control methods will be used: data entry will be performed by two study personnel and will be periodically range checked and means tested to check for data entry errors.

14.0 Privacy/Confidentiality Issues

All outlined procedures will be performed in private laboratory settings and medical facilities to respect the participants' right to privacy. Only approved members of the research team (listed on IRB personnel tab) will be present during procedures. While there is not absolute guarantee of confidentiality, every practical precaution will be taken.

Each study participant will be assigned a unique ID. Samples (including blood collection vials) and collected data will be de-identified using this unique ID. Information containing identifying data (e.g. Informed Consent Statements, Physician Clearance Forms) will be stored separately from de-identified data. All data will be stored either electronically on computers in locked rooms or in password protected files on department servers (OnCore, BoxHealth Account), or as paper copies stored in locked rooms within separately locked filing cabinets.

15.0 Follow-up and Record Retention

Study recruitment will be ongoing. The entire study is expected to last a duration of 24 months.

It is our intention to retain data collected indefinitely. Paper copies of study documents will be stored in locked rooms within separately locked filing cabinets separate from any identifying documents (e.g. Informed Consent Statements, Physician Clearance Forms). All electronic data will be stored on password-protected department servers (OnCore, BoxHealth Account) that are backed up daily. If recorded data are eventually deleted, paper copies will be shredded appropriately, all code keys deleted containing identifiable information, and remaining data permanently deleted from computers and portable storage mediums.

16.0 References

1. Leveille SG, Resnick HE, Balfour J. Gender differences in disability: Evidence and underlying reasons. *Aging (Milano).* 2000 Apr; 12(2): 106-112.

2. Rossouw JE, Manson JE, Kaunitz AM, Anderson GL. Lessons learned from the women's health initiative trials of menopausal hormone therapy. *Obstet Gynecol.* 2013 Jan; 121(1): 172-176. PMCID: PMC3547645.

3. Seals DR, Justice JN, LaRocca TJ. Physiological geroscience: Targeting function to increase healthspan and achieve optimal longevity. *J Physiol.* 2016 Apr 15; 594(8): 2001-2024. PMCID: PMC4933122.

4. Moreau KL, Ozemek C. Vascular adaptations to habitual exercise in older adults: Time for the sex talk. *Exerc Sport Sci Rev.* 2017 Apr; 45(2): 116-123. PMCID: PMC5357172.

5. Pierce GL, Eskurza I, Walker AE, Fay TN, Seals DR. Sex-specific effects of habitual aerobic exercise on brachial artery flow-mediated dilation in middle-aged and older adults. *Clin Sci (Lond).* 2011 Jan; 120(1): 13-23. PMCID: PMC3809822.

6. Mosca L, Barrett-Connor E, Wenger NK. Sex/gender differences in cardiovascular disease prevention: What a difference a decade makes. *Circulation.* 2011 Nov 8; 124(19): 2145-2154. PMCID: PMC3362050.

7. Taddei S, Virdis A, Ghiadoni L, Salvetti G, Bernini G, Magagna A, Salvetti A. Age-related reduction of NO availability and oxidative stress in humans. *Hypertension.* 2001 Aug; 38(2): 274-279.

8. Sanada F, Taniyama Y, Muratsu J, Otsu R, Shimizu H, Rakugi H, Morishita R. Source of chronic inflammation in aging. *Front Cardiovasc Med.* 2018 Feb 22; 5: 12. PMCID: PMC5850851.

9. Moreau KL, Hildreth KL, Meditz AL, Deane KD, Kohrt WM. Endothelial function is impaired across the stages of the menopause transition in healthy women. *J Clin Endocrinol Metab.* 2012 Dec; 97(12): 4692-4700. PMCID: PMC3513538.

10. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest.* 2003 Apr; 111(8): 1201-1209. PMCID: PMC152929.

11. Doel JJ, Benjamin N, Hector MP, Rogers M, Allaker RP. Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur J Oral Sci.* 2005 Feb; 113(1): 14-19.

12. Kapil V, Khambata RS, Robertson A, Caulfield MJ, Ahluwalia A. Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: A randomized, phase 2, double-blind, placebo-controlled study. *Hypertension.* 2015 Feb; 65(2): 320-327. PMCID: PMC4288952.

13. Bailey SJ, Varnham RL, DiMenna FJ, Breese BC, Wylie LJ, Jones AM. Inorganic nitrate supplementation improves muscle oxygenation, O(2) uptake kinetics, and exercise tolerance at high but not low pedal rates. *J Appl Physiol (1985).* 2015 Jun 1; 118(11): 1396-1405.

14. Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs AJ, Ahluwalia A. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension.* 2008 Mar; 51(3): 784-790. PMCID: PMC2839282.

15. 39. Gallardo EJ, Coggan AR. What's in your beet juice? nitrate and nitrite content of beet juice products marketed to athletes. *Int J Sport Nutr Exerc Metab.* 2018 Oct 9: 1-17.

16. 40. Coggan AR, Leibowitz JL, Kadkhodayan A, Thomas DP, Ramamurthy S, Spearie CA, Waller S, Farmer M, Peterson LR. Effect of acute dietary nitrate intake on maximal knee extensor speed and power in healthy men and women. *Nitric Oxide.* 2015 Aug 1; 48: 16-21. PMCID: PMC4362985.

17. Haider G, Folland JP. Nitrate supplementation enhances the contractile properties of human skeletal muscle. Med Sci Sports Exerc 2014; 46:2234-2243.

18. Kelly J, Fulford J, Vanhatalo A, Blackwell JR, French O, Bailey SJ, Gilchrist M, Winyard PG, Jones AM. Effects of short-term dietary nitrate supplementation on blood pressure, O_2 uptake kinetics, and muscle and cognitive function

19. ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories. ATS statement: Guidelines for the six-minute walk test. Am J Respir Crit Care Med. 2002 Jul 1; 166(1): 111-117.

17.0 Appendix

All supplemental materials have been uploaded to the protocol attachments section for Questionnaire (A+B), Participant Adverse Event Reporting Form, Recruitment Flyers, Participant Instruction Sheet, and Participant Dietary Recall Instruction Sheet.