

Study of the Utility of Oral Nitrite Therapy to Improve Skeletal Muscle Bioenergetics and Physical Capacity in Older Heart Failure Patients.

Daniel Forman, MD
Principal Investigator

Professor of Medicine
University of Pittsburgh Medical Center
Chair, Geriatric Cardiology Section

Version 7
September 2, 2016

TABLE OF CONTENTS

PROTOCOL SYNOPSIS.....	3
1. STUDY OBJECTIVE, SPECIFIC AIMS, BACKGROUND AND SIGNIFICANCE.....	8
1.1 OBJECTIVE.....	8
1.2 SPECIFIC AIMS.....	9
1.3 BACKGROUND.....	9
1.4 SIGNIFICANCE.....	10
2. RESEARCH DESIGN AND METHODS.....	10
2.1 CLASSIFICATION AND METHODOLOGICAL DESIGN.....	10
2.2 DETAILED DESCRIPTION OF STUDY DESIGN.....	11
2.3 STUDY ASSESSMENT.....	14
2.4 STUDY ENDPOINTS.....	21
2.5 STATISTICAL ANALYSIS.....	21
3. HUMAN SUBJECTS.....	22
3.1 SUBJECT POPULATION.....	22
3.2 INCLUSION CRITERIA.....	22
3.3 EXCLUSION CRITERIA.....	23
4. RECRUITMENT AND INFORMED CONSENT PROCEDURES.....	23
4.1 RECRUITMENT METHODS.....	23
4.2 INFORMED CONSENT PROCEDURES.....	24
5. POTENTIAL RISKS AND BENEFITS.....	24
5.1 POTENTIAL RISKS.....	24
5.2 ALTERNATIVE TREATMENTS.....	28
5.3 POTENTIAL BENEFITS.....	28
5.4 DATA SAFETY MONITORING PLAN.....	28
5.5 RISK MANAGEMENT PROCEDURES.....	32
6. COSTS AND PAYMENTS.....	33
6.1 COSTS.....	33
6.2 PAYMENTS.....	33
7. QUALIFICATIONS AND SOURCE OF SUPPORT.....	34
7.1 QUALIFICATIONS OF THE INVESTIGATORS.....	34
7.2 SOURCE OF SUPPORT.....	35
8. REFERENCES.....	36

PROTOCOL SYNOPSIS

Protocol Title:	Study of the Utility of Oral Nitrite therapy to improve Skeletal Muscle Bioenergetics and physical capacity in older heart failure patients.	
Protocol Number:	PRO15020481	
NCT Number:		
Version # and Date:	Version 7.0 / September 2, 2016	
Clinical Phase:	Phase II clinical investigation	
Investigational Drugs:	Nitrogen 14 Sodium Nitrite (¹⁴ N Sodium Nitrite)	
Trial Site:	Single-Center Trial	
IND Sponsor:	Mark T. Gladwin, MD Professor of Medicine Chairman, Department of Medicine University of Pittsburgh School of Medicine	
Investigator:	Daniel Forman, MD Professor of Medicine Chair, Geriatric Cardiology Section, University of Pittsburgh Medical Center University of Pittsburgh 3471 Fifth Avenue, Suite 500 Pittsburgh, PA 15213	
Sub-Investigators:	Mark T. Gladwin, MD Sruti Shiva, PhD John Gorcsan, MD Ana Mora, MD Marc Simon, MD Nicole Helbling, MS, RN Manisha Jhamb, MD, MPH	
Study Monitor:	Education and Compliance Office for Human Subject Research Research Conduct and Compliance Office University of Pittsburgh 3500 Fifth Avenue, Suite 303 Pittsburgh, PA 15213	
Research Facilities:	UPMC Montefiore Hospital CTRC 3459 Fifth Ave. Pittsburgh, PA 15213 UPMC Montefiore Hospital Endocrinology and Metabolism Research Center (EMRC) 3459 Fifth Ave. Pittsburgh, PA 15213	

