PennState

Study Protocol / Statistical Analysis Plan

NCT03380000

Acute Blood Pressure Lowering Effects of Beetroot Juice in Post-Menopausal Women with and without Hypertension

July 16, 2018

PENNSTATE

HRP-592 - Protocol for Human Subject Research with Use of Test Article(s)

Protocol Title:

Acute blood pressure-lowering effects of beetroot juice in postmenopausal women with and without hypertension.

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1.0 Objectives

1.1 Study Objectives

The proposed study aims to evaluate the acute vascular effects of dietary nitrates on the peripheral and central circulation, both at rest and during physiological stress in older women.

Aim 1: To determine the blood pressure-lowering effects of acute dietary nitrate supplementation in postmenopausal women.

Hypothesis: A one time dose(140 mL) of concentrated beetroot juice will lower resting arterial stiffness, brachial artery blood pressure, and/or derived aortic blood pressure in postmenopausal women with and without hypertension.

Aim 2: To determine the effects of acute dietary nitrate supplementation on myogenic vasoconstriction of leg blood vessels during simulated postural stress.

Hypothesis: A one time dose (140 mL) of concentrated beetroot juice will reduce the amount of vasoconstriction in leg blood vessels exposed to graded increases in vascular transmural pressure (i.e., single leg suction) in postmenopausal women with and without hypertension.

Aim 3. To determine the effects of acute dietary nitrate supplementation on the control of blood pressure responses during exercise.

Hypothesis: A one time dose (140 mL) of concentrated beetroot juice will lower blood pressure responses to static arm and dynamic leg exercise. Beetroot juice will reduce the concentration of blood pressure-raising metabolites in exercising muscles through improvements in muscle perfusion and/or reductions in the metabolic cost of muscle contraction.

1.2 Primary Study Endpoints

The primary endpoints of this study are blood pressure, arterial wave reflection characteristics, and muscle metabolic responses. These responses will be measured at rest and during exercises in post-menopausal women before and after beetroot juice consumption.

1.3 Secondary Study Endpoints

Blood pressure, arterial wave reflection characteristics, and muscle metabolic responses will also be measured in pre-menopausal women (visit 2 only) to allow age group comparison of these variables.

2.0 Background

2.1 Scientific Background and Gaps

High blood pressure is a major risk factor for cardiovascular morbidity and mortality. Following menopause, women are more likely to develop high blood pressure. Postmenopausal women also experience greater increases in blood pressure during physical activity/exercise, and are more susceptible to blood pressure-related complications compared with either premenopausal women or men of a similar age. In addition to experiencing a greater risk associated with elevated blood pressure, blood pressure management is also relatively poor in women after menopause; more than half of postmenopausal women with hypertension do not have their blood pressure successfully treated. Therefore, there is a clinical need to investigate novel interventions aimed at improving blood pressure regulation in postmenopausal women both at rest and in response to physical stresses encountered during activities of daily living (e.g., posture, carrying objects, &ambulation).

2.2 Previous Data

Nitric oxide (NO) plays a key contributing role in the modulation of blood vessel tone at rest and under conditions of increased metabolic demand, such as exercise [1–3]. As a result, there has been a growing interest in the use of dietary interventions that increase bioavailable NO as both cardiovascular health-promoting [4–6] and ergogenic aids [7–9]. In particular, dietary supplementation with inorganic salts or high-nitrate containing foods (e.g., beetroot juice) has recently grown in popularity for its potential blood pressure-lowering and aerobic exercise performance-enhancing effects. With regard to the former function, numerous investigators have shown the reduction in resting blood pressure with beetroot juice consumption in healthy adults [4,10–17]. However, only two publications reported blood pressure lowering effects of beetroot juice in older adults [13,18], and no study has investigated the influence of dietary nitrate supplementation on exercise pressor response in postmenopausal women who are known to have reduced nitric oxide bioavailability.

2.3 Study Rationale

Dietary nitrate supplementation is a natural means of increasing the molecule nitric oxide in the body, and an emerging intervention for improving cardiovascular health and exercise performance. Specifically, beetroot juice (a nitrate-rich food supplement) has been shown to have both vasodilatory and peripheral blood pressure lowering, as well as muscle metabolic effects. However, the effectiveness of dietary nitrates to lower resting blood pressure in postmenopausal women, as well as their ability to modulate blood pressure responses to physical stress have not been adequately explored. Therefore the purpose of the present study is to examine the effectiveness of an acute dose of nitrate-rich beetroot juice to lower resting blood pressure, arterial stiffness, and blood pressure responses to physical stress in postmenopausal women with and without hypertension.

3.0 Inclusion and Exclusion Criteria

3.1 Inclusion Criteria

1) Premenopausal women between the ages of 18 and 35 years old with resting blood pressure < 140/90 mm Hg, and who have had regular menstrual cycles for the previous 6 months.
2) Postmenopausal women between the ages of 55 and 80 years old.

3.2 Exclusion Criteria

1) Individuals with any overt cardiovascular, metabolic, hematologic, pulmonary, renal, musculoskeletal, and/or neurological disease(s).
2) Users of any tobacco and/or nicotine products (smokers, chewing tobacco, nicotine-containing patches/gum, smokeless cigarettes)
3) Individuals with a BMI > 35
4) Individuals with resting blood pressure > or = 160/100 mm Hg
5) Individuals with high blood lipids (total cholesterol > or = 240 mg/dl, LDL > or = 160 mg/dl, triglycerides > or = 200 mg/dl).
6) Premenopausal women with hyperglycemia (fasting glucose > or = 110 mg/dl)
7) Postmenopausal women with hyperglycemia (fasting glucose > or = 110 mg/dl and HbA1c >6.0%).
8) Individuals taking any of the following medications:
    a. blood pressure lowering medication (e.g., beta blockers, ACE inhibitors, angiotensin antagonists, calcium channel blockers, diuretics)
    b. lipid- lowering medication (e.g., statins)
    c. nitrates (e.g. nitroglycerin) for angina
    d. phosphodiesterase inhibitors (e.g., Viagra)
    e. anti-Inflammatory drugs
9) Individuals taking hormone replacement therapy, hormone-based contraceptives.
10) Premenopausal women who are pregnant
11) Individuals with a history of orthopedic problems including hip or knee surgery that could cause significant knee pain during the step test or knee extension exercise.

