Nitrites, Skeletal Muscle Mitochondrial Bioenergetics, and Physical Activity in Old Age

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PROTOCOL SYNOPSIS

Protocol Title:	**Nitrites, Skeletal Muscle Mitochondrial Bioenergetics, and Physical Activity in Old Age**
Protocol Number:	**STUDY20110334**
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Clinical Phase:	**Phase II Clinical Investigation**
Investigational Drugs:	**Nitrogen 14 Sodium Nitrite (^{14}N Sodium Nitrite)**
Trial Site:	**University of Pittsburgh Single-Center Randomized Trial**
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Study Rationale:	Old age is associated with declining skeletal muscle mitochondrial bioenergetics with related decrements in cardiorespiratory fitness (CRF) and physical function[1] that predispose to frailty, disability, and diminished quality of life.[2] While exercise training may moderate and possibly even reverse declines[3] in mitochondrial bioenergetics,[4] and bring about clinical benefit, the potential for such improvement is typically confounded by exercise intolerance with early fatigability that results from the same age-related mitochondrial declines.[5] Consequently, sedentariness is endemic and insidious among the growing population of older adults.[6] The principal rationale for the trial is innovative as it is oriented to sedentariness and related fatigability, and a pharmacological means to moderate them. It targets a mechanistic underpinning to fatigability as a symptom related to limitations in mitochondrial bioenergetics and vascular function and explores the utility of nitrite supplements as a novel means to improve the underlying physiology and clinical correlates.

Study Objectives:	To assess the impact of nitrite supplements on mitochondrial respiration, with associated focus on mitochondrial energetics and muscle perfusion, and to complete preliminary analysis on clinical correlates.
Study Hypotheses:	• We hypothesize that nitrite will increase skeletal muscle mitochondrial energetics. • We hypothesize that improved skeletal muscle mitochondrial respiration will relate to both direct enhancement of mitochondrial respiration as well as improved skeletal muscle perfusion. • We hypothesize that changes in skeletal muscle mitochondrial respiration will correspond to improved CRF, reduced fatigability and improved PA.
Study Aims:	**Aim 1:** To show efficacy of 12-weeks of sodium nitrite treatment to improve mitochondrial energetics. **Aim 2:** To show efficacy of 12-weeks of sodium nitrite treatment to improve skeletal muscle perfusion. **Aim 3:** To explore the effects of nitrite-induced changes in mitochondrial energetics on CRF and metrics of clinical function. Overall, this protocol lays a mechanistically-oriented foundation for future studies to definitively assess the utility of nitrite treatment as an important therapeutic option for the growing burden of sedentariness in old age
Study Design:	Prospective, randomized, placebo-controlled, double-blinded trial at 2 clinical sites to study the utility of a 12-week intervention (oral nitrite capsules vs. placebo) in older (age ≥70 years) healthy, sedentary adults (<1 hour of structured PA per week).
Planned Sample Size:	70 participants total
Duration of Treatment:	14 weeks (12 weeks of Intervention with 2 weeks of Post-intervention testing)
Major Inclusion Criteria:	• Age ≥70 years • Sedentary (<1 hour/week of volitional exercise activity) • Clinically stable (euvolemic; baseline HR <100 bpm) and without hospitalization or invasive cardiac procedure for 6 weeks
Major Exclusion Criteria:	**See complete list in section 3.3**

Study Endpoints:	Overall, study endpoints pre- and post- nitrites will include: 1. **Aim 1**: Mitochondrial energetics: ex vivo assessments of permeabilized skeletal muscle fiber respiration with Oroboros (primary outcome) and in vivo assessments of skeletal muscle ATP production with phosphorus-magnetic resonance spectroscopy (^{31}P-MRS). 2. **Aim 2:** Skeletal muscle perfusion: near infrared spectroscopy (NIRS) to oxygenation and perfusion**.** 3. **Aim 3**: Exploratory assessment of clinical correlates. Symptom-limited (maximal) CPET will be used to assess CRF (measured as peak oxygen consumption [VO_2]).[7] Submaximal CPET (resistance which corresponds to 60%-80% peak VO_2) to exhaustion to assess endurance. Steady-state walking test on a level treadmill (with gas exchange) to assess walking efficiency and fatigability.[7,8] Other functional measures include 400-m corridor walk, short physical performance battery (SPPB), handgrip strength, and daily physical activity (PA). [7,8]

1. STUDY OBJECTIVES, SPECIFIC AIMS, BACKGROUND, AND SIGNIFICANCE

1. STUDY OBJECTIVES

PRIMARY OBJECTIVE: To assess the impact of nitrite administration on mitochondrial respiration, with associated focus on mitochondrial bioenergetics and muscle perfusion.

1.2 SPECIFIC AIMS

Two-site randomized controlled trial to clarify the impact of a 12-week course of nitrite supplements versus placebo on mitochondrial energetics in older sedentary adults. We will take an integrative physiology approach to determine the effect of nitrite therapy on a comprehensive assessment of mitochondrial energetics, skeletal muscle vascular function, and whole-body physical function (cardiorespiratory fitness [CRF], exercise endurance, strength, balance, and daily physical activity [PA]) and fatigability.

Aim 1: To show efficacy of 12 weeks of sodium nitrite treatment to improve mitochondrial energetics.

- We hypothesize that compared to placebo, the participants receiving nitrite supplements will show an improvement in:

  H1a: Skeletal muscle mitochondrial respiration, efficiency (high resolution respirometry [primary outcome]) and adenosine triphosphate (ATP) production (phosphorus-magnetic resonance spectroscopy)

  H1b. Morphology and molecular underpinnings of muscle energetics (Direct Immunofluorescence and light microscopy of mitochondrial structure, RNA-Seq and Western Blot)

H1c: Platelet mitochondrial respiration (Seahorse), i.e., an analysis to clarify whether platelets can be used as a surrogate to skeletal muscle to assess equivalent changes in mitochondrial bioenergetics

Aim 2: To show efficacy of 12 weeks of sodium nitrite supplements to improve skeletal muscle perfusion.

- We hypothesize that, compared to placebo, the nitrite group will show a greater improvement in:

  H2a: Tissue oxygenation, as measured using near infrared spectroscopy (NIRS) during exercise.

Aim 3: To explore the effects of nitrite-induced changes in mitochondrial energetics on CRF and metrics of clinical function.

- We hypothesize that, compared to placebo, the participants receiving nitrite supplements will show a greater improvement in:

  H3a: Increased CRF (peak oxygen uptake [VO_2] achieved during progressive resistance on a cycle ergometer), increased endurance (duration of exercise at fixed resistance 60%-80% peak VO_2 intensity), increased efficiency of walking (decreased VO_2 at steady-state [1.5 mph] and self-paced walking), increased 400-meter corridor walk, increased SPPB score; increased handgrip strength, increased PA (accelerometry), and decreased fatigability (during steady-state walking and 400-meter corridor walk respectively).

  H3b: Changes in mitochondrial energetics and skeletal muscle perfusion induced by nitrite will be associated with changes in daily activity (measured by accelerometry), and fatigability (measured by rate of perceived exertion during steady-state [1.5 mph] walking).

1.3. BACKGROUND AND RATIONALE

Our multi-disciplinary team has published seminal work indicating that mitochondrial bioenergetics and CRF are significant determinants of physical function in older adults.[9] In parallel efforts, we showed efficacy of chronic nitrite supplements to improve mitochondrial bioenergetics in older sedentary adults. Only one month of nitrite supplements significantly improved ex vivo assessments of mitochondrial energetics in skeletal muscle biopsies, concomitant with increased skeletal muscle sirtuin-3 expression, a nicotinamide adenine dinucleotide (NAD) dependent lysine deacetylase and key regulator of mitochondrial metabolism. These key data reinforce the premise that nitrite enhances vital mitochondrial metabolism in older adults. Moreover, improvement in muscle energetics in nitrite-treated older adults was linked with increased exercise efficiency as evidenced by reduced VO_2 during submaximal steady-state walking. These data support our overarching hypothesis that nitrite supplements will make physical function easier such that PA will increase.

Older adults represent the fastest growing demographic in the population of the United States and constitute a unique and growing healthcare challenge.[10] Aging is associated with an inexorable decline in CRF[11] and decreased PA[12] with harmful implications[13] that include increased vulnerabilities to disease, frailty, reduced independence, diminished quality of life as well as escalating healthcare costs. Yet despite typical age-related declines, many older adults are able to mitigate the rate of decline in fitness and activity, and to thereby better preserve healthfulness and well-being.[14] While behavioral and community interventions are often pursued as vital strategies to optimize activity and health, for many older adults such good intentions are stymied by intrinsic age-related exercise intolerance and

early fatigability[15] that undermine goals to be active. Our protocol responds to such challenges by focusing on the utility of nitrite supplementation to enhance skeletal muscle mitochondrial energetics in older sedentary adults. We will build on our exciting preliminary data to clarify if mitochondrial benefits extend to improved physical function, including CRF, endurance, strength, balance, and PA.

1.4 SIGNIFICANCE

The protocol is conceptually innovative in multiple aspects:

- The principal rationale for the trial is innovative as it targets mechanistic underpinnings, i.e. mitochondrial energetics and vascular function, as integral means to foster physical function, PA, and to diminish fatigability.
- The application of oral nitrite supplementation is innovative. While seminal literature regarding nitrite focuses predominantly on vascular effects,[30] our pilot data raise important perspectives regarding its intrinsic effects on skeletal muscle mitochondria, which are the source of over 90% of adenosine triphosphate (ATP) needed for movement. Age-related changes in skeletal muscle mitochondrial function likely contribute to functional decline.[1,22,31-34]
- We will use high-resolution respirometry to examine the activity of electron transport system (ETS) in permeabilized intact muscle fibers from freshly collected muscle specimens. This allows us to assay ETS function in a highly controlled ex vivo experiment at the myocellular level, removed from other potentially limiting physiological factors including supplies of substrates and oxygen. Specifically, we will use an Oroboros Oxygraph 2k[35,36] to interrogate mitochondrial ETS function when reducing equivalents are channeled via complex I, complex II or electron transfer flavoprotein (fatty acids). This method preserves mitochondrial reticulum structure and function, which avoids erroneous mitochondrial dysfunction observed in isolated mitochondria[37,38] and will complement in vivo assessment of mitochondria.
- Mitochondrial energetic capacity (ATPmax) will be determined by (phosphorus-magnetic resonance spectroscopy [^{31}P-MRS]) of the non-dominant quadriceps muscle. ATPmax incorporates all aspects of mitochondrial content and function including oxygen consumption, efficiency, and Ca^{2+} handling, all working together to produce ATP.[39]
- The molecular underpinnings of improved skeletal muscle mitochondrial energetics will be determined via transcriptional RNA transcriptionomic profiling (RNA-seq), expression of key proteins (Western blots), and immunohistology (fiber type, capillary density).
- The skeletal muscle mitochondrial assessments will be coupled with an analysis of platelet mitochondrial energetics (Seahorse) to determine the utility of platelet measurements to be applied as (1) a novel and convenient biomarker of mitochondrial energetics.
- NIRS will be used to assess: (1) skeletal muscle mitochondrial metabolic performance profile during the hypoxic stress[40] by gauging perfusion changes pre- and 12-week post-nitrite versus placebo. Clinical and Physiological assessments:

  Fitness and Efficiency (1) CRF (peak VO_2) on a progressive intensity cycle ergometer will clarify how changes in mitochondrial respirometry translate into systemic oxygen utilization, highlighting physiological linkage between cellular respiration and whole-body oxygen utilization and putative benefits of nitrite to improve these relationships; (2) VO_2 at 60%-80% exercise resistance (watts) on a cycle ergometer until exhaustion will assess nitrite-medicated changes in endurance[41,42]; (3) VO_2

at a steady-state walking (1.5 mph) will assess nitrite-mediated changes in the efficiency of walking and associated perceived fatigability (changes in rated of perceived exertion); (4) 400-meter corridor walk (with portable gas exchange) will be used to assess overall capacity (disability), physical fatigability (slowing of walking speed), as well as the energy of walking.