	<p>Pepper Center –SMART Center Kaufmann Medical Building, Suite 1200 3471 Fifth Ave. Pittsburgh, PA 15213</p> <p>UPMC Presbyterian/Montefiore Echocardiography lab 200 Lothrop St Pittsburgh PA 15213</p>
Clinical Laboratories:	UPMC Presbyterian University Hospital 3477 Euler Way Pittsburgh, PA 15213
Study Rationale:	<p>Heart failure (HF) is epidemic with aging and prevalence of HF is steadily increasing (1) as the population of older adults expands. While age stands out as a leading risk factor for HF incidence as well as for poor HF prognosis (2), few HF trials focus specifically on aging physiology as a key determinant of the disease and as a pertinent therapeutic target. Consistently, HF trials have tended to focus primarily on central mechanisms of cardiac pumping dysfunction (3) despite the fact that HF-outcomes are strongly related to functional decrements that are largely mediated by age- (4) and disease-related (5) peripheral manifestations. HF-related skeletal muscle myopathy is a component of HF that diminishes physical function, and which is likely exacerbated by sarcopenia, vascular stiffening, and other aspects of aging such that exercise intolerance is disproportionate among older HF populations as well as its insidious clinical implications (6). In a pilot investigation, we will study older (age ≥ 70 years) adults, including patients with HF with reduced ejection fraction (HFrEF), patients with HF with preserved ejection fraction (HFpEF), and age-matched healthy controls, to study benefits of enteral nitrite therapy (in addition to established standards of HF care) to improve physical function. In this pilot analysis we will focus on the utility of daily nitrite supplements to moderate aerobic (maximal and submaximal) and strength (maximal, endurance, and power) indices as well as underlying skeletal muscle mechanisms (skeletal muscle mitochondrial performance, gene expression, and capillarity). Studies have demonstrated nitrite therapy increases ATP synthesis in skeletal muscle mitochondria concomitant with reduced whole-body oxygen cost during steady state exercise (15). Our own work has demonstrated safety and efficacy of a FDA-IND approved sodium nitrite (10 mg) tablet, and its utility to upregulate the SIRT3-AMP pathway of skeletal muscle of younger HF patients (16).</p>
Study Objectives:	<p>Studies have demonstrated nitrite therapy increases ATP synthesis in skeletal muscle mitochondria concomitant with reduced whole-body oxygen cost during steady state exercise (15). Our own work has demonstrated safety and efficacy of a FDA-IND approved sodium nitrite (10 mg) capsule, and its utility to upregulate the SIRT3-AMP pathway of skeletal muscle of younger HF patients (16). It now seems exceptionally logical</p>

	<p>and opportune to apply these insights to older HF patients and to delineate mechanisms of disease and aging that respond to nitrite therapy.</p>
<p>Study Hypothesis:</p>	<p>Oral supplementation with nitrite will intrinsically improve skeletal muscle performance in HF as a vital means to improve physical function and moderate effects of disease itself as well as well as to frailties and enfeeblements associated with the disease. This will potentially improve efficacy and quality of care, and also potentially mitigate the skyrocketing costs associated with aggregate HF management.</p>
<p>Study Aims:</p>	<ol style="list-style-type: none"> 1. To demonstrate that oral nitrite pills provide skeletal muscle physiological benefit in old HFrEF and HFpEF patients. 2. To show that oral supplements are manifest as increased plasma nitrite. 3. To show that increased plasma nitrite is associated with improved skeletal muscle (mitochondrial respirometry) and platelet (Seahorse XF) metabolism. 4. To demonstrate that improved metabolism is associated with shifts in skeletal muscle anabolic gene expression (FNDC5, PGC1α, Sirtuin 3) as well as reduced catabolic gene expression (ubiquitin [MuRF, Atrogin1]) and inflammation (TNFα, IL-1β, IL-6). 5. To demonstrate that improved skeletal physiology achieved using oral nitrate pills is associated with improved clinical indices in old HFrEF and HFpEF patients. 6. To show that oral nitrite supplements increase efficiency of work, i.e., reduced oxygen uptake (VO₂) required for the same work intensity.
<p>Study Design:</p>	<p>The study aims to assess the safety and feasibility of oral nitrite in adults with HFrEF, HFpEF or healthy control.</p> <p>Subjects who meet the Inclusion Criteria and none of the Exclusion Criteria will receive oral sodium nitrite for 4 weeks. We will stratify dosing as below:</p> <p>Study drug to be initiated and prescribed as below:</p> <ol style="list-style-type: none"> a. 20 mg tid to subjects who <ol style="list-style-type: none"> i. are \geq80 years of age or, ii. are age 70 to <80 years with recent history of low blood pressure or other factors that PI judges to be at higher risk for hypotensive response b. 40 mg tid to subjects who <ol style="list-style-type: none"> i. are age 70 to <80 with no recent history of low blood pressures or PI judges to be at lower risk for hypotensive response c. Subjects to ingest drug once in the morning and again in the early afternoon and again in the evening, approximately 6 hours apart.