3.3 Early Withdrawal of Subjects

3.3.1 Criteria for removal from study

Any subjects experiencing a serious adverse event felt to be related to the study intervention (beetroot juice supplementation) or procedures will be withdrawn from the study. Also, if a subject withdraws her consent to participate in the study or authorization to use her protected health information, she will be withdrawn from the study.

3.3.2 Follow-up for withdrawn subjects

A subject who decides to discontinue participate in this study will immediately be withdrawn from the study. Within 5 days, one of the study investigators will contact the subject for a follow-up interview. Subject who withdraw withdrawn may be replaced, at the discretion of the principal investigator.

4.0 Recruitment Methods

4.1 Identification of subjects

Premenopausal women will be recruited from the Penn State (University Park campus) student population and State College community by fliers and advertisements. Fliers will contain basic information about the study, as well as contact information for study personnel in the Vascular Aging and Exercise Laboratory (201 Noll Lab). Potential participants who show interest in the study by contacting study personnel will be sent an informed consent form in the mail with instructions to contact the one of the study investigators if she has any questions or would like to schedule a visit to Noll Lab for the screening and familiarization visit.

Postmenopausal women (both normotensive and hypertensive subjects) will be patients of the Penn State Hershey Medical Group in State College (i.e., the physician co-investigators listed on this protocol) or members of the State College community. Patients who meet the study's inclusion/exclusion criteria may be identified by their physician during the patient's routine visit to physician's medical office, and informed them of their potential eligibility for a research study at Penn State. They will also be provided with a flier that contains basic information about the study, as well as contact information for study personnel in the Vascular Aging and Exercise Laboratory (201 Noll Lab). Potential participants who show interest in the study by contacting Noll Lab personnel will be sent an informed consent form in the mail with instructions to contact the one of study investigators if she has any questions or would like to schedule a visit to Noll Lab to obtain informed consent. Postmenopausal women will also be recruited from the State College community by fliers and advertisements. Fliers will contain basic information about the study, as well as contact information for study personnel in the Vascular Aging and Exercise Laboratory (201 Noll Lab).

In addition to fliers and direct recruitment methods, a database search in the Penn State Hershey electronic medical record will be requested by Ms. Amy Behe, Penn State Hershey Medical Group (PSHMG) research coordinator. Search criteria will include postmenopausal women ages 55-80 seen by a PSHMG Family and Community (F&C) medicine physician between July 1, 2013 to present, non-smoker, BP less than 160/100mmHg with a BMI<35. Medications will be reviewed to exclude anyone currently taking lipid or blood pressure lowering medication or hormone replacement therapy. After potential subjects are identified, Ms. Behe will mail study recruitment letters, signed by one of the 4 physician co-investigators, to the patients. Ms. Behe will conduct a follow up phone call within 1-2 weeks of initial contact being made to answer any questions potential subjects may have. If the patient is interested in participating in the study, she will be asked to call the Vascular Aging and Exercise lab (201 Noll Lab) for further information. Potential participants who originally received a recruitment letter from PSHMG will not be contacted by phone since the original letter indicated that there would be no further contact.

4.2 Recruitment process

Members of the Vascular Aging and Exercise Laboratory will answer phone calls (office phone) or emails from potential participants. During these exchanges prospective participants may ask any questions regarding the study and/or schedule a visit to complete informed consent and screening process.

4.3 Recruitment materials

- Flyers/posters
- Posting on Website, Research At Penn State (www.research.psu.edu/volunteer)
- Database search in Penn State Hershey Electronic Medical Records
- Recruitment letters sent out by Ms. Amy Behe

4.4 Eligibility/screening of subjects

An initial screening will occur prior to the first visit to the laboratory. Potential subjects will be asked eligibility questions via phone call, and eligibility questions will contain inclusion and exclusion criteria. See the attached prescreening form.

5.0 Consent Process and Documentation

5.1 Consent Process

5.1.1 Obtaining Informed Consent

5.1.1.1 Timing and Location of Consent

Informed consent will be obtained either in the Vascular Aging and Exercise Laboratory (201 Noll Lab) or Clinical Research Center (CRC; 211 Elmore Research Wing, Noll Lab) after potential participants have made initial phone or email contact with one of the study investigators.

5.1.1.2 Coercion or Undue Influence during Consent

Potentially eligible (postmenopausal) participants for this study will be identified by the co-investigator physicians from their respective practices. While unlikely, it is possible that patients may feel compelled to participate in the study if they were informed by their physicians (i.e., Drs. Flanagan, Grine, Jiang, Fragin). Therefore, to minimize any potential feelings of coercion, a clinic nurse will inform patients of their potential eligibility for a research study looking at a dietary intervention on blood pressure, and then provide them with the informational brochure about the study. The brochure will clearly state that the patient's decision will have no bearing on their future care and not require more frequent or fewer physician visits. It would then be at the discretion of the patient to contact study personnel to express interest or request additional information.

It is possible that students enrolled in a course taught by the PI, advisor, or any of the study co-investigators, could express interest in participating in this study. Study investigators may inform students of the study, but will not entice or encourage them to participate. Moreover, participation in this study will not be a mandatory component of any of these courses, and participation (or lack thereof) will not influence a student course grade in any form.

5.1.2 Waiver or alteration of the informed consent requirement

The consent form will provide sufficient information about this study for subjects to make an informed decision about their participation in this study. During the first visit, one of the study investigators will explain all procedures in detail, and the subjects will be given an opportunity to ask questions. Subjects will be informed of potential risks and benefits involved in this study, and they will be assured that their participation is voluntary. The consent form must be signed by the subject and one of the investigators obtaining the consent.

5.2 Consent Documentation

5.2.1 Written Documentation of Consent

The consent form will provide sufficient information about this study for subjects to make an informed decision about their participation in this study. During the first visit, one of the study investigators will explain all procedures in detail, and the subjects will be given an opportunity to ask questions. Subjects will be informed of potential risks and benefits involved in this study, and they will be assured that their participation is voluntary. The consent form must be signed by the subject and one of the investigators obtaining the consent.

5.2.2 Waiver of Documentation of Consent

Not applicable.

5.3 Consent – Other Considerations

5.3.1 Non-English Speaking Subjects

Not applicable.

5.3.2 Cognitively Impaired Adults

Not applicable.

5.3.2.1 Capability of Providing Consent

Not applicable.