Additional functional measures: (1) short physical performance battery (SPPB) to assess strength, balance, and an index of frail[7]; (2) handgrip to assess strength; (3) daily activity (accelerometry).

Fatigability: Fatigability assessments are novel, and include both "perceived" (rate of perceived exertion [RPE])[43] during steady-state walking[8], as well as "performance" (energy consumed during a 400-meter corridor walk)[44] subtypes.

2. RESEARCH DESIGN AND METHODS

2.1 CLASSIFICATION AND METHODOLOGICAL DESIGNS

We will conduct a prospective, randomized, placebo-controlled, double-blinded trial to study the utility of a 12-week intervention (oral nitrite capsules vs. placebo) in older (age ≥70 years) healthy, sedentary adults (<1 hour of structured PA per week). The primary aims of this protocol are to demonstrate the utility of chronic nitrite treatment to enhance (1) mitochondrial energetics, (2) vascular perfusion and (3) to explore whether physiological improvements translate to clinical enhancements (physical function, physical activity, fatigability).

2.2 DETAILED DESCRIPTION OF STUDY DESIGN

Study Procedures and Visits:

Study Visits and procedures will take place in the UPMC Magnetic Resonance Research Center (MRRC), UPMC Montefiore Translational Research Center (TRC), University of Pittsburgh Geriatric Research Center (GRC), or UPMC Kaufmann at the University of Pittsburgh Pepper Center– Senior Mobility Aging Research Training (SMART) Center and UPMC Emphysema/COPD Research Center.

Water and snacks will be available to participants throughout non-fasting visits. Participants will be provided a meal during fasting visits

Reminder Calls:

Study staff will call participants to remind them of study visit details, and of medications hold plans. The study staff will remind participants to hold medications per their anti-platelet and anti-coagulation medications hold plan at reminder calls for Visit 4 and Visit 9. Participants with diabetes who are on hypoglycemic medications will be advised if they need adjustments to their usual regimen for fasting and functional testing visits. Participants with diabetes will be asked to bring their own glucometer to assess glucose as needed. If a participant does not provide their own glucometer, a study glucometer will be available.

2.2.1 Study Procedures and Assessments

Consent Visit

1. Obtain written informed consent using standardized procedures.

2. Medical histories will be obtained prior to the visit. At the study visit, details about the participants' medical histories will be reviewed. PI or a study clinician will evaluate the participants. Participants will be instructed to notify the study staff if there are any health or medication changes during their participation in the study. The Charlson Comorbidity Index and the Pittsburgh Fatigability Scale will be administered.
3. Weight, height, and vital signs (temperature, blood pressure (BP), heart rate (HR), respiratory rate (RR), SpO2). BP will be re-measured if it is initially outside of the inclusion range. This allows for common clinical variations attributable to anxiety, dehydration, timing of medications, and other factors.
4. Screening blood work by trained study staff will be completed be completed after consent and before randomization. See Study Timeline 2.2.2

5 ***7-Day Home Activity Monitoring***: Participants will be issued an accelerometer (ActiGraph) non-invasive wrist monitoring device to be worn for a full 7 days following visit to assess changes in daily activity, including level/intensity of activity and sleep. Serial assessments by ActiGraph over the weeks of the protocol provide key data to gauge changes in physical activity that may result from nitrite supplements over time. Participants will receive instruction in the use of the ActiGraphs and asked to maintain an ActiGraph physical activity/sleep diary for the days that they are wearing the device. Participants will be reminded to return their ActiGraph at the visit following their 7-day wear.

Pre-drug baseline study visits (Visits 1- 4)

Visit 1

Participants will be encouraged to have a light meal prior to arrival.

1. Interval history, vital signs, and a brief physical exam.
2. Exercise testing:
    a. Cardiopulmonary Exercise Testing (CPET)[46]: A symptom-limited (maximal) graded cycle ergometer exercise test in association with air-gas-exchange, an optimal gauge of aerobic capacity will be conducted by trained research exercise physiologists. A study clinician will be immediately available if needed. A computerized cycle ergometer will be used to generate a symptom-limited exercise stimulus. ACSM criteria for starting and stopping will also be utilized. A face mask or mouthpiece will be positioned over the subject's mouth and nose during the exercise for gas exchange assessments. VO_2, minute ventilation (VE)/exhaled carbon dioxide (VCO_2) slope, and respiratory exchange ratio (RER) will be measured as well as hemodynamics (max HR and BP), time, and ECG waveforms). Any unexpected abnormalities will be reported to the participant's primary provider(s); continued participation in the study will require the primary provider's clearance. The rate of perceived exertion (RPE) will be completed during the CPET.
    b. 400-meter corridor walk[8]: Participants will walk over a 400-meter course. During the 400-meter corridor walk, average VO_2 per lap during steady-state

(2 minutes of plateaued VO_2) will be used to calculate the energy cost of walking by normalizing the VO_2 to lap gait speed (ml/kg/m).

c. For participants with diabetes, they will be asked to bring their own glucometer to assess glucose before and after the CPET.

d. NIRS: NIRS will be worn during the CPET and 400-meter corridor walk. Sensors will be placed on the skin of one leg to allow the non-ionizing, LED light source to assess tissue oxygenation and perfusion via measurements of the optical absorption of hemoglobin.

e. Accelerometry: An ActiGraph wrist accelerometer will be worn during the 400-meter corridor walk to establish baseline activity parameters and during the CPET.

3. A battery of questionnaires will be administered.
    a. The following questionnaires will be administered:
    b. Community Healthy Activities Program for Seniors (CHAMPS)[48]
    c. Fried Frailty Index[50]
    d. EuroQol[49]
    e. Montreal Cognitive Assessment[51,22]
    f. Patient Health Questionnaire (PHQ-2)[52]
    g. PROMIS-Sleep Disturbance Short Form (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3261577/)
    h. Self-efficacy: Sullivan[54]

4. Participants who fail to meet applicable inclusion/exclusion criteria based upon the results of the screening assessment will be excluded from further study participation. Such participants will be paid for the visit.
5. Prior to departing, participants will be reminded to resume their usual medication regimen and clarify any modifications related to the study if indicated.

Visit 2

Participants will be encouraged to eat a light breakfast prior to arrival.

1. Interval history, vital signs, and a brief physical exam.
2. Steady-State Walking Test: A steady-state walking (1.5 mph) test will be completed on a treadmill. Participants will wear a portable CPET device during the steady-state walk. The rate of perceived exertion (RPE) will be completed during the steady-state walking test.
3. Submaximal CPET: the participant will pedal on a cycle ergometer with a fixed resistance (the resistance associated with 60%-80% peak VO_2 achieved during the initial symptom-limited CPET on visit one). The rate of perceived exertion (RPE) will be completed during the submaximal CPET.
4. Handgrip Strength Assessment
5. Short Physical Performance Battery (SPPB) will be completed:
    a. Sitting and standing out of a chair

b. Walking over a 4-meter course (to measure walking speed)

c. Standing with their feet in different positions to measure their balance

6. NIRS will be worn during the submaximal CPET. NIRS and ActiGraph will be worn during the steady-state walk.
7. Standard breath-by-breath CPET ventilatory indices VO_2, VE/VCO_2, and RER will be collected and used to characterize the performance during the submaximal CPET as well as the steady-state treadmill walk completed in this study visit.
8. Three-day food record (3DFR): participants will be provided forms to complete a 3DFR which will be analyzed for plant-based dietary nitrite and nitrate. Participants will be assigned dates for these self-assessments, including one weekend day. Participants will be asked to maintain a diet that is consistent throughout the trial. Major dietary modifications during the 16-week trial will be discouraged.
9. Prior to departing, we will remind diabetic participants to resume their usual medication regimen and clarify any modifications related to the study if indicated.

Visit 3

Participants will be encouraged to eat a light breakfast prior to arrival.

1. Vital signs will be completed by study or MRRC staff. Any concern for medical instability after collecting the vital signs and the interval history will prompt contact with a study clinician, and possible physical exam before proceeding.
2. Magnetic Resonance Spectroscopy (MRS):

   MRS is an in-vivo test to measure maximal mitochondrial capacity to generate ATP. It will be performed on the subject's leg as they complete a flexion-extension exercise within the bore of the magnet as described below.

   Prior to the exercise, the technician will collect a fully relaxed, high-resolution 31P spectrum image of the resting muscle.

   The exercise protocol will then be performed: Participants lay supine with the non-dominant knee (unless contraindicated) elevated at ~30º and straps are placed over the legs. Participants perform voluntary isometric contractions (kicking) repeatedly as hard and as fast as they can for two bouts each followed by a rest period. The protocol is designed to deplete PCR stores by 33-66% to ensure high signal to noise defining PCR recovery without inducing acidosis (pH <6.8), which inhibits oxidative phosphorylation.
3. Participant may complete any remaining functional testing from Visit(s) 1 or 2. Visit 3 and Visit 8 will not occur if the Subject is ineligible for the MRS.

Visit 4

Participants will be asked to fast for at least 8 hours prior to arrival.

Participants will be asked to hold their medications per the Anti-coagulation Medications Hold Plan.

1. Interval history, vital signs, and a brief physical exam

2. Staff will obtain verbal confirmation from participants of fasting for at least 8 hours prior to arrival and that Anti-coagulation Medications Hold Plan was followed.
3. An IV line will be placed
4. Skeletal Muscle Biopsy #1: Participants will lie comfortably in the supine position for biopsy of the vastus lateralis muscle of the non-dominant leg. Biopsy sites will be disinfected with chlorhexidine, lidocaine will then be administered to biopsy site. The physician investigator or study clinician will then make a small incision in the skin and insert a needle into the vastus lateralis to remove approximately 150 mg of muscle. Additional passes of the needle may be necessary for an adequate muscle sample. The wound site will be closed with Steri-strips™ (or a suture if subject is allergic to Steri-strips™) and a sterile pressure bandage.
    a. Muscle specimens will be immediately processed and stored. Muscle specimens will be divided into portions for specific analyses. Any extra tissue will be stored for future analyses.
    b. The biopsy wound will be compressed and dressed.
5. Randomization: Subject will be randomized following review of the participant safety status by PI.
6. Pharmacokinetics (pK)
    a. Nursing staff will measure BP, RR, HR, SpO_2, and MetHb to ensure subject is at a steady-state before administering the study drug. Baseline assessments of nitrite/nitrate will be collected. Participants will receive their first dose of study drug (20 mg Nitrite vs. Placebo). Then, the following will be assessed: BP(orthostatic hypotension assessment), HR and MetHb, RR and SpO_2 every 15 minutes for the 2 hours post-drug administration period per standard operating procedure. If high MetHb level or if subject is hypotensive or symptomatic, after the study drug is administered, the PI or study clinician will be informed and advise on plan for management.
7. Blood assessments:
    a. Nitrite/Nitrate Serum Levels and other Research Bloods: baseline (before study drug), 30 minutes, 60 minutes
    b. Platelet Mitochondrial Respirometry: baseline (before study drug), 30 minutes
    c. Research Inflammatory Indices: baseline (before study drug)
    d. Heparinized blood will be immediately centrifuged at bedside to separate plasma and red cells and then flash frozen for future analyses. Total volume of blood drawn for 3 samples will be about 80 ml (about 5.4 tablespoons).
8. Skeletal Muscle Biopsy #2 immediately after the 30-minute blood sample is drawn.
9. A breakfast will be provided after the skeletal muscle biopsy #2.
10. If the study drug is well tolerated, the PI will be contacted and IDS orders for enough study drug/placebo will be ordered to sufficiently cover dosing for participants as described in drug distribution section.
11. Drug supply dispersal

a. Participants will receive sufficient study drug/placebo supply. We will instruct participants to return all of the study drug packaging, including unused study drug and empty packaging at each study visit and/or at the time of discontinuation from treatment.