	<p>Subjects will be evaluated as outpatients with the first dose of study drug. Blood pressure, heart rate, methemoglobin levels and plasma nitrite will be measured in the first two hours after dosing. Every week safety visits will occur by phone to assess symptoms, adverse events (AE), interval histories and medication compliance review.</p> <p>Subjects will also be contacted by the study investigator and/or the study coordinator by phone for a follow-up assessment one week following completion of Visit 5.</p>
Planned Sample Size:	30 subjects
Duration of Treatment:	4 weeks
Major Inclusion Criteria:	<p>Inclusion Criteria HF Subjects</p> <ul style="list-style-type: none"> • NYHA class II or III for the previous three months despite a minimum of 6 weeks of treatment. Echo criteria will be confirmed as part of the initial study assessment. • Age ≥ 70 years • HFrEF patients left ventricular ejection fraction [LVEF] $< 40\%$ • HFpEF patients LVEF $\geq 40\%$, may also include $E/E' > 8$, left atrial size > 40 mL/m² • Optimal therapy according to AHA/ACC and HFSA HFrEF guidelines, including treatment with ACEI and beta-blocker therapy (for at least 6 weeks), or have documented reason for variation, including medication intolerance, contraindication, patient preference, or personal physician's judgment. • Patients using aspirin (ASA) will be eligible, but asked to hold the medication for 48 hours prior to biopsy. This technique has previously been used with consistent safety. Patients will also be asked to avoid non-steroidal anti-inflammatory medications (NSAIDs) for 48 hours prior to the biopsy. • Patients using anti-thrombin and anti-platelet therapy will plan to modify prior to muscle biopsies individually in coordination with the participant's primary provider or cardiologist. • Baseline BP $\leq 170/95$: subjects with either systolic or diastolic over stated level are ineligible. • Subjects who report using PDE-5s will be asked to hold the medication 24 hours before initial nitrite dose and for the entire period on study drug. <p>Inclusion Criteria Age-Matched Control Population</p> <ul style="list-style-type: none"> • Age ≥ 70 years • Absence of clinically significant chronic disease processes (including cardiovascular disease, diabetes, cancer, CKD, liver disease, COPD, and/or osteoarthritis). Non-significant

	<p>cardiac rhythm abnormalities and stable hypertension are acceptable for inclusion</p> <ul style="list-style-type: none"> • Baseline BP \leq170/95: subjects with either systolic or diastolic over stated level are ineligible. • Subjects who report using PDE-5s will be asked to hold the medication 24 hours before initial nitrite dose and for the entire period on study drug. • Patients using aspirin (ASA) will be eligible, but asked to hold the medication for 48 hours prior to biopsy. This technique has previously been used with consistent safety. Patients will also be asked to avoid non-steroidal anti-inflammatory medications (NSAIDs) for 48 hours prior to the biopsy. • Patients using anti-thrombin and anti-platelet therapy will plan to modify prior to muscle biopsies individually in coordination with the participant's primary provider or cardiologist. <p>Exclusion Criteria All Participants</p>
<p>Major Exclusion Criteria:</p>	<ul style="list-style-type: none"> • Allergy to lidocaine • Anemia: Hgb $<$11.0 in men and $<$10.0 in women • Dementia • End-stage malignancy • Orthopedic exercise limitation • Chronic use of oral corticosteroids or other medications that affect muscle function. • Chronic ETOH or drug dependency. • 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). • Psychiatric hospitalization within the last 3 months • Chronic nitrate use • Baseline BP $<$100/60 (either value) • Refusal to participate in muscle biopsy <p>Exclusion Criteria HF Population</p> <ul style="list-style-type: none"> • Major cardiovascular event or procedure within the prior 6 weeks. • HF secondary to significant uncorrected primary valvular disease (except mitral regurgitation secondary to left ventricular dysfunction). If valve replacement has been performed, patient may not be enrolled for 12 months after this procedure. • Severe valvular heart disease • Mechanical valve replacement requiring warfarin • Currently taking clopidogrel or other anti-platelet medication for a recent stent placement and/or a complex atherosclerotic lesion such that holding clopidogrel (or similar medications) creates disproportionate risk. This

	<p>decision will always be made by the patient's own primary provider or cardiologist.</p> <ul style="list-style-type: none"> • ICD device with heart rate limits that prohibit exercise assessments. Referring physicians will be provided with an opportunity to reprogram devices so that patients can participate.
Study Endpoints:	<p>Overall, study endpoints pre- and post- nitrites will include:</p> <ol style="list-style-type: none"> 1. Skeletal muscle bioenergetics: Mitochondrial function will be assessed using respirometry. Polymerase chain reaction (PCR) to assess pertinent gene expression (ubiquitin [MuRF, Atrogin1, FoxO], FNDC5, PGC1α, Sirtuin 3). 2. Serology: plasma BNP, inflammatory markers, plasma nitrite and platelet bioenergetics (using Seahorse XF analysis) (40), i.e., including glycolytic as well as basal and maximal respiratory rates. 3. Measures of physical function will include CPX indices, gait speed, handgrip strength, and balance. 4. Quality of life: Kansas City Cardiomyopathy Questionnaire or SF-12.