5.3.2.2 Adults Unable To Consent

Not applicable.

5.3.2.3 Assent

Not applicable.

5.3.3 Subjects who are not yet adults (infants, children, teenagers)

Not applicable.

5.3.3.1 Parental Permission

Not applicable.

5.3.3.2 Assent

Not applicable.

6.0 HIPAA Research Authorization and/or Waiver or Alteration of Authorization

6.1 Authorization and/or Waiver or Alteration of Authorization for the Uses and Disclosures of PHI

Check all that apply:

- ☐ Authorization will be obtained and documented as part of the consent process.
- ☒ Partial waiver is requested for recruitment purposes only *(Check this box if patients' medical records will be accessed to determine eligibility before consent/authorization has been obtained)*
- ☐ Full waiver is requested for entire research study *(e.g., medical record review studies)*
- ☐ Alteration is requested to waive requirement for written documentation of authorization

6.2 Waiver or Alteration of Authorization for the Uses and Disclosures of PHI

6.2.1 Access, use or disclosure of PHI representing no more than a minimal risk to the privacy of the individual

6.2.1.1 Plan to protect PHI from improper use or disclosure

PHI will only be accessible by Amy Behe. Amy will maintain a contact list for the purposes of the follow up phone call. The list will be stored on a password protected computer in the office of Amy Behe, located at 303 Benner Pike, Suite 1.

6.2.1.2 Plan to destroy identifiers or a justification for retaining identifiers

The list with the names of potential participants who have been mailed a letter will be kept until completion of the study. This is done to ensure that no potential participant is reached out to more than once while the study is being conducted.

6.2.2 Explanation for why the research could not be practicably be conducted without access to and use of PHI

The inclusion and exclusion criteria for our postmenopausal group are relatively restrictive. In addition, potential subjects need to have recent measurements of their resting blood pressure

and blood lipids. To enhance our ability to identify and recruit such subjects, we have 1) formed a collaboration and 2) requested a data-base search in the Penn State Hershey electronic medical record.

6.2.3 Explanation for why the research could not practicably be conducted without the waiver or alteration of authorization

Given the inclusion and exclusion criteria for postmenopausal women, it is difficult to recruit sufficient number of subjects with the current methods in place. By targeting individuals who meet the criteria, we are enhancing our ability to identify and recruit potential subjects.

6.3 Waiver or alteration of authorization statements of agreement

Protected health information obtained as part of this research will not be reused or disclosed to any other person or entity, except as required by law, for authorized oversight of the research study, or for other permitted uses and disclosures according to federal regulations.

The research team will collect only information essential to the study and in accord with the 'Minimum Necessary' standard (information reasonably necessary to accomplish the objectives of the research) per federal regulations.

Access to the information will be limited, to the greatest extent possible, within the research team.

All disclosures or releases of identifiable information granted under this waiver will be accounted for and documented.

7.0 Study Design and Procedures

7.1 Study Design

The proposed study will examine age- and dietary nitrate supplementation induced effects on blood pressure and other cardiovascular variables in women. To do so, we will recruit groups of healthy premenopausal (18 – 35 yrs) and postmenopausal (55 – 80 yrs) women from State College, Pennsylvania and surrounding communities. A strength of our study design will be: 1) double-blind placebo-controlled study intervention and 2) our careful control for overt diseases and disease risk factors, medication use, and extremes in physical activity/inactivity to minimize their potential confounding influence on our study outcomes.

7.2 Study Procedures

7.2.1 Visit 1. Screening/Familiarization Visit

Subjects will participate in this visit without having consumed caffeine for the previous 12 hours, alcohol or dietary supplements for the previous 48 hours, or participating in any exercise workouts (e.g., weight lifting or aerobic exercise > 15 minutes) for the previous 24 hours prior to arrival. Premenopausal women will also report to this visit without consuming food (except water) for 12 hours prior to arrival, and will provide a fasted blood sample (taken by Clinical Research Center [CRC] nurse) from an arm vein to determine whether they meet study inclusion/exclusion criteria. For postmenopausal women whose blood results indicated a fasting blood glucose level between 110 and 125 mg/dl a fasted blood sample (approximately 6 ml) will

be taken from an arm vein to determine Hemoglobin A1c level. After the blood draw, they will be given a small snack to alleviate feelings of hunger. All participants will then complete health history and physical activity questionnaires, and have their resting heart rate and blood pressure measured. All participants will then perform a sub maximal exercise test to estimate their aerobic fitness. During this test, participants will be asked to step on and off of a 30 cm box for 5 minutes, while their heart rate is monitored. Participants will then be familiarized with the measurements that will be performed during subsequent study visits.

Daily Urine Collection and Menstrual Tracking: To test menstrual cycle hormones, participants will be asked to collect a urine sample daily when they first get up in the morning for one complete menstrual cycle (if regularly-menstruating) or one 30-day monitoring period (if postmenopausal). Written and verbal instructions on urine collection will be provided to the participant *(Document titled "Daily Urine Collection Instructions").* A urine collection will occur every morning using a small urine cup that is provided by the lab. Samples will be stored in the freezer by the participant and transported to the lab using supplies that the lab provides. Premenopausal participants will be asked to begin these collections on the first day of their next menstrual cycle and continue to collect daily samples until the first day of the following menstrual cycle. Postmenopausal participants (i.e. those who have gone more than 12 months without menses) will be asked to collect a urine sample daily for a 30-day monitoring period, starting on a random day. Participants will keep track of menses and times of urine collection on a menstrual and urine calendar provided by the lab *(Document titled "Menstrual and Daily Urine Log").* Collection, processing, and recording of this urine sample will take about 5 minutes per day. Urine analysis will be prepared and performed in rooms 116, 117, and 124 of Noll Laboratory. Measurement of a urinary derivative of estrogen, estrone glucocoronide (E1G), will be used to assess estrogen levels.

7.2.2 Visits 2-4. Experimental Visits

For all remaining visits (Visits 2-4) participants will arrive in a fasted state, without having consumed food or caffeine for the previous 12 hours; consumed alcohol, supplements or high-nitrate containing foods for the previous 48 hours; or participated in any exercise workouts (e.g., weight lifting or sustained aerobic exercise > 15 minutes) for the previous 24 hours prior to the visit.