12. Study staff will review medication administration and use of the drug diary card with all participants. Participants will be asked to record the time they take the drug each day and any symptoms with severity rating and/or health events during the dosing period.
13. The study staff will review biopsy site care with participants and if indicated, remind participants to resume their normal anti-coagulation/anti-platelet/anti-diabetic medications.
14. Participants will be called 2 days after biopsy for assessment of biopsy healing. All participants will be given the option of returning for an Interim Visit for direct assessment if symptomatic.
15. Daily activity: Participants will be encouraged to begin or extend a daily walking routine with specific instructions and encouragement to progress in time and length as tolerated.

In order to track these changes over the course of the 12-week intervention, participants will be asked general questions about activity level, exertion level, and barriers to activity. Furthermore, the study nurse will ask about any progress as part of the interim study on-site and phone-based assessments.

The Daily Activity questions are not a study endpoint, but rather they are pragmatic tools to encourage motivation and behaviors of activity. Only the ActiGraph data are study endpoints.

Midway Drug Intervention Assessment (Visit 5)

Visit 5 (Midway): On-site assessment to assess safety and abbreviated functional assessment during the 12-week intervention phase. Participants will be asked to fast for at least 8 hours prior to arrival.

The following procedures and assessments will be completed:

1. Interval history, vital signs, and a brief physical exam.
2. IV line will be placed
3. Dose Monitoring:
    a. Subject will take morning dose of study drug
    b. Research Blood assessments at baseline (before study drug), 30 minutes, 60 minutes:
        i. Nitrite/Nitrate Serum Levels and other Research Bloods: baseline, 30 minutes, 60 minutes
        ii. Platelet Mitochondrial Respirometry: baseline (before study drug), 30 minutes
        iii. Research Inflammatory Indices: baseline (before study drug)
        iv. CBC (for safety assessment)
4. pK assessments as in Visit 4 with orthostatic hypotension assessment Blood Pressures.
5. Functional evaluation:
    a. Participants will complete the following:

i. Steady-state walk, 400-meter corridor walk (with VO_2 assessment), SPPB, Handgrip Strength
ii. Participants will complete the Steady-state walk test 30minutes(+/-10 minutes from the 30 minute blood collection)

6. Additional study drug capsules distributed if needed
7. A breakfast will be provided after the 60-minute blood draw
8. 7-Day Home Activity Monitoring

Post-Drug Assessments (Visits 6-9)

The procedures and assessments are generally similar to those performed Pre-drug baseline study visits.

Visit 6

Participants will be encouraged to have a light meal before arrival. Participants will be asked to hold their AM study drug prior to arrival.

See Visit 1 for details.

Study staff will update concomitant medication list and complete a symptom assessment. The drug diary cards and empty drug boxes for completed weeks on study drug/placebo will be collected. Compliance of the study drug will be reviewed.

1. Interval History, vital signs, and a brief physical exam.
2. The participant will take their study drug 30 minutes before the CPET.
3. Cycle Ergometer symptom-limited CPET
4. 400-meter corridor walk
5. Questionnaires
6. Prior to departing, we will remind diabetic participants to resume their usual medication regimen and clarify any modifications related to the study.
7. Blood work collected by trained study staff. (See Study Timeline 2.2.2)
8. Prior to departing, we will remind diabetic participants to resume their usual medication regimen and clarify any modifications related to the study if indicated.

Visit 7

Participants will be encouraged to eat a light breakfast prior to arrival. The participants will be asked to take their study drug 30 minutes prior to the submaximal CPET.

Interval history, vital signs, and a brief physical exam.

See visit 2 for details.

1. Steady-State Walking Test
2. Submaximal CPET
3. Handgrip Strength Assessment.

4. SPPB
5. 3DFR will be provided to subject and collected at following visit.
6. Prior to departing, we will remind diabetic participants to resume their usual medication regimen and clarify any modifications related to the study if indicated.

Visit 8

Participants will be encouraged to eat a light breakfast prior to arrival. The participant will be asked to hold the AM dose of the study drug prior to arrival. The participant will take the study drug ~30 minutes prior to start of the MRS.

See Visit 3 details.

1. MRRC Staff will collect vital signs. Any concern for medical instability after collecting the vital signs and the interval history will prompt contact with a study clinician, and possible physical exam before proceeding.
2. MRS
3. Participant may complete any remaining functional testing from Visit(s) 6 or 7
4. Participant will take the afternoon dose of study drug 30 minutes prior to completing any remaining functional testing from visit 7.

Visit 9

Participants will be asked to fast for at least 8 hours prior to arrival. Participants will be asked to hold their AM dose of the study drug prior to arrival.

See Visit 4 for details.

1. Interval history, vital signs, and a brief physical exam.
2. Staff will also confirm participant has been fasting for at least 8 hours prior to arrival and that any customary anti-platelet or anti-coagulation medications were held as instructed.
3. An IV line will be placed
4. Skeletal Muscle Biopsy #1
5. Participant will self-administer AM dose of study drug.
6. Research Blood assessments at baseline (before study drug), 30 minutes, 60 minutes, as per Pre drug biopsy visit.
7. Skeletal Muscle Biopsy #2 immediately after the 30-minute blood sample is drawn.
8. Pharmacokinetics (pK) with orthostatic hypotension assessment Blood Pressures every 15 minutes as noted in Visit 4.
9. A breakfast will be provided after the second skeletal muscle biopsy.
10. Remaining study drug/placebo, 3DFR, daily activity questions, drug diary cards, and/or Actigraphy and diary will be collected from subject prior to discharge.
11. Participants will be called 2 days after biopsy for assessment of biopsy healing. All participants will be given the option of returning for an Interim Visit if they prefer a direct assessment.

12. The study staff will review biopsy site care with participants and if indicated, remind participants to resume their normal anti-coagulation/anti-platelet/anti-diabetic medications
13. Following safety monitoring the study staff will schedule final follow up phone call for 1 week after visit 9.

Weekly Telephone Calls

Following Visit 4, the study staff will contact participants by phone each week to review any symptoms. Staff will continue to call weekly until 1 week after visit 9. Each call will last approximately 5-10 minutes. We will ask participants if they have missed any study drug doses and if there have been any changes in their medications or medical history, including emergency room visits or hospitalizations since their study visit. We will ask participants to review their drug diary card to report what they wrote down for the week to help them remember.

We will also remind participants to contact the study staff or doctor anytime their health care team suggests a change in medications or if they have any symptoms that cause concern. There will be study personnel available to address participant's questions or concerns 24 hours/7 days a week**.**

During the drug intervention period, participants will also be asked about their activity levels, exertion levels, activity enjoyment, and barriers to activity as part of the weekly calls.

2.2.2 Study Timetable

Study Phase	Pre-Drug Assessments					Midway Assess-ment	Post-Drug Assessments			
	Con-sent Visit	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9
Approximate Time between Study Visits		Same day to +30 days	Visit 1 +1 day to ±14days	Same day to +7 days	Previ-ous Visit +1 day to±14days	6 weeks ±5 days	6 weeks ±5 days	Visit 6 days ±14 days	Same day to +7 days	Previous Visit to ±14days
Approximate Duration of each visit	1-2 hours	3-5 hours	3-4 hours	1-2 hours	3-4 hours	1-2 hours	3-4 hours	3-4 hours	1-2 hours	3-4 hours
Fasting prior to arrival					X	X				X
Consent	X									
Medical History	X									
Physical exam	X	X	X	X[+*]	X	X	X	X	X[+*]	X
Vital Signs	X	X	X	X						
Vital Signs with Methemoglobin					X	X	X	X	X	X
Max CPET*		X					X			
Sub-Max CPET*			X					X		
400-Meter Corri-dor Walk*#		X				X	X			
Steady-state walk*#			X			X		X		
SPPB			X			X		X		
Handgrip			X			X		X		
Home Actigraph	X	*					X			
Questionnaires	X	X					X			

3 Day Food Record (3DFR)++			X					X		
MRS				X					X	
Randomization					X					
Muscle Biopsies 1 & 2					X					X
Blood Draw Approximate total quantity	**20 ml+**				**83 ml**	**81 ml**	**20 mL**			**81 ml**
CBC	**X+**					X	X			
Complete Metabolic Panel (CMP)	**X+**						X			
25-hydroxy vitamin d (Vitamin D)	**X+**						X			
HbA1c	**X+**						X			
TSH	**X+**									
Nitrite/Nitrate					X	X				X
Inflammatory Indices					X	X				X
Additional Research Bloods					X	X				X

***Assessment with NIRS**

#Assessment with ActiGraph

+ Blood draw may be collected after signing consent and before randomization, blood draw may not include CMP and/or CBC/d if collected within the last 4 weeks

++ 3 Day Food Record distributed at Visit 1 and Visit 6 and collected from subject at Visit 2 and Visit 7

+* If deemed necessary

2.3 STUDY DRUG

Dr. Gladwin has developed nitrite capsules for oral dosing prepared using 14-nitrogen formulation in collaboration with the University of Iowa's drug development program and FDA approved under IND 115,926. Greater than 99.9% of naturally occurring nitrite exists as 14N nitrite.

2.3.1. Study Drug Preparation and Dispensing

The institutional Investigational Drug Service (IDS) pharmacy at the University of Pittsburgh will select study drug/placebo by the randomization sequence number. The independent research pharmacist at IDS will package all drugs/placebos to be similar in physical appearance. The study drugs are oral formulation of 20 mg sodium nitrite and matching placebo. Sodium nitrite of dose strengths and matching placebo will be supplied as capsules for oral administration. All capsule formulations will be identical in appearance (size, shape, color) and smell. The packaging and labeling will be designed to maintain blinding to the Investigator's team and to participants.

2.3.2. Drug Administration

IDS will be provided an updated order for dispensing drug/placebo at baseline and PRN. Participant will be instructed to bring remaining supply to follow up visit for interim monitoring counts.

2.3.3. Dose Selection

In the 2001 National Toxicology Program (NTP) Report summarizing 2-year rodent drinking water studies, there was no evidence of carcinogenic activity of in male or female F344/N rats exposed to up to 130 mg/kg/day in males and 150 mg/kg/day in females, or in male B6C3F1 mice exposed to up to 220 mg/kg/day. There was equivocal evidence of carcinogenic activity of sodium nitrite in the highest dose of 165 mg/kg/day in female B6C3F1 mice based on the positive trend in the incidences of squamous cell papilloma or carcinoma (combined) of the forestomach. Exposure to sodium nitrite in drinking water resulted in increased incidences of epithelial hyperplasia in the forestomach. However, no chromosomal damage (genetic toxicity) was observed in three studies conducted in rats and mice in vivo.[55] Taken together; these findings suggest minimal carcinogenic nitrite-mediated risk.

Multiple studies now demonstrate the predominantly positive safety profile of nitrate therapy in humans (most typically administered as beetroot juice).[56] At the University of Pittsburgh, several pilot studies show safety and efficacy of nitrite, the active metabolite of nitrate, and show strong physiological rationale for its use.

Kara Hughan is a co-investigator on this protocol. She has been studying utility of oral nitrite supplements (administered as capsules) to improve insulin sensitivity over 12 weeks in adults aged 18-60 years with metabolic syndrome in an NHLBI K23 funded study. Hughan's first subject (healthy) was started on nitrate 1,000 mg once daily (with NO3 supplying a more prolonged source of plasma nitrite across the day via enterosalivary circulation until next dosing) in combination with nitrite 20 mg once daily.