1. OBJECTIVE, SPECIFIC AIMS, BACKGROUND, AND SIGNIFICANCE

1.1 OBJECTIVE

Studies have demonstrated nitrite therapy increases ATP synthesis in skeletal muscle mitochondria concomitant with reduced whole-body oxygen cost during steady state exercise (15). Our own work has demonstrated safety and efficacy of a FDA-IND approved sodium nitrite (10 mg) capsule, and its utility to upregulate the SIRT3-AMP pathway of skeletal muscle of younger HF patients (16). It now seems exceptionally logical and opportune to apply these insights to older HF patients and to delineate mechanisms of disease and aging that respond to nitrite therapy.

1.2 SPECIFIC AIMS

Hypothesis:

Oral supplementation with nitrite will intrinsically improve skeletal muscle performance in HF as a vital means to improve physical function and moderate effects of disease itself as well as well as to frailties and enfeeblements associated with the disease. This will potentially improve efficacy and quality of care, and also potentially mitigate the skyrocketing costs associated with aggregate HF management.

Specific Aims:

Aim 1: To demonstrate that oral nitrite pills provide skeletal muscle physiological benefit in old HFrEF and HFpEF patients

Aim 2: To show that oral supplements are manifest as increased plasma nitrite.

Aim 3: To show that increased plasma nitrite is associated with improved skeletal muscle (mitochondrial respirometry) and platelet (Seahorse XF) metabolism.

Aim 4: To demonstrate that improved metabolism is associated with shifts in skeletal muscle anabolic gene expression (FNDC5, PGC1 α , Sirtuin 3) as well as reduced catabolic gene expression (ubiquitin [MuRF, Atrogin1]) and inflammation (TNF α , IL-1 β , IL-6).

Aim 5: To demonstrate that improved skeletal physiology achieved using oral nitrite pills is associated with improved clinical indices in old HFrEF and HFpEF patients:

Aim 6: To show that oral nitrite supplements increase efficiency of work, i.e., reduced oxygen uptake (VO₂) required for the same work intensity.

BACKGROUND

1.3. Longevity is increasing in the United States and much of the world, and the older adult population is growing as more people are surviving into old age (13). Since aging is also associated with predictable morphological and physiological changes that are conducive to cardiovascular disease (CVD), prevalence of CVD is increasing as the older adult population expands (6,17). Consistently, prevalence of heart failure (HF) with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF) are both growing (18). HFrEF and HFpEF are associated with high morbidity and mortality among older adults, as well as with decreased quality of life and increased dependency. Vulnerability to and consequences from exercise intolerance are especially significant in older HF patients. Both HFrEF and HFpEF are associated with reduced type 1 skeletal muscle fibers (7,8,9), and clinical manifestations are compounded by age-related reductions of type 2 muscle fibers (19) as well as associated predispositions to increased interstitial fat and inflammation in skeletal muscle (20,21). Exercise intolerance in HFrEF and HFpEF is common and insidious, and predisposes to HF's notoriously poor outcomes and associated (high) costs (stemming from increased hospitalizations, increased dependency, and increased frailty). Interventions to intrinsically improve skeletal muscle health may prove vital therapeutic benefit and also help moderate today's mounting healthcare expenditures.

Peripheral mechanisms of exercise intolerance in HFrEF have been recognized for many years. Seminal work by Sullivan, Massie, and Hambrecht (6) and other preeminent investigators have demonstrated diminished type I skeletal muscle fibers, mitochondria (size and density), and capillarity in HF patients. Related work has demonstrated increased inflammation (particularly TNF α) (22) and increased ubiquitin proteolysis, myostatin, and other catabolic factors in HFrEF (23). While skeletal muscle changes associated with HFpEF have often seemed more ambiguous than in HFrEF, recent work by Kitzman et al. demonstrates decrements in Type I fibers in HFpEF similar to those seen in HFrEF, and correlation to similar reductions in peak oxygen uptake (VO₂) (9).

There has been much enthusiasm about the utility of exercise training to modify HF skeletal muscle changes (24, 25). Benefits have been established in HFrEF and HFpEF, but training for either is also similarly confounded by the high prevalence of comorbidities, especially those who are older and who are also inherently prone age-related pulmonary disease (COPD, pulmonary hypertension, sleep apnea), metabolic disease (diabetes), osteoarthritis depression, and other morbidities, as well as to geriatric syndromes (frailty, falls, incontinence, sensory impairments, poor sleep) and logistic impediments (transportation, financial) (13). Even HF-ACTION, a well-

funded trial that included significant behavioral components to reinforce exercise compliance in a large HFrEF study population, achieved notably suboptimal exercise adherence (14).