VISIT 2. Baseline Study Visit - Participants will have approximately 16 mL (~1 tbsp.) of blood sample taken by a CRC nurse, and be given a small snack. Blood samples will be used to assess nitrate, nitrite, estradiol 17-beta, and cGMP concentrations. Measurement of estradiol levels will be performed in the lab of Dr. Mary Jane De Souza, located in 116 Noll laboratory. Premenopausal will take a urine pregnancy test before the blood sample is taken. If the pregnancy test is negative, they will provide the blood sample, and continue with the remainder of the measurements in this study visit. However, if the pregnancy test is positive, they will immediately be withdrawn from the study and will be asked to schedule an appointment with a physician to confirm the positive test. After the blood draw, the following procedures will be performed:

1) Heart Rate (HR) - Three ECG electrodes will be placed on the participant's chest, and a small inflatable cuff will be placed around a finger. Participants will lie on a bed for 20 minutes while their heart rate and finger pulse are recorded.

2) Pulse Wave Velocity (PWV) - While lying flat on a bed, a blood pressure cuff will be placed around the upper thigh while a researcher holds a pen-sized sensor on the participants' neck. The blood pressure cuff placed on the thigh will inflate and then deflate over 1-2 minutes, while sensors in the cuff and on the neck measure how fast each pulse of blood travels between these two sites.

3) Blood Pressure (BP) - Blood pressure will be measured with an automated blood pressure device. An inflatable cuff will be placed on the participant's upper arm while they sit upright in a chair. The cuff will then inflate and deflate two times each over 1-2 minutes. As the cuff deflates, sensors inside of the cuff will detect the blood pressure in the upper arm, and the device will use a validated equation to also calculate the blood pressure in the aorta. Three to four measurements will be performed.

4) Leg Suction (LS) - While lying on a bed, participants will have one leg sealed up to the mid-thigh inside of a closed box. Using a vacuum, we will apply up to 4 different levels of suction to the leg inside the box for one minute at a time. Each level of suction will be applied 2-4 times each separated by a 3-4 minute rest period. We will measure blood flow into the leg inside and outside of the box with a Doppler ultrasound machine.

5) Static Handgrip Exercise (SHE) - A blood pressure cuff will be placed on the subject's upper arm and forearm. The cuff on the upper arm will be inflated to above systolic blood pressure. The subject will rest with the cuff inflated for a period of 2 minutes, after which the cuff on their forearm will rhythmically inflate and deflate at a rate of 30 inflations/min for a period of 1 minute (upper arm cuff to remain inflated) or their wrist will be flexed and extended by a member of the Vascular Aging lab. After this 3 minute period, the upper arm cuff will be deflated and the subject will be given a 10-15 minute resting period. Participants' maximal voluntary contraction (MVC) will be determined by having them squeeze a handgrip device as hard as they can. Subjects will then squeeze the handgrip device intermittently at a rate of 30 contractions/min at 10-20% MVC. This intermittent exercise will be performed twice: once without inflation of the blood pressure cuff and once with gradual cuff inflation (approximately 12 mmHg per minute). In both intermittent handgrip trials, every 1 to 3 minutes participants will be asked their rating of perceived exertion (RPE) on a scale from 6 to 20. Exercise will be terminated when the participant reports an RPE value of 19 or 20, or when there is a >90% reduction in blood flow through the brachial artery. Five seconds prior to the termination of exercise, the blood pressure cuff will be inflated above systolic blood pressure for 3 minutes. During the last minute of this 3 minute period, the blood pressure cuff on the subjects forearm will be rhythmically inflated and deflated for a 1 minute period or their wrist will be flexed and extended by a member of the Vascular Aging lab. Forearm blood flow will be measured by Doppler ultrasound, and muscle pH will be measured by a near-infrared sensor placed over the skin on the forearm.

6) Dynamic Leg Exercise (DLE) - While seated in semi recumbent position, participants will have one leg placed in a knee-exercise device. We will then passively move their leg for them in a kicking motion while a cuff is inflated on their upper thigh. Participants will then perform 9 minutes of active knee extensions (3 min no resistance; 6 minutes moderate resistance) with the thigh cuff deflated, followed by 3 minutes of rest with the cuff inflated. Leg blood flow will be measured by Doppler ultrasound, and muscle pH will be measured by a near-infrared sensor placed over the skin on the thigh.

<u>Premenopausal Visit 3. Estradiol assessment visit (Premenopausal women only). –</u> Subjects will be asked to return to the lab on one day between days 9-11 of their menstrual cycle for a blood draw. Approximately 10 ml of blood will be taken from an arm vein during this visit. This blood draw will be used to assess their serum estradiol content at that point in their cycle. The results of this test will be used to schedule their second experimental visit (Premenopausal visit 4). Subject visits will be scheduled so that no more than 2 blood draws occur in a given week.

<u>Postmenopausal Visit 3. Beetroot Juice Study Visit (Postmenopausal women only)</u> - A CRC nurse will take a blood sample from an arm vein. Participants will then undergo measures of HR, PWV, and BP. Next, participants will consume 140 mL (9.5 tablespoons) of either nitrate-rich beet juice or beet juice with nitrates removed with a small snack, and will remain at the CRC

for 1.5 hours. 1.5 hours after consuming the beet juice, a second blood sample will be taken and the participants will undergo measures of HR, PWV, BP, LS, SHE, and DLE. Once these measurements are completed, a third blood sample will be taken.

Premenopausal Visit 4. Experimental Study Visit- Procedures will be identical to those during Premenopausal Visit 3.

Postmenopausal Visit 4. Beetroot Juice Study Visit (Postmenopausal women only) - Procedures will be identical to those during visit 3 except that participants will consume whichever drink supplement they did not receive during visit 3.

7.3 Duration of Participation

Visit 1 will take approximately 1.5 hours.
Visit 2 will take approximately 4 hours.
Premenopausal Visit 3 will take approximately 30 minutes.
Premenopausal Visit 4 will take approximately 4 hours.
Postmenopausal Visits 3 and visit 4 (postmenopausal women only) will each take approximately 6 hours.

For premenopausal women the total duration of all the research sessions (Visit 1-4) will be approximately 10 hours.

For postmenopausal woman the total duration of all the research sessions (Visits 1-4) will be approximately 17.5 hours.

All postmenopausal experimental study visits (Postmenopausal Visits 2-4) will be separated by no less than 5 days each. Therefore, it is expected that all postmenopausal participants will be able to complete all study visits (up to 3) over 2-6 weeks following their screening visit. Premenopausal women will be able to complete their visits 1-2 over 2-4 weeks.