During the first subject's drug course, PK plasma nitrate and nitrite concentrations, MetHb and BP data were assessed every other week. Given that only minor increases in MetHb were de-

tected (that remained well within the safety threshold) and that the plasma nitrite rise after ingestion then fell to baseline after ~3-6 hours with nitrite dosing, requests were made to the IRB to increase nitrite dosing to 20 mg two times daily (bid) this does not pose undue risk to participants.

2.3.4. Treatment Period

Participants will undergo study drug treatment duration of up to 16 weeks of nitrite vs. placebo three times daily; study drug during the 12 week intervention period and over the 2-4 week post intervention period. The two to four additional weeks during post intervention period are included to:

- allow continuation of the study drug/placebo through the final outcome collection series of visits
- Range of two to four post intervention weeks accounts for minimum length of time between Visits 6 and Visits 9 and the maximum length of time allowed between Visit 6 through Visit 9.

This is considered appropriate to study benefits of oral nitrite therapy.

2.3.5. Breaking the Blind

All participants and study personnel (except the study statistician, the dedicated providers, our safety officer and the study adjudicator) involved in assessments will be blind to treatment assignment.

2.3.6. Medication Compliance

At Visit 4, participants will self-administer their first dose of study medication under the supervision of the physician investigator or study clinician in the outpatient facility following completion of all baseline clinic assessments. After the pK (and participant is stable after first dose), participants will be dispensed their study medication for home use in prefilled seven-day labelled pill boxes to help with compliance and to help reduce burden or confusion. On the weekly phone calls, the study coordinator will review the daily diary card (study drug and symptoms), inquire about missed doses and review symptom assessments. Participants will return empty pill boxes when they return for study visits. Medication adherence will be reviewed. Participants will return any remaining capsules at their Visit 9 for final accountability. In the event that the adherence rate is <80% at any time reported by the subject, participants will be re-educated on medication adherence. If medication compliance repeatedly falls outside of the acceptable range, the study investigators will discuss subject eligibility for continued participation in the study. Of note, adherence during Dr. Forman's pilot study was excellent with no subject approaching the 80% level of concern.

2.3.7. Medication Storage and Accountability

The study investigators or the study coordinator will document the amount of study drug/placebo dispensed and/or administered to participants, the amount returned by participants, and the amount received from and returned to the UPMC-IDS. The study drug/placebo accountability records will be maintained throughout the course of the clinical trial.

2.3.8 Concomitant Medications

Potential participants will be asked during phone screening if prescribed medication on the exclusion criteria list. Potential participants will be asked to provide a list of concomitant medications at screening Visit 1. All candidates using vasoactive medications, unable to withhold warfarin (or use bridging therapy) prior to muscle biopsy, or unable to withhold thienopyridine medications for 5 days prior to muscle biopsy will be excluded. Participants taking daily aspirin, nonsteroidal anti-inflammatory medications, or direct oral anticoagulants will be asked to hold 3

days prior to biopsy. If unable to hold aspirin, PI/study clinician will hold pressure after biopsy to prevent further bleeding for an additional 3 minutes.

2.3.9 Rescue Medications

Not applicable

2.3.10 Randomization and Blinding:

We will use the high quality pseudo-random deviate generator in SAS® (SAS Institute, Inc., Cary, North Carolina) to randomize participants to NO or placebo in 1:1 ratio. Because of the moderately large sample size and the resulting opportunity for participant characteristic imbalance between the arms and the threat it would pose to internal validity, we will employ an adaptive randomization scheme to ensure balance by design rather than chance with respect to some key characteristics. Specifically, stratified by study site, we will use a variation of the minimization algorithm that can account for both continuous and categorical variables to ensure balance with respect to gender and walking speed with 80% biased coin probability; and simultaneously guard against heavy sample size imbalance.[68] Such adaptive schemes have been shown to be valid alternatives in trials. Study personnel will call in all prescriptions to the independent research pharmacist at the institutional Investigational Drug Service (See Resources and Environment), who will package all drugs/placebos to be similar in physical appearance. We have successfully employed the same process in other recently completed trials. Therefore, all participants and study personnel (except the study statistician and the dedicated provider of clinical care related to study safety) involved in assessments will be blind to treatment assignment.

2.4 STATISTICAL ANALYSIS

2.4.1 Sample Size Justification

The principle focus of the proof of mechanism trial is the impact of nitrite on mitochondrial energetics. To detect a significant difference in mitochondrial state 3 respiration in permeabilized fibers (the primary outcome for Aim I), the planned sample size is 35 per group (a total of 70 participants). Respiration data from a previous multi-site RTC that was co-led by Dr. Coen, showed a baseline values of 270.30±99.80 pmol/min/mg (mean ± standard deviation) for State 3 respiration and a pre- to post-intervention change of 68.5±97.61 pmol/min/mg[58] We conservatively anticipate a dropout rate of approximately 14%. Using published methods[59-62] implemented in commercial software (PASS 2012®, Number Cruncher Statistical Systems, Kaysville, Utah), with 70 patients and 60 anticipated completers, we will be able to detect statistical significance of a difference as small as 71.80 pmol/min/mg in pre- to post-nitrite change with 80% power in a 2-tailed test at the α=0.05 level. The criterion for a minimally clinically important difference (MCID) has not been authoritatively established for the measure, but the above detectable difference corresponds to a moderate to large effect size (*d*=0.74) by Cohen's criterion.[63] Therefore, the planned sample size affords an appropriate level of statistical sensitivity to address the aims in a mechanistic study with proximal biological outcomes such as ours.

For Aim 3, with the anticipated 30+30 completers, we will be able to detect statistical significance of correlations as small as 0.49 and 0.35, respectively, between changes in mitochondrial energetics and changes in physical activity/function and fatigability with 80% power in two-tailed tests at α=0.05 conducted without and with stratification by treatment group. As such, the planned sample size will afford sufficient statistical sensitivity to detect even moderate associations between mitochondria respiration and clinical endpoints.

2.4.2 Data Analysis

Overview: All statistical analyses will be performed and overseen by the study statistician Dr. Perera (see LOS) using SAS® version 9 (SAS Institute, Inc., Cary, North Carolina). Participant

flow will be summarized using a CONSORT diagram. Data will be summarized by intervention arm and time point as well as baseline to follow-up change using appropriate descriptive statistics. First, the baseline participant characteristics will be compared between the two arms. Any significant differences will be noted and accounted for as additional covariates in the sensitivity analyses. Second, main analyses to address the aims will be performed as outlined below using multiple imputation for missing data. The primary analysis will be performed to test the primary hypotheses H1a about the primary outcome of mitochondrial respiration (high resolution respirometry). Secondary analyses will be performed for remaining hypotheses, secondary outcomes and other exploratory analyses. Third, we describe below our primary approach to missing data and a set of sensitivity analyses by including additional covariates and ignoring missing data.

Baseline Comparison: Due to the adaptive randomization scheme, it is unlikely that baseline participant characteristics will be significantly different between the arms. If we do find any, they will be included as additional covariates in the sensitivity analyses. We will not alter the primary analytic strategy to preserve its a priori nature and predictability. We will use independent samples t- or Wilcoxon rank sum tests, as appropriate based on distributional properties, to compare continuous baseline characteristics between the intervention arms. For categorical baseline participant characteristics, we will use chi-square and Fisher's exact tests, as appropriate. Statistical significance of the intervention term will be interpreted as indicating the need to include them as covariates in sensitivity analyses.

Aim 1 Analysis: Analysis of covariance (ANCOVA) model using the SAS® GLM procedure with baseline to follow-up change in mitochondrial respiration (high resolution respirometry) as the dependent variable, intervention arm (nitrite/placebo) as the effect of main interest, and the baseline value of mitochondrial bioenergetics as a covariate. The magnitude and statistical significance at $\alpha=0.05$ of the nitrite vs placebo contrast will serve as the formal test of the primary hypothesis H1a.

We will employ the same analytic strategy described above for the primary outcome mitochondrial respiration for each of our remaining secondary continuous outcomes of mitochondrial bioenergetics, skeletal muscle perfusion and clinical measures collected only pre- and post-intervention. The magnitude and statistical significance of the nitrite vs placebo contrasts will serve as formal tests of the hypotheses H1b-c and H2a-b.

Aim 2: For physical function and activity measures additionally assessed midway, and hypothesis H3a, we will instead employ a linear mixed model to accommodate multiple post-randomization assessments and the resulting stochastic non-independence of observations. Specifically, we will fit a series of linear mixed models with change from baseline in each measure of physical function/activity as the dependent variable; intervention arm (nitrite/placebo), follow-up time (mid-course assessments and12 weeks) and their interaction as fixed effects; baseline value of the dependent variable as a fixed-effect covariate; and a participant random effect to account for multiple observations from each participant. We will appropriately construct means contrasts to make comparisons between intervention arms at each of the follow-up time-points, and interpret their statistical significance as supporting hypothesis H3a.

Aim 3 (hypothesis H3b) Analysis: We will examine whether changes in mitochondrial energetics, muscle perfusion and physical function/activity/fatigability tend to move at least partially in unison. We will compute Pearson and Spearman rank correlations between pre- to post-treatment changes in the above measures over the treatment period. We will consider correlations both with and without stratification by treatment group. We will also consider adjusting for baseline values of the measures considered and obtain partial correlation coefficients to isolate the associations in changes above and beyond any cross-sectional associations at baseline and comparing the correlations between groups using the Fisher's z-transformation. Finally, we will explore multivariable linear regression models with change in measures of physical function/ac-

tivity as the dependent variable; treatment group, and baseline and changes in measures of mitochondrial energetics as independent variables, taking care not to exceed the number of independent variables that could be realistically afforded by the effective sample size.

Sensitivity Analyses: We will perform a series of sensitivity analyses to ensure the robustness of our results against various assumptions. One such analysis will involve inclusion of gender and baseline participant characteristics significantly different between groups as additional covariates in the ANCOVA and linear mixed models above. Another will involve sensitivity of results to missing data handling techniques. In addition, if analyses of residuals from the models show violations of statistical assumptions, we will consider fitting models after Box-Cox transforming the continuous variables.

2.4.3 Safety Analysis

We will describe and descriptively compare proportions of those with clinical endpoints/adverse events such as hospitalizations, urgent clinic/ER visits, and death between the treatment groups, as using chi-square and Fisher's exact tests for dichotomous outcomes with such a small number of participants is unlikely to reliably demonstrate statistical significance.

2.4.4 Handling Missing Data

The best approach for handling missing data is prevention. Despite our best efforts, missing data will occur. To minimize missing data, we will use a multi-pronged approach:

1. Adherence to protocol measurement completion by participants. We will compensate participants for participation in trial by assessment.

2. Completion of protocol measurements by staff. We employ an experienced group of research staff, familiar with the measurement protocols described. We utilize a double check system during participant visits to assure all data are collected. We have available back-up systems for most of the outcome assessments, should we experience equipment failure. Staff is cross-trained to allow for planned or inadvertent absences.

3. We will clearly document those with missing data and reasons in the CONSORT diagram and compare those with missing data to complete data with respect to available data. Statistical guidelines for handling missing data recommend methods such as multiple imputation, which consider the uncertainty involved in imputing missing data. Multiple imputation is arguably the best available objective method to analytically account for missing data under the ignorable or missing-at-random (MAR) assumption. Specifically, we will generate M=5 imputed values for each missing value, analyze the 5 datasets as though complete, and finally combine the results appropriately so that they reflect the uncertainty involved in imputation. SAS® MI and MIANALYZE procedures will be used. Other approaches to missing data, including the naïve approaches of ignoring the missing values and last-value-carried-forward (LVCF) will also be considered in sensitivity analyses and robustness of the results to using these approaches will be examined.