Nitrite therapy is a relatively novel concept to potentially modify skeletal muscle bioenergetics and which may thereby improve physical function. Shiva et al demonstrated the capacity of nitrite to be converted nitric oxide (NO) in response to ischemic stress. NO then can bind to cytochrome c oxidase of the mitochondrial electron transport chain, reducing electron transport chain and utilization (26, 27). Key clinical correlates in multiple animal and human trials include increased exercise time and decrease oxygen uptake (VO_2) efficiency (i.e., reduced VO_2 for an equivalent workload). In one such trial Larsen et al. compared healthy men before and after dietary nitrate supplements. Gross efficiency increased from 19.7 ± 1.6 to $21.1 \pm 1.3\%$, with no differences in heart rate, lactate, ventilation (VE), VE/VO_2 , or the respiratory exchange ratio (RER) indicating this was mediated purely as an intrinsic skeletal muscle change (28). Nitrite rich beetroot juice supplementation has been demonstrated to improve time required to achieve a pre-specified work level for several sports and to also improve performance during intense intermittent exercise (29).

1.4 SIGNIFICANCE

The proposed research responds to a critical need to elucidate peripheral mechanisms that contribute to functional limitations of older HF in older adults (i.e., abnormal skeletal muscle bioenergetics) and to assess the related benefits of a novel therapy that can potentially mitigate these abnormalities, and thereby enhance vital functional attributes of HF patients. Theoretically, both HFrEF and HFpEF patients may benefit. Potential benefits pertaining to HFpEF patients are particularly exciting to consider, as currently there is no established therapy for this common disease.

2. RESEARCH DESIGN AND METHODS

2.1 CLASSIFICATION AND METHODOLOGICAL DESIGNS

The study is a 4-week open-label, single-center, non-randomized drug treatment study of 3 groups: HFpEF, HFrEF, healthy age-matched controls.

2.2 DETAILED DESCRIPTION OF STUDY DESIGN

Subjects will undergo a screening visit to determine that eligibility requirements are met. Prior to receiving the study drug, subjects will be scheduled within 4 weeks of screening for a baseline assessment.

Subjects who meet the Inclusion Criteria and none of the Exclusion Criteria will be provided study drug. The study population ($n=30$) will receive oral sodium nitrite combination for 4 weeks:

Drug: oral formulation of sodium nitrite 20 or 40 mg oral three times daily or TID (once in the morning and again in the early afternoon and again in the evening)

Subjects will complete a 4-week outpatient treatment period. During the treatment period, subjects will be evaluated at weekly intervals. Throughout the study period, subjects will be asked to remain diligent in recording the time of study drug dosing and any associated symptoms (diary cards will be pre-dated).

2.2.1. Study Drug Preparation and Distribution

The nitrite formulations will be prepared at and obtained from the Investigational Drug Pharmacy Service at the National Institutes of Health (NIH-IDS). The University of Pittsburgh Medical Center Investigational Drug Pharmacy Service (UPMC-IDS) will be utilized for dispensing of the study drug.

The daily study drug is:

¹⁴N Sodium nitrite

Standard sodium nitrite will be supplied as one or two 20 mg capsules, three times daily oral administration at the dose strength of 60 or 120 mg qd.

2.2.2. Dose Selection

The Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) proposed an Acceptable Daily Intake (ADI) for nitrate in 1990 based on harmless daily intakes of ≤ 500 mg of sodium nitrate/per kg of body weight in rats and dogs. They divided this dose by an uncertainty factor of 100 to yield an ADI of 5 mg of sodium nitrate or 3.7 mg nitrate ion per kg of body weight. An ADI for nitrite ion was derived and revised in 2003 based on intakes of 10 mg of sodium nitrite or 6.7 mg nitrite ion per kg of body weight in rats. They divided this dose by an uncertainty factor of 100 and rounded to yield an ADI for sodium nitrite of 0.1 mg/kg of body weight.

A recent modeling of the fruit and vegetable consumption patterns of The Dietary Approaches to Stop Hypertension (DASH) diet was done choosing foods that were low or high in nitrate. The "high nitrate" DASH diet pattern would result in consumption of 1,222 mg nitrate ion and 0.351 mg nitrite ion daily. The "low nitrate" DASH diet would result in consumption of 174 mg nitrate ion and 0.41 mg nitrite ion daily. The hypothetical high-nitrate DASH diet pattern exceeds the WHO's ADI for nitrate ion by 550% for a 60-kg adult. Subjects on this diet exhibited lower blood pressure with no adverse effects.

In a recent study, single dose potassium nitrate (KNO₃) capsule ingestion in healthy adult volunteers in doses of 2,424 mg KNO₃ (or 1,488 mg nitrate) and 1,212 mg KNO₃ (or 744 mg nitrate) resulted in dose dependent reductions in BP over a 24 hour period without adverse effects.