7.4 Test Article(s) (Study Drug(s) and/or Study Device(s))

7.4.1 Description

For our proposed study, we will be using a Beet Juice supplement which is high in dietary nitrates. Also, we will use a Beet Juice supplement that has had the nitrates removed to provide a placebo for our proposed research study. The developer of both drinks is James White Drinks in Ipswich, UK. James White Drinks has created many different Beet Juice varieties as a part of their "Beet It" line of drinks. We will use their Beet It Sport Shot for the treatment (contains dietary nitrates).

7.4.2 Treatment Regimen

Participants will consume orally 140 mL (9.5 tablespoons) of either nitrate-rich beet juice or beet juice with nitrates removed with a small snack on visits 3 and 4, and will remain at the CRC for 1.5 hours.

7.4.3 Method for Assigning Subject to Treatment Groups

Not applicable.

7.4.4 Subject Compliance Monitoring

Subjects will consume beetroot juice under the supervision of a Clinical Research Center Nurse.

7.4.5 Blinding of the Test Article

Treatment order will be randomly assigned to participants by Clinical Research Center (CRC) staff using computer-based mathematical software (Microsoft Excel). The generated list that determines each participants treatment order will be kept in a secure location in the CRC which cannot be accessed directly by research team members.

7.4.6 Receiving, Storage, Dispensing and Return

7.4.6.1 Receipt of Test Article

Product will be shipped from James White Drinks, Ipswich, UK. We will use the Beet It Sport Shot and its nitrate-removed placebo for our study. Dr. David Proctor will be responsible for accountability of the supplement.

7.4.6.2 Storage

In a locked cabinet located in the Clinical Research Center of Noll Laboratory at a temperature maintained between 22 and 25 degrees Celsius.

7.4.6.3 Preparation and Dispensing

Research nurses with the Clinical Research Center will dispense the supplements to participants on the two study visits. No preparation is necessary. Labels for [70 ml] bottles of nitrate-containing and nitrate-removed (i.e., placebo) beetroot juice are identical with the exception of a color-coded ring at the neck of the bottle. The color coding for the two forms of beetroot juice will be unknown to study participants.

7.4.6.4 Return or Destruction of the Test Article

Empty bottle will be disposed.

7.4.6.5 Prior and Concomitant Therapy

Not applicable.

8.0 Data and Specimen Banking For Future Undetermined Research

8.1 Data and/or specimens being stored

The screening venous blood sample from premenopausal women will be analyzed by Quest diagnostics to measure blood lipid profiles, glucose levels, and a complete blood count. The combination of these measurements will provide us the information necessary to determine if the prospective participant meets our inclusion/exclusion criteria and/or is healthy enough to be included in our study.

Each venous blood sample taken during the baseline and experimental study visits (visits 2-4) will be tested for plasma concentrations of nitrate, nitrite, estradiol 17-beta, and cyclic guanosine monophosphate (cGMP; a marker of nitric oxide activity).

Urine from premenopausal women (Visit 2) will be tested for human chorionic gonadotropin, a marker used to determine pregnancy, by dipstick urinalysis.

Daily urine samples will be stored with the subject ID and date of collection. The data will be labeled with subject ID and date of data collection.

8.2 Location of storage

Blood samples from the baseline and experimental study visits (visits 2-4) will be stored in a low-temperature freezer located in 118 or 120 Noll Laboratory.

Blood samples used to assess estradiol concentrations will be kept in a low temperature freezer in the lab of Dr. Mary Jane De Souza located in room 2, 117, 121 of Noll Laboratory.

Urine samples used to determine pregnancy will be collected in the Clinical Research Center of Noll Laboratory; however, they immediately discarded (within 5 minutes of testing) on the same day as the study visit.

Daily urine samples used to assess estrogen exposure will be stored in -20 freezers. These freezers are located in room 124, room 121, room 116, room 117, room 2, room 3, or in the basement hallway in Noll Lab.

8.3 Duration of storage

5 years

8.4 Access to data and/or specimens

Only approved study personnel of the Vascular Aging and Exercise Laboratory (Dr. Proctor, Director), and its co-investigators on this protocol will have access to the blood samples.

For data, members of the Vascular Aging and Exercise Laboratory research team will have access to the link associating the participants to the data, which will be stored in a secure file in 201 Noll Laboratory.

8.5 Procedures to release data or specimens

Not applicable.

8.6 Process for returning results

Not applicable.

9.0 Statistical Plan

9.1 Sample size determination

We calculated 6 subjects per group will be sufficient to detect a meaningful physiological differences in resting blood pressure of mildly hypertensive older adults after acute dose of nitrate-rich beetroot juice.

The effect size of 77% was calculated based on published date comparing resting blood pressure of grade 1 hypertension patients before and after beetroot juice consumption [19]. With an alpha = 0.05 and power = 0.80, the projected sample size needed (G*Power 3.1, [20] is approximately 6 for our within-between group comparison (ANOVA, power = 0.08, $\alpha = 0.05$).

9.2 Statistical methods

Comparisons of arterial stiffness and arterial wave characteristics between the placebo and beetroot juice visits will be performed using paired t-tests. Paired t-tests will also performed to compare plasma nitrate and nitrite concentrations between the two visits for all data points. Two-factor ANOVA with repeated measures will be used to assess blood pressure lowering effects of beetroot juice at rest and during exercise in postmenopausal women. A value of $p<0.05$ will be considered statistically significant for all experiments.

10.0 Confidentiality, Privacy and Data Management

10.1 Confidentiality

Confidentiality will be maintained with all data collected. Participants will be assigned an identity code that will be used to identify all data collected during the study. The identity code will be only known by study personnel and will not contain any information that can be used to identify the participant. Paper and computer forms of data will be stored within a specific study binder and on a dedicated research laboratory computer, respectively. The study binder is always stored within the laboratory and the laboratory door is locked outside of work hours. The research computer is located inside the laboratory and cannot be accessed unless a correct username and password is provided.

10.1.1 Identifiers associated with data and/or specimens

There will not be a direct identifier associated with data and/or specimens.