2.4.5 Data Management

The PI will oversee all aspects of data management. With the consultation of the Data Center (DC), the PI and coordinator will develop an operations manual to standardize all procedures and staff training in areas such as patient recruitment, measurement, assessment, and data entry, management, and security.

Data management will follow the policy and procedures of the DC. The data entry system will be created using .NET 2.0, Microsoft Access, or SQL. We will create a tracking database that will be linked to the data entry database that will monitor enrollment, and track follow-up rates and the data entry process, providing up to date status reports. The database will include routine data edit checks for consistency both within and between forms. DC will work closely with investigative team and other study personnel to ensure that protocols are being followed, data integrity and confidentiality are maintained, and that the data contains a minimum amount of missing data. All study files residing in designated network folders will be backed-up daily and archived weekly. The weekly data are stored in a safety deposit off site (> 1 mile off campus). The weekly archived files are maintained for 1 year until the data are erased. All screened study participants will be assigned unique study identifiers that will appear on all data collection instruments, tapes, documents, and files used in the statistical analysis and manuscript preparation. Only authorized team members will have access to personal information needed for tracking and informed consent. Other data quality assurance measures will include detailed documentation of computer operations and data editing procedures and regular meetings with project staff to review any changes in procedure. The DC also has specific data quality measures that will be implemented. These include data verification, built in data validation mechanisms such as logic and out of range data checks, and repeated evaluation of the data collection and entry process.

3. HUMAN SUBJECT

3.1 PARTICIPANT POPULATION

Potential participants who meet the inclusion criteria, and have none of the exclusion criteria, will be enrolled without restriction as dictated by the study protocols. We will make efforts to enroll participants in this research in a distribution that mirrors the study population of the Pittsburgh area.

3.2 INCLUSION CRITERIA

- Age ≥70 years
- Sedentary (<1 hour/week of volitional exercise activity)
- Clinically stable (euvolemic; baseline HR <100 bpm) and without hospitalization or invasive cardiac procedure for 6 weeks

3.3 EXCLUSION CRITERIA

- Blood pressure <110 or >160/95 mmHg
- Participant is at greater risk of hypotensive adverse events caused by sodium nitrate following review of past medical history and physical exam.
- Participants with GDPD deficiency in whom sodium nitrate might increase the risk of hemolytic crisis.
- Orthopedic or other chronic condition which limits physical activity or exercise testing assessments
- If valve replacement has been performed, patient may not be enrolled for 12 months after this procedure
- Severe peripheral or pulmonary artery disease
- Anemia: Hgb <11.0 (♂),10.0 (♀) gm/dl
- Participants with diabetes whose HgbA1c >10.0%

- Chronic alcohol (>14 drinks ETOH a week) or drug (any cocaine, methamphetamine, and cannabis ≥4 x week) dependency
- Allergy to lidocaine; Allergy to Red Dye
- Chronic use of oral corticosteroids or other medications that affect muscle function
- Current use of organic nitrates or phosphodiesterase type 5 (PDE5) inhibitors
- Unable to hold warfarin, direct-acting oral anticoagulants (DOACs), non-steroidal anti-inflammatory medications (NSAIDs) or aspirin for 3 days prior to muscle biopsy, or to hold thienopyridine medications for 5 days prior to muscle biopsy. Participants unable or unwilling to hold will follow the modified ASA hold plan
- Any bleeding disorder that would contraindicate biopsy such as history of clinically significant bleeding diathesis (e.g., Hemophilia A or B, Von Willebrand's Disease or congenital Factor VII deficiency)
- Unstable psychiatric diagnosis that would affect adherence and ability to complete the protocol
- Dementia or inability to give informed consent or follow study protocol
- End-stage disease
- Other chronic unstable disease such as active neoplasm, end stage chronic kidney, liver or other organ disease

Relative Exclusions

- Participants with a non-3T MRI compatible pacemaker, implantable cardiac defibrillator, stent or other identified metal in their body will be excluded from the Magnetic Resonance Spectroscopy study.
- Subject who use PDE5s for erectile dysfunction and are willing to withhold use 24 hours prior and during entire dosing period may be enrolled.

4. IRB APPROVAL AND FDA AMENDMENTS

The Investigator will obtain, from the University of Pittsburgh Institutional Review Board (IRB), prospective approval of the clinical protocol and corresponding informed consent form(s); modifications to the clinical protocol and corresponding informed consent forms, and advertisements (i.e., directed at potential research participants) for study recruitment.

The only circumstance in which a deviation from the current IRB-approved clinical protocol/consent form(s) may be initiated in the absence of prospective IRB approval is to eliminate an apparent immediate hazard to the research subject(s). In such circumstances, the Investigator will promptly notify the University of Pittsburgh IRB of the deviation. The Investigator should also notify the sponsor of this event.

The University of Pittsburgh IRB operates in compliance with FDA regulations at 21 CFR Parts 50 and 21 CFR 56, and in conformance with applicable International Conference on Harmonization (ICH) Guidelines on Good Clinical Practice (CGP).

In the event that the University of Pittsburgh IRB requires, as a condition of approval, substantial changes to a clinical protocol submitted under an FDA-accepted IND application, or in the event of the Investigator's decision to modify the previously accepted clinical protocol:

- For Phase 2 and 3 clinical studies: The Sponsor will submit (i.e., in advance of implementing the change) a Protocol Amendment to the IND describing any change to a Phase 2 or Phase 3 protocol that significantly affects the safety of participants, the scope

of the investigation, or the scientific quality of the study. Examples of Phase 2 and 3 clinical protocol changes requiring the submission of a Protocol Amendment include:

i. Any increase in drug dosage or duration of exposure of individual participants to the investigational drug beyond that described in the current protocol, or any significant increase in the number of participants under study.

ii. Any significant change in the design of the protocol (such as the addition or deletion of a control group).

iii. The addition of a new test or procedure that is intended to improve monitoring for, or reduce the risk of, a side effect or adverse event; or the dropping of a test intended to monitor the safety of the investigational drug.

5. RECRUITMENT AND INFORMED CONSENT PROCEDURES

5.1 RECRUITMENT METHODS:

A variety of mechanisms will be used to recruit participants for this protocol

a. Potential candidates will be identified through screening of inpatients at the UPMC Presbyterian, UPMC Montefiore, UPMC Mercy, and UPMC Shadyside hospitals. Potential candidates will be identified through screening of inpatients' medical records for subjects 70 years and older with past medical history relative to inclusion and exclusion criteria. Recruiter will request bedside nurse to ask patient if it is okay to approach and explain study. Those interested in participating further will be provided the study brochure and study contact information or the option to complete the initial screening in person.

b. UPMC patients can be referred by their physician and/or cardiologist from the UPMC Cardiology Center, General Cardiology clinic, Benedum Geriatric Center, the Comprehensive Pulmonary Hypertension (PH) Program or other UPMC or community facility. For UPMC Presbyterian/Montefiore-based clinics, we will obtain physician and HRPO approval for screening of subject medical records for identification of eligible candidates. Dr. Forman currently already has and will confirm continued approval from UPMC physician groups to screen their patients. He will engage with additional cardiologists and geriatricians for permission to screen from those clinics. Once identified as preliminarily eligible, the study coordinator/staff will reaffirm with the subject's physician on the clinic visit day that recruitment of the subject is medically appropriate. Staff will also request direction from the attending physician as to the timing of speaking to the subject, i.e., before or after the subject has seen the physician for their visit. This will be done on a case-by-case basis. Study staff will speak with candidates at clinic visits or by telephone call to assess interest and review medical history to assess eligibility.

   Participants referred from facilities outside the UPMC Epic medical record system will be provided a medical record release for their cardiologist/physician to release needed documents to confirm eligibility.

   Participants can also be identified from the inpatient Cardiology service. The study coordinator will follow the same procedure as for outpatients by screening the inpatient service for participants 70 years of age and older. Such participants will need to be followed for 6 weeks of disease stability before enrollment, but recruitment and ascertainment of interest can be initiated, with consideration of medical circumstances in each case. As noted, permission to speak with participant will be obtained from the Cardiology attending.

c. Research Registries

Two research registries are available to the PI. The HRPO submission will include the request to use the Clinical Translational Science Institute's PittPlusMe Research Registry, which includes a database of over 90,000 individuals who have indicated their interest in participating in research studies. The PittPlusMe initiative includes the use of engaging participants via social media.

Second, the Claude D. Pepper Center has a Research Registry of over 3000 community dwelling participants that are >60 years of age and is also available once HRPO and Pepper Registry approves.

d. Advertisements

An approved study flier and recruitment brochure will be placed in key places and/or be distributed to physician offices, related clinics, or on other occasions/venues that present as an opportunity to recruit (e.g., a PI speaking engagement or a community outreach event to reach minority participants). Potential participants can self-refer by contacting the study staff via a telephone number/email address that is provided on these advertisements. Study staff will utilize the approved phone screening script when responding to interested candidates. With subject permission, they will be screened on the phone to make a preliminary assessment of eligibility. We will obtain permission to access their medical records in the UPMC database or request records from their provider as needed to further document eligibility.

Advertisement, such as on radio, television, or print copy in newspapers, or bus signs may also be utilized depending on recruitment rates.

e. Once participants have been determined to be eligible from medical record review and attending approval, recruitment and enrollment procedures will then follow, including:

i. Confirmation of eligibility by PI

ii. Updating participants' cardiologist and confirming management of holding anti-coagulants medication pre-muscle biopsy per protocol, if needed.

iii. Setting dates for patient visits with subject and staff will also occur prior to screening visit to maintain timeliness of visits per protocol

Scheduling outpatient screening visits (Consent and Visit 1) where study risks and potential benefits, and rights as a research subject will be described in detail, informed consent will be obtained and where final eligibility will be confirmed

5.2 INFORMED CONSENT PROCEDURES

Participants must provide informed consent. The information about this study will be given to the subject in language understandable to them. Either the physician investigator or a non-physician member of the research team will present the study. They will verbally present a general outline of the research plan, including inclusion and exclusion criteria, to the prospective participant. The consent form, outlining the design of the study, will include the risks and benefits of participating, and will be reviewed and the physician investigator and/or non-physician member of the research team will answer any questions. Prospective participants may take as much time as required to make an informed decision. Written informed consent will be obtained from each participant and the physician investigator prior to performing any research study procedures.

6. POTENTIAL RISKS AND BENEFITS

6.1 POTENTIAL RISKS

6.1.1 Risk of Experimental Drug Intervention

Nitrite: Numerous studies have evaluated acute, subacute and chronic drinking water exposures of nitrate and nitrite in laboratory animals and drinking water and dietary exposures in humans. Recent studies are available using high doses of nitrite by oral route in the form of beet root juice. Recent studies have evaluated acute exposures of oral preparations of nitrite and nitrate on PK and BP and are characterized below. More extensive human data is available on parenteral sodium nitrite as it is currently available and approved by the FDA for use in the emergency treatment of cyanide poisoning. It is also notable that neutrapharmaceutical preparations are currently being sold with levels of nitrite (12.7 mg per tablet) and nitrate (3.9 mg per tablet).