Hunault et al. reported an open-label pharmacokinetic crossover study in 9 healthy adult subjects who received two single high dose oral sodium nitrite solutions equivalent to 290-380 mg and 140-190 mg sodium nitrite, respectively, and one intravenous sodium nitrite dose of 290-380 mg. Mild headache occurred in 44-55% and up to 22% experienced nausea, which subsided within half an hour during each treatment session. Lowered blood pressure accompanied by increased heart rate were observed after each higher dose treatment, whereas the lower dose did not induce notable changes.

Chronic dietary supplementation of inorganic sodium nitrate in obese, hypertensive, insulin resistant mice lacking the gene for endothelial NO synthase (eNOS) (20) was studied. With a 10 week treatment of a modest dose of sodium nitrate of 0.1 mmol/kg/day (8.5 mg/kg/day) in the drinking water, significant reductions in blood pressure, fasting blood glucose, HbA1C, and proinsulin to insulin ratio were demonstrated, indicating reduced demands on the β -cell for insulin secretion.

Sodium nitrate drug is considered a source of nitrite through salivary nitrate reduction. Despite the plasma nitrite rise following a single oral sodium nitrate dose, Drs. Gladwin, Hughan and Shiva recently demonstrated in a phase I PK/PD study in 10 normal volunteers that no persistent blood pressure reduction with sodium nitrate 1,000 mg. In contrast, in the same phase I PK/PD study in

10 normal volunteers, they showed blood pressure decline in systolic, diastolic and mean arterial pressures over 3 hours with sodium nitrite 20 mg. The oral sodium nitrite half-life was about 40-minutes and the effects of nitrite only lasted ≤ 6 hours. This was demonstrated by the plasma nitrite levels returning to baseline levels between 3 and 6 hours after nitrite dosing (i.e. the drug was given and gone out of the circulation within 6 hours). Specifically, mean baseline (pre-nitrite dosing) blood nitrite concentrations were 0.129 micromolar with a peak at 30 minutes of 5.5 micromolar, and return to baseline levels between the 3- (0.176 micromolar) and 6-hour (0.103 micromolar) monitoring time points. In addition, there were no serious adverse events when these normotensive individuals received sodium nitrite.

Next, a recently published study (Mohler III ER, Hiatt WR, Gornik HL, Kevil CG, Quyyumi A, Haynes WG, Annex BH. Sodium nitrite in patients with peripheral artery disease and diabetes mellitus: safety, walking distance and endothelial function. *Vascular Medicine* 2014; 19(1):10-17) randomized adults 1:1:1 to oral sodium nitrite 40 mg BID vs. 80 mg BID vs. placebo for 10 weeks in >50 adults (age 35-85 years) with peripheral arterial disease +/- diabetes. The dose was escalated after the 10 weeks for 1 additional week in all 3 groups (dose-doubling) to 80 mg BID vs. 160 mg BID vs. doubling of placebo, respectively. The dosing was well tolerated, especially in the 40 mg BID and 80 mg BID randomized dosings. (Our proposed study dosing of 20-40 mg TID (60-120 mg total daily) falls just below and in the middle of the above two total daily doses of 80 mg and 160 mg daily.) Safety was monitored throughout all aspects of the study. No serious adverse events occurred in the two sodium nitrite groups. There were no imbalances in GI, musculoskeletal, respiratory, vascular or skin disorders nor shifts in liver function or creatinine levels. Pertinent safety outcomes revealed that methemoglobin changes never exceeded 3% in any nitrite dose regimen and no clinically significant change in resting blood pressure.

A current phase II study of inhaled nitrite in patients with pulmonary arterial hypertension (PAH) at the University of Pittsburgh uses a dose of 80 mg, and is approved by the University of Pittsburgh IRB and FDA. Also, phase II studies went as high as 90 mg three times a day, approved by the FDA. So, our dose of 20-40 mg TID will not be out of line of approved dosing.

Nine of 10 control subject enrollees into this pilot study have tolerated 40 mg po tid without incident; the one exception was a subject who had a decrease in systolic BP at pK but was asymptomatic. In the three heart failure subjects enrolled to date we have observed a vasoactive response at the pK with the 40 mg dose, such that low blood pressures or symptoms have been observed in the 2 hour monitoring period. This includes subjects with preserved and reduced ejection fraction. Therefore, we postulate the heart failure subjects appear to have a greater vasodilatory response to nitrite and could require a stratified dose based on age and baseline risk for hypotension. To further ameliorate risk, we will serve a standardized meal prior to drug dosing at Visit 3 so that lack of po intake is not a causative factor of hypotension.