10.1.1.1 Use of Codes, Master List

The list linking the code numbers to participants will be created and stored in a secure file cabinet, the password-protected lab computer inside of the Vascular Aging and Exercise Laboratory (201 Noll Laboratory) which will also be locked when not occupied by lab personnel. The list linking code numbers to participants will be destroyed within 5 years of completing data collection.

10.1.2 Storage of Data and/or Specimens

Data collected for this study will be stored in Noll 201 for hard-copies and password-protected folders on the secured PSU server for electronic copies.

Plasma samples from the study visits will be stored in a low-temperature freezer located in 118 or 120 Noll Laboratory.

Daily urine samples used to assess estrogen exposure will be stored in -20 freezers. These freezers are located in room 124, room 121, room 116, room 117, room 2, room 3, and in the basement hallway in Noll Lab. Samples will be destroyed within one year of enrollment in the study.

10.1.3 Access to Data and/or Specimens

Only members of the Vascular Aging and Exercise Laboratory and co-investigators approved on this protocol will have access to the data.

10.1.4 Transferring Data and/or Specimens

Approximately 1 ml of plasma from each blood sample will be sent to Dr. Daniel Kim-Shapiro at Wake Forest University for measurement of plasma nitrate/nitrite concentrations. The frozen samples will be shipped overnight.

Approximately 5 ml of blood will be given to Dr. Mary Jane De Souza, also located in Noll lab, for measurement of serum estradiol concentrations. The frozen samples will be stored in the freezers located in the lab of Dr. Mary Jane De Souza in Noll laboratory (rooms 2, 117, 121).

10.2 Privacy

Throughout the study duration, researchers will maintain a professional manner. Regarding health and/or personal information, researchers will only obtain this information via questionnaires and lab results. Moreover, researchers will only discuss this information with participants as it relates to their participation in the study (i.e., their eligibility). Participants will also be reminded that any information that they provide is voluntary, and can refuse to provide any information they wish.

During the study visits, subjects' privacy will be maintained by performing all procedures in a private laboratory room(s). These rooms will be shut during all study visits, and will not be visible to persons in the building corridors.

11.0 Data and Safety Monitoring Plan

11.1 Periodic evaluation of data

Not applicable

11.2 Data that are reviewed

Not applicable

11.3 Method of collection of safety information

Not applicable

11.4 Frequency of data collection

Not applicable

11.5 Individual's reviewing the data

Not applicable

11.6 Frequency of review of cumulative data

Not applicable.

11.7 Statistical tests

Not applicable.

11.8 Suspension of research

If abnormal physiological responses to any part of the protocols or dietary nitrate supplementation are observed, the research will be suspended immediately.

12.0 Risks

Blood Sampling - This may cause some mild discomfort and bruising where the needle is inserted into the arm. There is also a very small risk of developing an infection or a clot at the insertion site.

Step-based Fitness Test - During the exercise test, participants may experience muscle fatigue, shortness of breath, or a side cramp. It is also possible to experience light-headedness, chest discomfort, or irregular heartbeats during this test. There is also a small risk of participants losing their balance and/or falling during the test.

Heart Rate - It is possible that the adhesive on the sticky electrodes may irritate participants' skin. There is also a minimal risk that an allergic reaction could occur from the adhesive.

Blood Pressure/Pulse Wave Velocity - During inflation of the cuffs, participants may feel a numb and/or tingling sensation in their hands/feet that will go away quickly after the cuffs are deflated. During the pulse wave velocity test, they may also experience some temporary discomfort (pressure) while the researcher holds a small sensor over an artery in their neck.

Leg Suction - During the suction participants feel temporary sensations of swelling in theirleg. There is also a small risk that they may experience temporary feelings of light-headedness. The vacuum will tend to pull participants' legs leg into the box, and as a result they may feel a moderate pressure pushing against their upper thigh.

Doppler Ultrasound - There is a minimal risk that the ultrasound probe and/or gel will irritate participants' skin. Participants may feel minor discomfort (pressure) when the researcher is pressing the ultrasound sensor against their upper arm or upper thigh.

Near-Infrared Spectroscopy - There are no known risks associated with use of the near-infrared device. However, it is possible that the adhesive on the sticky plastic sensor may irritate the skin.

Static Arm Exercise - Participants may feel temporary fatigue in the muscles of the exercising arm. It is also possible to experience soreness in these muscles within 24-48 hours following this study visit. There is a small risk that participants may develop a bruise from the inflation of the cuff on the upper arm. It is also possible to experience a numb, tingling sensation in the arm while the cuff is inflated. There is also some risk of the appearance of petechia (small pink blotches on the skin) as a result of the increased venous blood pressure in the occluded forearm during exercise. If present, these very small pink dots may persist for several days but are not associated with any pain or long term adverse effects.

Dynamic Leg Exercise - Participants may feel temporary muscle fatigue in the thigh muscles of the exercising leg. It is also possible to experience soreness in these muscles within 24-48 hours following this study visit. There is a small risk that participants may develop a bruise from the inflation of the cuff on the upper thigh. YIt is also possible to experience a numb, tingling sensation in the leg while the cuff is inflated.

Consumption of beet juice - There are no known health risks associated with consumption of nitrate-rich beet juice, a commonly sold health drink/supplement in Europe. The most common side-effect of beet juice consumption is pinkish-colored urine (known as "beeturia") and/or stool. This can occur after consuming either the nitrate-rich or placebo version of the drink.

13.0 Potential Benefits to Subjects and Others

13.1 Potential Benefits to Subjects

Postmenopausal participants will receive more regular monitoring of their resting blood pressure. Throughout the duration of the study protocol, participants will have their untreated blood pressure measured by trained research personnel on 4 separate occasions, and these measurements will be provided at no cost to them and/or their insurance provider. Postmenopausal participants will also be provided information about their aortic blood pressure, a measurement which may have more predictive value for future cardiovascular events than arm blood pressure measurements alone. With patient

permission, all blood pressure-related information will be shared with their primary care physician.

There are no direct benefits to premenopausal women for participating in this study other than receiving information about their cardiovascular risk factors (i.e., blood pressure, blood cholesterol, fitness level, etc.).

13.2 Potential Benefits to Others

This research may further our understanding about the potential for nitrate-rich food/dietary supplements to help control blood pressure without (or with less) medication(s) in a population with increased blood pressure-related cardiovascular risk (i.e., postmenopausal women).