Sodium nitrite has been used commercially as a food preservative, an anti-corrosive agent, a coloring agent, and an anti-anginal agent, with additional uses in laxatives, burn ointments, and liniments. Amyl nitrite has been inhaled or ingested as a euphoric stimulant. Nitrite has also been found as a contaminant in well water. Literature searches generated case reports of nausea, vomiting, abdominal pain, dizziness, headache, flushing, cyanosis, tachypnea, dyspnea, hypotension and death attributed to excess nitrite (high-dose) exposure from these sources as a consequence of methemoglobinemia due to oxidation of heme-iron in oxyhemoglobin. Normal background methemoglobin production is 1-3%. If levels of methemoglobin rise above approximately 30% of total hemoglobin, a subject may appear cyanotic and experience dyspnea, due to the reduced oxygen carrying capacity of hemoglobin (methemoglobin cannot bind oxygen). Levels above 50% can cause seizures, hypotension, coma and death. Sodium nitrite administration for cyanide poisoning at the labeled dosage of 300 mg causes methemoglobinemia, a desirable effect, as methemoglobin binds to cyanide, thus protecting cellular mitochondria. A standard dose of nitrite used for cyanide poisoning is 300 mg up to 600 mg. Note that methemoglobin levels have never risen higher than 3% at the currently used therapeutic doses (<75 mg) in 80 volunteers in phase I studies at the NIH.

The Sponsor of this study protocol, Dr. Gladwin, has previously held an IND for sodium nitrite (IND # 70,411) for cardiovascular applications and currently has an approved IND for the use of sodium nitrite for lung transplant recipients (IND # 111,643). The cardiovascular IND involved the administration of sodium nitrite to 69 normal volunteers in 4 phase I-II clinical trials without observed adverse effects. He has also treated 11 participants with sickle cell disease on this IND without observed adverse effects. The lower doses of nitrite used in these investigational treatment regimens – 60-120 mg daily or 20-40% of the dose (300 mg) used in the emergency treatment of cyanide poisoning–do not produce methemoglobin levels greater than 3% and have not been associated with clinically significant hypotension. There have been no adverse events noted in the 80 treated normal human volunteers and patients with sickle cell disease.[67] In another study by Gladwin et al., 18 healthy adults received an infusion of sodium nitrite totaling 75 mg (15 minutes each x 2 infusions). This was associated with a 7 mm Hg decrease in mean arterial pressure, a peak methemoglobin of less than 3% and no other significant effects. Note this single dose is 1.9 times the larger single dose (tid) we plan to use in this trial.

In Dr. Forman's current pilot study, 20 mg tid were used, which were lower or similar to the doses of nitrite cited and used safely in any of the noted studies.

To summarize, we anticipate the following symptoms by organ system and likely frequency of risk:

Gastrointestinal

- Common: none
- Frequent: none
- Infrequent: dry mouth
- Rare: nausea, abdominal pain and vomiting

Hematologic

- Common: none
- Frequent: none
- Rare: methemoglobinemia

Cardiovascular

- Common: none
- Frequent: hypotension in older participants on higher nitrite doses
- Rare: flushing, tachycardia, hypotension in healthy controls

Neurologic

- Common: none
- Frequent: none
- Rare: headache, dizziness, seizure, coma

Respiratory

- Common: none
- Frequent: none
- Rare: tachypnea, dyspnea, cyanosis

Renal

- Nocturia: infrequent

6.1.2 Risk of Study Procedures

Risks of exercise testing assessments: (stress test on cycle, 6-minute walk, steady-state on treadmill, strength and balance test):

These tests require people to apply effort. Although careful medical supervision will help to lessen the difficulty of doing these tests, some people might still find them unpleasant. The exercise test may cause muscle soreness or fatigue (common). Some people get anxious while breathing through a mouthpiece or mask(common) or experience a fall (rare). Another risk is redness, skin chafing or irritation from the EKG electrodes used during exercise testing. If an abnormal rise in blood pressure or changes in the electrical pattern of the participants heart is noted, or if the participant develops chest pain, the exercise will be stopped immediately. Rarely, exercise may cause muscle sprains, muscle strains, or broken bones. Other risks include abnormal blood pressure (infrequent), fainting, dizziness (infrequent), disorders of heart rhythm (infrequent), and in very rare instances, heart attack, stroke, or even death (rare). In adults without a known history of heart disease, the risk of heart attack or death from maximal or sub-maximal exercise bouts is rare. The relative risk of exercise testing for older adults has not been clearly defined. However, a survey of more than 2,000 clinical exercise testing laboratories, in which more than 600,000 tests were performed, showed a death rate of approximately 0.5 per 10,000. The PI/ study clinicians, and study staff are trained to minimize risks and to treat appropriately if any instability should develop.

Cardiopulmonary and Functional Assessments:

The cardiopulmonary exercise test (CPET) entails symptom limited (maximal) exercise provocation. The exercise stimulus is associated with a 1 in 10,000 chance of significant untoward outcome (e.g., myocardial infarction, arrhythmia), including the possibility of death. However, all enrolled will have a physical exam immediately before the test to best insure they are stable. All physicians and study exercise testing personnel are ACLS trained and a code cart is in the immediate vicinity. In addition, patients' cardiologists will be notified before patients are enrolled, and will be asked to approve only those patients they deem to be stable.

During the other physical activity assessments (e.g., 400-meter corridor walk, 5 minute steady-state treadmill walk, SPPB, and handgrip) there are also inherent exercise-associated risks, yet since CPET will be completed first, that initial assessment provides some certainty that participants are stable for the walking based assessments thereafter.

Skeletal Muscle biopsy

- Muscle biopsies are associated with a chance of bruising (about 1 in 100). Participants are instructed in what signs or symptoms at the biopsy site warrant contacting the study coordinator post biopsy.
- Vasovagal reaction due to anxiety,
- Bleeding and infection are rare side effects. Participants will be provided with signs and symptoms, educated on when to contact the study team and when to seek emergency medical treatment.
- Muscle biopsies are also associated with a small chance of infection (less than one in 1000) and pain.

  Infection: Careful steps to keep the area free of germs will minimize infection risks. At the biopsy visit, participants will be given instructions for care and signs or symptoms of infection for which they should contact study staff.

  Pain: lidocaine numbing medicine is used to reduce any possibility of pain, but sometimes the lidocaine itself produces a brief burning feeling as it enters through the skin. Additional numbing cream may be used in participants who report lower pain thresholds or inadequate response to numbing in the past. The PI/study clinician will evaluate the need and safety for this in select cases as needed.
- Holding of anti-coagulant and anti-thrombin medications. With the permission of the prescribing physician (if deemed necessary), blood-thinning medicines such as aspirin (81 mg - 3 days) as well as warfarin (3 days) or others will be held before the muscle biopsy, and restarted post muscle biopsy. However, if the primary cardiologist prefers, bridging therapy using a different blood thinning medication (enoxaparin) will be used while the aspirin and warfarin are on hold. Holding the anti-coagulant regimen which is in place for prevention of cardiovascular events theoretically increases the risk of cardiovascular events, albeit a very small amount.
- Additional unusual risks include allergic reactions to the elastic bandage wrap, leg numbness that would indicate the elastic bandage had been applied too tightly; and skin redness, irritation, and chafing from the Steri-strip™. Participants allergic to the Steri-strip™ will require a stitch.

Risks of withholding anti-diabetic medication.

Participants may be asked to withhold their anti-diabetic medication before or on the day of the exercise tests, biopsies. It will depend on the type of medication (and for the exercise test, current glycemic control), anticipated time of test and next meal. This may result in a transient increase in blood glucose which will self-resolve and does not carry long term risk.

Genetic testing

As a broad concept, results of genetic testing on muscle and blood are thought to influence future employment or insurability for participants or their blood relatives if new diagnoses are identified. The results of these genetic tests are de-identified, subject to privacy laws and are not available to future employers or insurers. Further, because the results of the genetic tests performed in this study will only be linked to a disease condition that is already known, it is unlikely that the results of these genetic tests will have any significant impact on participants' current health profile. The usefulness of these results in providing treatment for medical conditions has not been determined because they are research tests.

Risk of lidocaine

This FDA-approved numbing medicine, lidocaine, is injected into the site before the biopsy procedure. Participants with prior difficulty with this local anesthetic will be excluded from the study. A possible side-effect is an anaphylactic reaction. Participants will be questioned before enrollment about prior experiences with the local anesthetic lidocaine. While the risks of having a reaction to lidocaine used for this purpose are rare, it is always possible to have an unexpected serious reaction to any medication.

Magnetic resonance spectroscopy

- The MRRC has extensive experience with MRS studies in diverse populations and is well-trained to reassure participants. During these MRS sessions, even with previously scanned participants, measures are taken to insure subject comfort. If any participant exhibits any significant clinical findings on any of the exams (e.g., tumor on MRI), they will be referred to appropriate clinicians. In the case of an emergency during participation in the study, the staff will initiate the appropriate emergency procedures as per standard operating procedure at UPMC. All staff at UPMC involved in MRI/MRS scanning are trained yearly in safety and emergency protocols.
- MRS is not associated with any known adverse effect except for people with metal or magnetic implants (such as metallic clips in the brain or cardiac pacemakers).

  Metal objects within the body can heat to potentially dangerous temperatures or possibly move in the patient's body.

  Some types of tattoos (home-made) can also heat and cause discomfort. Any participants with the possibility of these risk factors will not be tested in the MRI/MRS studies.

  Metal objects can also become projectile when placed near the magnetic field. This has been reported, but it is a very rare occurrence. Protection from magnetic objects can be safeguarded by the usual safety techniques that are practiced in MRI/MRS sessions, including having participants and researchers take all metal objects off of the subject before entering the environment.
- Thus, any candidate who meets inclusion/exclusion criteria with the exception of a metal or possibility of metal in their body will be invited to participate, but will forego the MRI testing.

  Double screening of participants, by the study coordinator and again on the day of testing will be completed for thorough assurance that the subject is safe for MRI.
- Another potential risk is claustrophobia caused by being in the enclosed space of the MRI/MRS scanners. Participants who may become anxious or uncomfortable during any part of the procedure will be immediately taken out of the scanner and not tested. Participants are instructed to talk to the experimenter if they feel uncomfortable for any reason

during the testing procedures. If participants feel uncomfortable, they are immediately taken out of the scanner.

- Noise levels in the magnet can be uncomfortable for participants and they will be wearing earplugs through all MRI/MRS procedures. There is also microphone in the bore of the magnet. As above, participants who feel uncomfortable are taken out of the scanner.

Near Infrared Spectroscopy (NIRS)

- Participants may feel uncomfortable during the placement of the NIRS device onto their legs although it is not painful. There are some risks for minor skin irritation, redness, and chafing associated with the use of the doubled-sided tape to attach the NIRS sensors to the thighs. Though no other adverse effects from non-ionizing LED light source have been reported, it is possible that effects not yet reported may occur.

Actigraphy

- Participants will be instructed on wearing an ActiGraph GT3X+, non-invasive activity monitor for one week at the beginning and end of the study and during the cycle CPET and all walking testing performed. There may be the inconvenience of having to wear a device around the wrist, and a small chance of causing a rash or irritation of the skin but should be no more than that of watch wristband. It has a small flashing light that may slightly bother some people.

Questionnaires

- Patients will answer questionnaires as part of the study assessments which require responses about daily activities, diet, quality of life, medication, health history, cognitive ability and sleep which in some cases may be a source of emotional distress. Efforts will be made to keep the environment and support by staff to be reassuring and pleasant.

Peripheral Blood sample

- Blood samples for nitrite and nitrate monitoring as well as serological assessments for the study entail needle sticks and/or IV placement. There is a small chance of infection and pain. Careful steps to keep the area free of germs will minimize infection risks.
- Common risks of blood sampling by venipuncture or intravenous line placement include temporary pain, bruising which may last for several days, redness, and swelling. Infrequent risks include feeling lightheaded or faint when blood is drawn. This is usually due to nervousness (not due to the amount of blood), and it is not usually serious. Rare risks include infection and bleeding.
- A risk of drawing blood is anemia, from a lower hemoglobin level. A common symptom of anemia is fatigue (feeling tired or weak). The amount of blood to be drawn over the course of this investigation is an approximate maximum of 224 ml over approximately 16 weeks. This is not likely to cause anemia, especially with blood draws extended over 6-8 weeks. Also note that we are screening and excluding for anemia at Visit 1.