In summary, based on the above findings, inclusive of:

- The Joint FAO/WHO ADI for sodium nitrate and nitrite,
- the above DASH diet patterns,
- the recently observed human and rodent effects of nitrate and nitrite on blood pressure and glucose homeostasis, including our PK/PD trial of single doses of oral nitrate and nitrite,
- the open label trial safety and BP findings following a total of 10 subjects' completion,
- the existing inhaled nitrite trial,
- the preliminary findings of the current nitrite protocol with regard to blood pressure in heart failure subjects,

the study investigators believe that a safe and likely therapeutically effective dose of sodium nitrite will be 20-40 mg three times daily (~0.21-0.42 mg/kg body weight per day for a 95 kg subject). The 40 mg sodium nitrite dose selection is equivalent to 12.6 times the ADI.

2.2.3. Treatment Period

Subjects will undergo study treatment duration of 4 weeks of 14N sodium nitrite 20-40 mg three times daily. This is considered appropriate to study benefits of oral nitrite therapy to improve physical function in patients with HFrEF and HFpEF and age-matched controls.

2.2.4. Safety Monitoring:

Subjects will be contacted by phone weekly and this will include symptom review, assessing adverse events, interval histories and medication compliance.

In addition to these phone evaluations, subjects will be encouraged to immediately contact the study investigator and/or the study coordinator with questions, concerns, or to report new symptoms that occur during their study participation. If there are particular concerns that need to be addressed the subject will be asked to return to the EMRC or SMART Center as soon as possible for evaluation. Evaluations will be planned on an individual basis (depending on each individual's circumstances) and may include weight, vital signs, methemoglobin %, medication compliance review, and assessment by the study physician. If appropriate, based upon the evaluation, medical treatments will be provided to subjects, including appropriate referral to physicians or other services at the UPMC.

2.2.5. Medication Compliance

Subjects will self-administer their first dose of study medication under the supervision of the CTRC nurse (physician investigator will be immediately available) in the outpatient CTRC during Visit 3, following completion of all baseline clinic assessments. Subjects will also be dispensed their study medication (42-day supply) for home use, including four 7-day prefilled and labelled pill boxes and drug/symptom diary cards to help with compliance and to help reduce burden or confusion. (Visit 4 may be scheduled up to and on the 42nd day from visit 3 (25-35±5 days); additional drug will be distributed with the initial dispensation if needed to meet the need.) Subjects will return the previously dispensed study medication when they return for Visit 4 approximately 4 weeks later (+1 week if need be for flexibility of scheduling) and medication compliance will be assessed. The study coordinator will review the daily diary card with each phone call evaluation and also assess medication compliance with queries regarding empty pill compartments in the current 7-day box and remaining boxes. In the event that the compliance rate is <80% (subjects' report of doses taken/total doses prescribed at that date & time *100), subjects will be re-educated on medication compliance. If medication compliance repeatedly falls outside of the acceptable range, the study investigators will discuss subject eligibility for continued participation in the study.

2.2.6. Medication Accountability

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

Dose reduction

There is a rare possibility that subjects will have a high methemoglobin level or low blood pressure from the study drug dose during visit 3 or while they are on the drug. If this happens, they may experience lightheadedness or dizziness.

In CTRC:

If this occurs in the CTTC, 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 it is appropriate for the subject to proceed with study participation prior to discharge from CTTC. This will include what dose is appropriate and drug dispensing. The same process will be applied to subjects who are initially initiated on 20 mg; however, in these subjects there is relatively greater consideration of withdrawal as no lower dosing options are available.

As outpatient:

Blood pressure and related symptoms. If subjects experience lightheadedness or dizziness after visit 3 while they are on the drug, we request that they call the study coordinator who will inventory their symptoms, compare to baseline levels (measured at pK), and discuss need for interim visit with study physician. If a visit is indicated, we will measure blood pressure, RR, heart rate, and check methemoglobin level by a finger probe. A study physician will evaluate each subject, including:

1. review of all clinical measures,
2. subject history of symptoms,
3. blood pressures pre- and while on drug.

The physician will confirm a management plan including the possibility of lowering dose to 20 mg tid or need to discontinue and drop from the trial. Subjects will be reassessed via phone each day for the next 2 days thereafter to ensure that the patient is stable on the new dose. If stable, subjects will continue with the half dose. However if there are continued symptoms on lower doses, the physician investigator will reevaluate the patient's suitability for continued participation based on symptoms and/or hemodynamics.

If subjects initiated at Visit 3 on the 20 mg dose report symptoms as described above, the same process will be followed; however, the physician may have to withdraw the subject since a dose lower than 20 mg tid is not an option.