14.0 Sharing Results with Subjects

In the event that abnormal lab test results are obtained during initial screening or subsequently throughout this study, the subjects will be informed as quickly as possible of these results and instructed to contact your private physician for further assessment. The lab test results will be made available to your private physician at your request.

Individual data will be shared with the subjects upon requests. However, the study results will not be shared until the data are published (e.g. journal articles).

15.0 Economic Burden to Subjects

15.1 Costs

None.

15.2 Compensation for research-related injury

It is the policy of the institution to provide neither financial compensation nor free medical treatment for research-related injury. In the event of injury resulting from this research, medical treatment is available but will be provided at the usual charge. Costs for the treatment of research-related injuries will be charged to subjects or their insurance carriers.

16.0 Number of Subjects

The total number of subjects who are expected to be enrolled and screened is 60, and the minimal number of subjects needed to complete the research procedures is 24.

17.0 Resources Available

17.1 Facilities and locations

201 Noll Laboratory and the Clinical Research Center (Located in Noll Laboratory, Elmore Wing).

116 Noll Laboratory (Dr. Mary Jane De Souza)

124 Noll Laboratory (Biological Specimen Processing and Storage Laboratory): This lab is divided into several workstations for processing and long-term storage of urine and blood samples and includes: six

-20 freezers, 1 refrigerator, 1 Forma Scientific Centrifuge, 1 IEC Centra CL2 centrifuge, and a temperature controlled Eppendorf 5804-R centrifuge.

Penn State Hershey Medical Group Park Avenue (Drs. Flanagan &Jiang): 1850 East Park Avenue, Suite 312 State College, PA 16803

Penn State Hershey Medical Group Windmere Centre (Dr. Grine): 476 Rolling Ridge Drive, Suite 101 State College, PA 16801

Penn State Hershey Medical Group Colonnade (Dr. Fragin):32 Colonnade Way State College, PA 16803

All screening/familiarization and study visits will take place in Noll Laboratory's Vascular Aging and Exercise Lab (201 Noll Laboratory) and Clinical Research Center (CRC). All pregnancy testing (young women only), supplement consumption (older women only), and blood sampling (all subjects) will be performed at the CRC. All other experimental procedures including fitness testing will be performed in the Vascular Aging and Exercise Lab.

The Vascular Aging and Exercise Lab contains several noninvasive research devices used to assess cardiovascular health and function at rest and during exercise. Devices used in our proposed study will include electrocardiography (ECG), Doppler ultrasound, a plethysmography-based continuous blood pressure monitor (Finometer midi, Finapres Medical Systems), a central Blood pressure/vascular profiling device (SphygmoCor XCEL, AtCor Medical), and a rapid-inflation cuff system (Hokanson). Exercise devices include a commercially-available hand dynamometer (Stoelting Co.) and a custom-built single-leg knee extension ergometer.

17.2 Feasibility of recruiting the required number of subjects

Our lab have successfully recruited required number of healthy premenopausal women utilizing same recruitment methods.

Postmenopausal women (both normotensive and hypertensive groups) will be patients of the Penn State Hershey Medical Group in State College (i.e., the physician co-investigators listed on this protocol). We are not able to determine how many postmenopausal women are registered as patients in Penn State Hershey Medical Group without accessing the electronic medical records. However, the physician co-investigators listed this study have confirmed that sufficient subjects are available in their combined practices to meet the subject sample size requirement of this study.

17.3 PI Time devoted to conducting the research

PI, Dr. David Proctor, will oversee and advise conducting and completing the research while two graduate students, Jin-Kwang Kim and Megan Barrett, take the lead in subject recruiting, data collecting, data analysis, and manuscript preparation. Jin-Kwang is a post-comprehensive exam doctoral student, and Megan is a Master's student. Both graduate students will devote most of their time conducting and completing this study.

17.4 Availability of medical or psychological resources

Clinical research center (CRC) staffs will be available for any medical emergencies during experimental visit as well as University Health Services.

17.5 Process for informing Study Team

Prior to the first subject's experimental visit, numerous pilot studies and protocols, will be performed with investigators responsible for data collection. In addition to multiple practices, investigators will strictly follow study flow sheets for each study visit.

18.0 Other Approvals

FDA approval for dietary nitrate supplement -beetroot juice, Beet It Organic Shot by James White Company- is already obtained (IND # 119978).

19.0 Subject Stipend (Compensation) and/or Travel Reimbursements

Participants will be paid for the studies as outlined below. If a subject withdraws from the study, they will still be compensated for visits completed prior to withdrawal. The compensation provided for completion of each visit is outlined as follows:

Premenopausal subjects:
Visit 1: No compensation provided
Visit 2: $20
Visit 3 and 4: $20

Postmenopausal subjects:
Visit 1: No compensation provided
Visit 2: $20
Visit 3: $30
Visit 4: $30

No travel reimbursement is applicable for this study.

20.0 Multi-Site Research

20.1 Communication Plans

Not applicable.

20.2 Data Submission and Security Plan

Not applicable.

20.3 Subject Enrollment

Not applicable.

20.4 Reporting of Adverse Events and New Information

Not applicable.

20.5 Audit and Monitoring Plans

Not applicable.

21.0 Adverse Event Reporting

21.1 Adverse Event Definitions

For drug studies, incorporate the following definitions into the below responses, as written:	
Adverse event	Any untoward medical occurrence associated with the use of the drug in humans, whether or not considered drug related
Adverse reaction	Any adverse event caused by a drug
Suspected adverse reaction	Any adverse event for which there is a reasonable possibility that the drug caused the adverse event. Suspected adverse reaction implies a lesser degree of certainty about causality than "adverse reaction". • *Reasonable possibility.* For the purpose of IND safety reporting, "reasonable possibility" means there is evidence to suggest a causal relationship between the drug and the adverse event.
Serious adverse event or Serious suspected adverse reaction	Serious adverse event or Serious suspected adverse reaction: An adverse event or suspected adverse reaction that in the view of either the investigator or sponsor, it results in any of the following outcomes: Death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.
Life-threatening adverse event or life-threatening suspected adverse reaction	An adverse event or suspected adverse reaction is considered "life-threatening" if, in the view of either the Investigator (i.e., the study site principal investigator) or Sponsor, its occurrence places the patient or research subject at immediate risk of death. It does not include an adverse event or suspected adverse reaction that had it occurred in a more severe form, might have caused death.
Unexpected adverse event or Unexpected suspected adverse reaction.	An adverse event or suspected adverse reaction is considered "unexpected" if it is not listed in the investigator brochure, general investigational plan, clinical protocol, or elsewhere in the current IND application; or is not listed at the specificity or severity that has been previously observed and/or specified.