Risks of fasting

8 hours before the fasting visits indicated in Study Flowchart section 2.2.1

Fasting for blood work is common and carries little risk. Participants may feel tired, hungry or irritable until they are served the standardized breakfast upon arrival.

Participants who are diabetic have relatively greater risks of hypoglycemia, but the PI/study clinician will guide these individuals, and possibly adjust their medications as needed to minimize these risks.

Overall subject burden

- Subject burden is a possibility due to the number of visits and particularly during the comprehensive physical assessment battery that is completed as part of the protocol. However, as noted in the Methods, precautions have been integrated with the assessments to minimize risk to the participants. Rest periods are provided after each active functional assessment, moreover, active functional assessments alternate with ones that are completed at rest (questionnaires). Furthermore, at the end of each assessment and before the next functional assessment, and the rate of perceived exertion (RPE) is reassessed to evaluate fatigability and to make sure that the subject has returned to baseline of physiological and subjective parameters prior to the next test. The consent language includes that participants may withdraw at any time. The consent form includes language and PI and the study coordinator will be sure that participants understand the number, duration and activity for each visit, so as to fully inform potential participants on this issue.

6.2 ALTERNATIVE TREATMENTS

The alternative treatments for the participants participating in this investigation are to continue their medical care under the direction of their primary physicians.

6.3 POTENTIAL BENEFITS

There may be no direct medical benefit to participants in the study. It is hoped that researchers will learn more about the effectiveness of nitrite capsule supplements to improve strength, gait speed and balance. Oral nitrite may also increase efficiency of work by reducing their oxygen uptake required for the same work intensity. We hope the information learned from this study will benefit other older patients in the future.

7. RISKS MANAGEMENT PROCEDURES

7.1 PROTECTION AGAINST RISKS

General Risks of Study Protocol and Procedures

All research interventions/activities will be conducted in private patient care areas. The collection of sensitive information about participants is limited to the amount necessary to achieve the aims of the research, so that no unneeded sensitive information is being collected.

All demographic and clinical information about the subject will be stored in an electronic password-guarded study database under the supervision of the Investigator for this protocol.

The Center for Research on Health Care Data Center (CRHC-DC) at the University of Pittsburgh

Data will be maintained by the CRHC Data Center with MS SQL Server. SQL Server is designed for large relational databases and is a stable platform in which to house large volumes of data. All tables within SQL will be linked with a key identifier. SQL enables the system programmers to quickly generate custom datasets based on specific analyses. Additionally, SQL has a reporting mechanism that enables one of our system programmers to quickly generate reports as needed for the study. Data entered by clinical site personnel will be automatically added to the relational database stored on Data Center servers.

The EWI (Enterprise Web Infrastructure) is backed up with the Symantec NetBackup system. The backup images are processed through an IBM ProtectTier appliance and stored on IBM DS8870 storage arrays. All EWI servers get a daily operating system backup in the form of a full or differential backup. The backup schedule has daily differential and bi-weekly full backups for all EWI servers. The backup images have a retention period of 60 days. They are replicated to a secondary IBM DS8870 storage array located at Posvar Hall. This protects the backups images in the event of a catastrophic event at the RIDC data center.

EWI sites with a backend database server also receive a daily backup. All EWI database servers have a daily full backup and daily log backups. EWI database servers running MS SQL Server utilize a specialized NetBackup SQL backup agent. Any EWI database server running MySQL have a daily backup in the form of daily local dump and then have the exported database files backed up during the daily operating system backup. These again are replicated to the Posvar site. The backup methods used are proven methods for recovery. Partial file restores and full site restores have been performed upon a request from a department.

The Data Center is complaint with the FDA 21 CFR part 11 protocols. Access to data will be controlled through password and authentication policies. Only approved individuals will have access to data. Password policies control the length and variability of the user password selection. Audit trails will be implemented to log date, time, individual, changed values, and rationale for all data changes.

We have three high-end machines dedicated to statistical analyses. These machines contain up to 8 Xeon processors and up to 32gb or RAM with stripped raided SCSI high end performance hard drives. This provides a framework to manipulate and analyze very large datasets in an efficient and time effective manner.

The CRHC-DC biostatistical group uses current versions of SAS, R, and Stata for all data manipulation, statistical analyses, and reporting with set guidelines for code documentation and reproducibility. In addition, the statisticians use M-Plus and StatXact for specific types of analyses or simulations.

Specimens will be stripped of subject identifiers and stored according to a similar coding protocol as described above. These specimens will be stored safely in the custody of the Investigator responsible for the individual assays. The Investigators will limit future access to any remaining sample to only those investigators with prior IRB approval for their studies.

All staff involved in this study are properly credentialed and instructed in the areas of testing, confidentiality, and safety.

The Investigator will retain the data for the entire period of this study and will retain the specified records and reports for up to two years after the marketing application is approved for the investigational drug; or, if a marketing application is not submitted or approved for the investigational drug, until two years after investigations under the IND have been discontinued and the FDA so notified. The Investigator may continue to use and disclose participants' de-identified information for the purpose of this study for a minimum of seven years after final reporting or publication of the study. If the subject and/or legal representative decide to withdraw or be withdrawn from study participation, they may request that the study data and samples be destroyed. Subject names or other directly identifiable information will not appear on any reports, publications, or other disclosures of clinical study outcomes.

If applicable, any clinical study data that will be recorded directly on the CRF or electronic database whereupon the CRF/electronic data is to be considered the Source Data. Source Data are the clinical findings and observations, laboratory and test data, and other information contained

in Source Documents. Source Documents are the original records (and certified copies of original records; including, but not limited to, hospital medical records, physician or office charts, physician or nursing notes, subject diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, x-rays, etc. When applicable, information recorded on the CRF shall match the Source Data recorded on the Source Documents. Describe the procedures for accounting for any missed, unused, and/or spurious data.

7.2 PROTECTION AGAINST POTENTIAL RISKS OF EXPERIMENTAL INTERVENTION

- Involvement by trained staff/investigators with experience in the administration of the study drug: Dr. Forman, PI, and physician Co-I, Dr. Hughan are principally responsible for monitoring and protecting the safety of participants' use of sodium NO2. Their combined experience in the use of the study drug is adequate to protect against (the relatively low) risks.
- Continuous monitoring by the Data and Safety Monitoring Board.
- Required Education in the Protection of Human Research Participants: The Principal Investigator and all sub-investigators listed on the University of Pittsburgh HRPO approved protocol are required to complete the Collaborative Institutional Training Initiative (CITI) courses in research fundamentals, including Human Participants Research and Responsible Conduct of Research. Staff will complete these courses as well, prior to any subject contact. Investigators and staff with subject contact will also complete the Good Clinical Practice module. Investigators with an identified Conflict of Interest (COI) will complete the COI module. These web-based tutorials are a requirement of the HRPO for investigators prior to protocol submission

8. ADVERSE EVENTS

The proposed study will use the FDA definition of adverse events (AE) and serious adverse events (SAE). Any SAE, which is unexpected and related to study intervention, will be reported immediately to the IRB and FDA and will include all known details regarding the nature of the SAE. In the event that a participant either withdraws from the study or the investigators decide to discontinue a participant due to a SAE, the participant will be monitored by the PI and treating investigator until (a) a resolution is reached (e.g., the problem has resolved or stabilized with no further change expected), (b) the SAE is determined to be clearly unrelated to the study intervention, or (c) the SAE results in death. Outcomes of SAEs not previously reported will be reported to the sponsor, IRB and FDA via a follow-up report. A summary of the SAEs that occurred during the previous year will be included in the FDA annual progress report as well as in the annual IRB renewal.

The severity of adverse changes in physical signs or symptoms will be classified as follows:

- Grade 1 (Mild): asymptomatic or mild symptoms; clinical or diagnostic observation only; intervention not indicated.
- Grade 2 (Moderate): minimal, local or noninvasive intervention indicated; limiting age-appropriate ADL (Activities of Daily Living).
- Grade 3 (Severe): medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care/ADL.
- Grade 4 (Life-threatening): consequences; urgent intervention indicated.

- Grade 5 (Death): event is a direct cause of death.

8.1 REPORTABLE ADVERSE EVENTS

For this study, a serious adverse event is any untoward clinical event that is thought by either the Principal Investigator, Dr. Forman or the sponsor, Dr Gladwin to be unexpected and at least possibly related to the study and results in any of the following:

1. Death
2. A life-threatening adverse event
3. Inpatient hospitalization or prolongation of an existing hospitalization
4. A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
5. A congenital anomaly or birth defect
6. Important medical events that may not result in death, be life threatening, or require hospitalization.
7. 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 serious outcomes listed above.

If clinically important and unexpected adverse experiences or clinically important study-related adverse experiences occur, they will be recorded on the adverse event case report form.

8.2 ADVERSE EVENTS REPORTING TIMELINES

Life-threatening or fatal unexpected adverse events associated with the use of the study drug or procedures must be reported to the DSMB Chair and to the IRB within 24 hours of discovery of the incident with subsequent follow-up submission of a detailed written report.

The FDA will be notified by telephone or facsimile transmission of a human adverse event that is fatal or life-threatening no later than 7 calendar days after receiving the respective human adverse event information, followed by the subsequent submission of a written IND Safety Report.

Serious and unexpected adverse events associated with the use of the study drug or procedures will be reported within 48 hours to the DSMB Chair and the IRB with subsequent follow-up submission of a detailed written report in accordance with the respective policies and procedures of the IRB. 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 investigator-sponsor's receipt of the respective adverse event information. All AE and SAE will be reported to the DSMB quarterly.

A summary report of the findings will be prepared and submitted to the regulatory agencies as required.

9. DATA SAFETY MONITORING

9.1 DATA SAFETY MONITORING BOARD

The DSMB will also be expected to meet as needed, but not less than, every six months to provide an overall summary status report to the regulatory agencies. An emergency meeting of the DSMB may be called at any time by the Chair should participant safety questions or other unanticipated problems arise**.**

In addition, the DSMB Report addressing the following information will be submitted to the IRB at the time of continuing review annually or more often as required:

- A list of the research personnel who participated in the data and safety monitoring.
- The frequency of monitoring that took place during the renewal intervals and/or the dates that data and safety monitoring was conducted.
- A summary of cumulative data related to unanticipated problems (including adverse events) including a determination of causality and whether the risk to benefit assessment has changed.
- If appropriate, a summary of pertinent scientific literature reports, therapeutic developments, or results of related studies that may have an impact on the safety of study participants or the ethics of the research study.
- A summary of the outcome of reviews conducted to ensure subject privacy and research data confidentiality.

Final conclusions regarding changes to the anticipated benefit-to-risk assessment of the study participation and final recommendations related to continuing, changing, or terminating the study.

9.2 DATA SAFETY MONITORING PLAN

Monitoring of safety and data quality in the proposed study will be the responsibility of all personnel on the project, with primary responsibility and supervision by the Investigator. The Institutional Review Board will approve the Statement of Informed Consent for the study and provide institutional oversight of data and safety issues. The study protocol will be approved prior to recruiting or obtaining consent from any participants. Moreover, the study will be reviewed at a minimum of annual basis (or more frequently as deemed necessary) by the IRB committee. Each participant will sign the Informed Consent Form described above prior to participating in the study. To ensure participant safety, once participants are enrolled in the study, study staff will immediately report all adverse and serious adverse events to one of the Investigators. The Investigator will, per institutional requirements, report them to the IRB for their review. These events should also be communicated to the sponsor of the IND. With regards to the monitoring of data quality and protected health information, all required personnel proposed for this project will have the required human participants and confidentiality training, which includes information about maintaining data integrity and security. Confidentiality will be guarded using established procedures such as storing data in locked cabinets within locked offices or locked data rooms, coding by study identification numbers rather than any personally identifying information to avoid revealing the identity of participants, and aggregating data across participants. The key linking names and study identification numbers will be kept separately from the data sets with limited access by study personnel. Only study personnel will have access to the data sets on protected servers. In order to maintain the highest standard of data entry quality, all data will be double entered, with discrepancies highlighted so that they can be reviewed by the project coordinator. Oversight of all aspects of data management will occur with the Investigator.

9.3 PARAMETERS TO BE MONITORED

The following progress will be monitored throughout the course of the research to ensure the safety of participants as well as the integrity and confidentiality of their data.

- An evaluation of the progress of the research study, including subject recruitment and retention, and an assessment of the timeliness and quality of the data.

- A review of collected data (including adverse events, unanticipated problems requiring reporting and those captured on the non-compliance log, and subject withdrawals) to determine whether there is a change to the anticipated benefit-to-risk assessment of study participation and whether the study should continue as originally designed, should be changed, or should be terminated.
- An assessment of external factors or relevant information (e.g. pertinent scientific literature reports or therapeutic development, results of related studies) that may have an impact on the safety and study participants or the ethics of the research study.
- A review of study procedures designed to protect the privacy of the research participants and the confidentiality of their research data.

9.4 FREQUENCY OF MONITORING

Dr. Forman will review subject safety data as it is generated. Dr. Forman and the research staff will meet with bi-weekly and/or monthly (as they get further into the study) intervals to re-evaluate study goals, subject recruitment, data coding and retention, documentation and identification of adverse events, complaints and confidentiality of participants. There will be an evaluation of the progress of the research study, including assessments of data quality, timelines, participant recruitment, accrual, and retention. Dr. Forman will also review the outcome and adverse event data to determine whether there is any change to the anticipated benefit-to-risk ratio of study participation and whether the study should continue as originally designed or should it be re-evaluated and changed

10. WITHDRAWAL OF PARTICIPANTS AND STOPPING CRITERIA

Life-threatening or fatal unexpected adverse events associated with the use of the study drug or procedures must be reported to the HRPO within 24 hours of discovery of the incident with subsequent follow-up submission of a detailed written report. The FDA must be notified by telephone or facsimile transmission of a human adverse event that is fatal or life-threatening no later than 7 calendar days after receiving the respective human adverse event information, followed by the subsequent submission of a written IND Safety Report.

Serious and unexpected adverse events associated with the use of the study drug or procedures must be reported to the HRPO with subsequent follow-up submission of a detailed written report in accordance with the respective policies and procedures of the HRPO. 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 investigator-sponsor's receipt of the respective adverse event information.

All reports noted in this section will also be provided to the NIA Program Officer and the DSMB.

Consent: During the consent process and when deemed appropriate by study staff throughout study, participants will also be informed that they are free to withdraw from the study at any time without any consequences.

Intervention Phase:

Outpatient: If participants report change in skin tone and dizziness or other signs of methemoglobinemia for ≥24 hours, the need for an interim visit with the above described steps will be followed. The visit can be conducted in the UPMC Montefiore GRC or TRC, or in the Pepper Center-SMART Center.

At this visit, methemoglobin will be assessed. If greater than 5%, the PI will be unblinded to drug or placebo assignment. If participants are on nitrite, PI will evaluate data and make a determination regarding discharge on 20 mg tid. If discharged on 20 mg tid, participants will be contacted via phone for the next 2 days for symptom re-assessment. If symptoms remain resolved, participants will continue on 20 mg tid. If symptoms persist, participants will be requested to come in for repeat methemoglobin testing after 3 days. If level persists above 5%, participation in the study for the subject will be discontinued.

Hypotension and related symptoms

During pK:

If this occurs while in the clinic, care will be administered, including fluids and monitoring, to ensure that the problem resolves, and patient is stable. The physician investigator will factor relevant subject symptoms and hemodynamics and then decide whether un-blinding is indicated and if it is appropriate for the subject to proceed with study participation prior to discharge.

As outpatient:

If participants experience lightheadedness or dizziness after visit 4 while they are on the study drug/placebo, we request that they call the study coordinator who will inventory their symptoms, compare to baseline levels (measured at pK), and discuss need for an interim visit with study physician. If a visit is indicated, participants will be asked to repeat drug dosing of 20 mg with follow up methemoglobin and BP pressure monitoring. A study physician will factor relevant subject symptoms and hemodynamics and will confirm a management plan including the:

-need for un-blinding,

-need to discontinue and drop from the trial.

If participants proceed, they will be reassessed via phone each day for the next 2 days thereafter to ensure that the patient is stable. However, if there are continued symptoms, the physician investigator will reevaluate the patient's suitability for continued participation based on symptoms and/or hemodynamics.

If participants who were initiated report symptoms as described above, they will be asked to complete an interim visit, methemoglobin and BP pressure assessment; the physician may have to withdraw the subject since a dose lower than 20 mg tid is not an option.

Discontinuation of the Clinical Trial

Stopping Rule:

Extensive published literature and considerable local experience at University of Pittsburgh indicates nitrite is not inherently dangerous at the doses targeted in this trial. Certain side effects of nitrite are idiosyncratic in older frail patients and it is anticipated that there will be participants who do not tolerate the treatment who may need to be withdrawn from the study. Nonetheless, if a subject enrolled in the study experiences a fatal event that is directly attributable to the NO_2, the overall study treatment will be discontinued.

11. COSTS AND PAYMENTS

11.1 COSTS

Study drug/placebo and all research testing will be supported by ongoing research grants. All medications, lab tests, and any procedures described will not be billed to the participants and/or their health insurance company.

11.2 PAYMENTS

Participant Reimbursement Consent Visit and V1 (Baseline)	$50
Participant Reimbursement V2	$50
Participant Reimbursement V3	$50
Participant Reimbursement V4	$100
Participant Reimbursement #1	**$250**
Participant Reimbursement V5	$50
Participant Reimbursement #2	**$50**
Participant Reimbursement V6	$50
Participant Reimbursement V7	$50
Participant Reimbursement V8	$50
Participant Reimbursement V9	$100
Participant Reimbursement #3	**$250**
TOTAL:	**$550**

Participants who require any interim visits (including the separate Consent visit if necessary) will be provided parking and compensated up to an additional $40 total at final reimbursement.

12. QUALIFICATIONS AND SOURCE OF SUPPORT

12.1 QUALIFICATIONS OF THE INVESTIGATORS

Sponsor:
Mark Gladwin, MD, is a Professor of Medicine, University of Maryland School of Medicine. He is an internationally recognized authority in the field of sodium NITRITE including both the basic science and a broad range of clinical applications in cardiovascular disease. He is a current IND holder for the investigation of sodium NITRITE in lung transplant.

Principal Investigator:
PI: Daniel Forman, MD, is a Professor (Pending) of Medicine at the University of Pittsburgh School of Medicine, Department of Medicine and Chair, Geriatric Cardiology Section, Divisions of Geriatrics and Cardiology. Dr. Forman has a well-established track record of translational work focused on the interplay between skeletal muscle and physical function in older HF patients. Moreover, he is an expert in functional assessments in older HF patients. He is responsible for all aspects of this investigation and will work directly with research staff to screen patients, coordinate data collection and quality, and he will personally complete all muscle biopsies and supervise all functional evaluations. He will play a primary role in data analysis and publications.

Sub-Investigators:

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Sruti Shiva, PhD is an Associate Professor in the Department of Pharmacology and Chemical Biology and VMI at the University of Pittsburgh. Her research lab focuses on the mechanisms by which reactive nitrogen species (particularly nitrite and nitric oxide) regulate mitochondrial function, the factors that influence this regulation and the implications of this regulation on pathology.

Nancy Glynn, PhD is an Assistant Professor, Epidemiology, who completed formative work that established and validated indices of performance and perceived indices of fatigability, i.e., performance fatigability which refers to the mechanistic property of declining function during a constant physical activity stimulus, and perceived fatigability as a perception of tiredness associated with a physical activity stimulus.

Subashan Perera, PhD is a Professor, Division of Geriatric Medicine and Department of Biostatistics at the University of Pittsburgh and has over 20 years of experience in providing statistical support to health science research at major academic medical institutions. He is a Senior Statistician and Co-Leader of Data Management, Analysis and Informatics Core of the NIA-funded Pittsburgh Claude D. Pepper Older Americans Independence Center. In addition, he is the principal source of statistical support to numerous clinical trials and intervention studies funded by NIH, PCORI, AHRQ, CMS and VA. Dr. Perera will play a key role in randomization, data analyses and publications.

Kara Hughan, MD is an Assistant Professor in the University of Pittsburgh School of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology and Diabetes. Dr. Hughan has designed and led the Phase I and early Phase II human subject trials with our IND-approved oral NITRITE at the University of Pittsburgh. She has extensive experience with evaluation of pK/pD, safety and efficacy of oral NO_2. As Co-Investigator, she will play a role in data collected on oral NITRITE drug metabolism (plasma nitrate/nitrite concentrations, RBC nitric oxide concentrations), safety (BP, MetHb) and efficacy (platelet and muscle mitochondrial function) and perform related statistical analyses on the data gained on these outcomes. Dr. Hughan will also assist with related trial regulatory/DSMB reporting. Dr. Hughan will work with Dr. Forman and his team

on implementing the nitrite therapy in this study. Dr. Hughan will provide leadership in protocol refinement, manuscript writing and publications.

Anne Newman, MD, MPH is the Chairperson of the Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh and has been a seminal leader in understanding the relationship between lean body mass, relative proportions and configuration of fat and related impact on strength and health. Dr. Newman will interpret related data from the study and participate in manuscript preparation.

Theodore Huppert, PhD is an Associate Professor in the Department of Radiology and has extensive experience in the area of non-invasive optical imaging (NIRS) both within the area of the human brain as well as muscle. He is the director of the NIRS Brain Imaging Laboratory at University of Pittsburgh Dr. Huppert is an biomedical engineer and will work with Dr. Forman and his team on implementing the NIRS measures in this study. Dr. Huppert will provide leadership in protocol refinement, manuscript writing and publications, and will participate on other study committees as needed. Dr. Huppert will also work closely with Dr. Forman on the collection and novel examination of NIRS data in association to fatigability.

Frank Sciurba, MD is an Associate Professor of Medicine at the University of Pittsburgh School of Medicine and the Medical Director of the Pulmonary Physiology Laboratory in the Division of Pulmonary, Allergy and Critical Care Medicine. Dr. Sciurba's current leadership positions include his role as a Principle Investigator of the Network Management Core of the new National Heart, Lung, and Blood Institute-sponsored Pulmonary Trials Consortium which manages the execution of pragmatic, “real world” studies in a variety of chronic pulmonary conditions; and his role as academic chair of the COPD Biomarker Qualification Committee, a group of academic, foundation and industry partners that work with U.S. Food and Drug Administration leadership to address the need for new biomarkers to facilitate development of drugs and devices for chronic pulmonary conditions

Paul Coen, PhD is an Associate Investigator at the Translational Research Institute Advent Health, Orlando, FL. He is the site PI at AdventHealth for this trial. Dr. Coen is an expert in mitochondrial bioenergetics both in respect to ex vivo and in vivo analytic techniques. He leads the Advent Hospital study team, and he will work closely with Dr. Forman in all aspect of analysis, presentation, and publication for this trial.

12.2 SOURCE OF SUPPORT

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