21.2 Recording of Adverse Events

All adverse events (serious or non-serious) and abnormal test findings observed or reported to study team believed to be associated with the study drug(s) or device(s) will be followed until the event (or its sequelae) or the abnormal test finding resolves or stabilizes at a level acceptable to the investigator.

An abnormal test finding will be classified as an adverse event if one or more of the following criteria are met:

- The test finding is accompanied by clinical symptoms
- The test finding necessitates additional diagnostic evaluation(s) or medical/surgical intervention; including significant additional concomitant drug treatment or other therapy
  Note: Simply repeating a test finding, in the absence of any of the other listed criteria, does not constitute an adverse event.
- The test finding leads to a change in study drug dosing or discontinuation of subject participation in the clinical research study

The test finding is considered an adverse event by the investigator.

21.3 Causality and Severity Assessments

The investigator will promptly review documented adverse events and abnormal test findings to determine 1) if the abnormal test finding should be classified as an adverse event; 2) if there is a reasonable possibility that the adverse event was caused by the study drug(s) or device(s); and 3) if the adverse event meets the criteria for a serious adverse event.

If the investigator's final determination of causality is "unknown and of questionable relationship to the study drug(s) or device(s)", the adverse event will be classified as associated with the use of the study drug(s) or device(s) for reporting purposes. If the investigator's final determination of causality is "unknown but not related to the study drug(s) or device(s)", this determination and the rationale for the determination will be documented in the respective subject's case history.

21.4 Reporting of Adverse Reactions and Unanticipated Problems to the FDA

21.4.1 Written IND Safety Reports

The Sponsor-Investigator will submit a written IND Safety Report (i.e., completed FDA Form 3500A) to the responsible new drug review division of the FDA for any observed or volunteered adverse event that is determined to be a serious and unexpected, suspected adverse reaction. Each IND Safety Report will be prominently labeled, "IND Safety Report", and a copy will be provided to all participating investigators (if applicable) and sub-investigators.

Written IND Safety Reports will be submitted to the FDA as soon as possible and, in no event, later than 15 calendar days following the Sponsor-Investigator's receipt of the respective adverse event information and determination that it meets the respective criteria for reporting.

For each written IND Safety Report, the Sponsor-Investigator will identify all previously submitted IND Safety Reports that addressed a similar suspected adverse reaction experience and will provide an analysis of the significance of newly reported, suspected adverse reaction in light of the previous, similar report(s) or any other relevant information.

Relevant follow-up information to an IND Safety Report will be submitted to the applicable review division of the FDA as soon as the information is available and will be identified as such (i.e., "Follow-up IND Safety Report").

If the results of the Sponsor-Investigator's follow-up investigation show that an adverse event that was initially determined to not require a written IND Safety Report does, in fact, meet the requirements for reporting; the Sponsor-Investigator will submit a written IND Safety Report as soon as possible, but in no event later than 15 calendar days, after the determination was made.

21.4.2 Telephoned IND Safety Reports – Fatal or Life-threatening Suspected Adverse Reactions

In addition to the subsequent submission of a written IND Safety Report (i.e., completed FDA Form 3500A), the Sponsor-Investigator will notify the responsible review division of the FDA by telephone or facsimile transmission of any unexpected, fatal or life-threatening suspected adverse reaction.

The telephone or facsimile transmission of applicable IND Safety Reports will be made as soon as possible but in no event later than 7 calendar days after the Sponsor-Investigator's receipt of the respective adverse event information and determination that it meets the respective criteria for reporting

21.5 Reporting Adverse Reactions and Unanticipated Problems to the Responsible IRB

In accordance with applicable policies of The Pennsylvania State University Institutional Review Board (IRB), the investigator will report, to the IRB, any observed or reported harm (adverse event) experienced by a subject or other individual, which in the opinion of the investigator is determined to be (1) unexpected; and (2) probably related to the research procedures. Harms (adverse events) will be submitted to the IRB in accordance with the IRB policies and procedures.

21.6 Unblinding Procedures

At the end of our study, the CRC nurse (coordinator - Tracy) will provide a member of the Vascular Aging and Exercise Laboratory with a randomization sheet that has information about who received what supplement on each visit.

21.7 Stopping Rules

Not applicable.

22.0 Study Monitoring, Auditing and Inspecting

22.1 Study Monitoring Plan

22.1.1 Quality Assurance and Quality Control

The principal investigator, David Proctor, will be responsible for monitoring the conduct of the study. The study will be monitored on a weekly basis.

All subjects will be provided a consent form describing this study and provided sufficient information for subjects to make an informed decision about their participation in this study. Data will be collected only by members of the Vascular Aging and Exercise Laboratory and those individuals listed as co-investigators on the IRB. Any and all modifications to this protocol will be submitted to the IRB. Members of the Vascular Aging and Exercise research team will have access to the link associating the participants to the data, which will be stored in a secure file in 201 Noll Laboratory.

Confidentiality will be practiced with all data collected. Participants will be assigned an identitiy code that will be used to identify all data collected during the study. The identity code will only be known by study personnel and will not contain any information that can be used to identify the participant. Paper and computer forms of data will be stored within a specific study binder and on a dedicated research laboratory computer, respectively. The study binder is always stored within the laboratory and the laboratory door is locked outside of work hours. The research computer is located inside the laboratory and cannot be accessed unless a correct username and password is provided.

22.1.2 Safety Monitoring

The **Principal Investigator** will confirm that all adverse events (AE) are correctly entered into the AE case report forms by the coordinator; be available to answer any questions that the coordinators may have concerning AEs; and will notify the IRB, FDA, sponsor and/or DSMB of all applicable AEs as appropriate. All assessments of AEs will be made by a licensed medical professional who is an investigator on the research.

The **research coordinator** will complete the appropriate report form and logs; assist the PI to prepare reports and notify the IRB, FDA, and/or DSMB of all Unanticipated Problems/SAE's.

The **Monitor** will confirm that the AEs are correctly entered into the case report forms. The Monitor will confirm that the adverse events are consistent with the source documents and are reported to the appropriate regulatory bodies, as required.

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24.0 Appendix