Methemoglobin. If subjects report change in skin tone and dizziness or other signs of methemoglobinemia for ≥ 24 hours, an interim visit will be requested. Methemoglobin will be assessed. If greater than 5%, a nitrite dose reduction is proposed from 40 mg three times daily to 20 mg three times daily. Subjects will be contacted via phone for the next 2 days for symptom re-assessment. If symptoms remain resolved, subjects will continue on 20 mg tid. If symptoms persist, subjects 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.

Safety of holding anti-platelet or antithrombotic medications

The research physician and the staff under his auspice will guide holding of the antiplatelet and anti-thrombotic medications. In each case, this will be discussed doctor to doctor by the research doctor and the patient's PCP or cardiologist.

2.3 STUDY ASSESSMENT

The complete study assessment and procedures are outlined in Table 1.

2.3.1. Study Visits and Procedures

Visit 1 (Screening)

The outpatient screening will take place in either the Montefiore Clinical and Translational Research Center (CTRC) or the Endocrinology Metabolism Research Center (EMRC) at the UPMC Montefiore Hospital and will last approximately 2-3 hours.

- Obtain written informed consent
- Complete history and physical examination to include body weight and height
- Medical history obtained prior to visit will be reviewed with subject. Subjects will be asked questions about any heart disease and related diseases in first degree relatives; no identifying information will be obtained. Dr. Forman will review risk factors for hypotension in subjects who are 70 to less than 80 years of age at the physical exam, in preparation for dose selection. Subjects will be asked while they are in the study to report promptly any changes in their health or medications so that the PI can evaluate any implications with study drug or participation.
- Hemoglobin and hematocrit to assess for anemia
- Blood pressure and other vital signs (temperature, pulse oximetry, breathing rate, heart rate) Blood pressure may be re-measured multiple times if out of inclusion range. This is to allow for influences of medication activation, subject nervousness, lack of fluid intake, and known factors which contribute to clinical variation.
- Transthoracic Echocardiogram (performed by trained study staff) - results of echo will confirm group placement. If subjects have had an echocardiogram within 4 weeks of the consent date, the records will be obtained prior to visit 1 and the test will be reviewed and interpreted by a study physician. Trained study staff doing echos will consist of cardiologists doing advanced echo training at UPMC under the supervision of Dr. Gorcsan. Drs. Gorcsan, Simon and Forman are responsible for all echocardiography procedures and interpretations. The subject may be transported to a lab in the UPMC Presbyterian/Montefiore system for the echocardiogram.

Screening evaluations will include a hemoglobin and hematocrit by the Presbyterian Hospital clinical automated testing lab and are to be covered by research funds. The total volume of blood drawn is approximately 1 teaspoon (4 mL). If laboratory results exclude low hemoglobin (<11.0 g/dL males, <10.0 females) the subject will be called to arrange the baseline assessments.

Subjects who fail to meet applicable inclusion/exclusion criteria based upon the results of the screening assessment will be excluded from further study participation.

An important component of the protocol is the muscle biopsy. Subjects who refuse the first biopsy will be excluded at screening. Participants who undergo the first biopsy at Visit 3 may refuse a second pass. (Participants may also refuse a second biopsy pass at Visit 5). However, participants who refuse to complete a second biopsy at Visit 5 (prior to starting sodium nitrite) will be withdrawn from the study. Alternatively, patients who refuse the 2nd biopsy after they have completed the 4 weeks of nitrite therapy will still be included.

Visit 2 (First Baseline Assessment Visit)

Subjects will be scheduled for an outpatient EMRC and Pepper SMART Center visit within 4 weeks of screening. The subject will be asked to refrain from caffeine the morning of this visit and to limit breakfast to a lighter meal prior to coming in. The visit will last approximately 3 hours.

Interval history and brief physical exam: temperature, blood pressure, pulse oximetry, breathing rate, heart rate and body weight

Physical Function Battery:

- Cardiopulmonary Stress Exercise Testing: Exercise testing in association with air-gas-exchange, an optimal gauge of aerobic capacity will be conducted by trained research exercise

physiologists. Dr. Forman or another physician will be immediately available if needed. A computerized cycle ergometer using a ramp protocol will be used to generate a symptom limited exercise stimulus. A lightweight disposable pneumotach device face mask will be positioned over the subject's mouth and nose during the exercise for gas exchange assessments of VO_2 , VE/VCO_2 slope, and respiratory exchange ratio (RER) will be measured as well as hemodynamics (max heart rate [HR] and blood pressure [BP]), time, and ECG waveforms). Any unexpected abnormalities will be reported to a patient's cardiologist or primary care physician; continued participation in the study will require physician clearance. Both the Borg Rate of Perceived Exertion (RPE) and the Modified Borg Scale for Perceived Dyspnea (Shortness of Breath) will be completed during the CPX. The EMRC has performed similar tests in more than 1,000 volunteers ranging from elderly women in their 80's to highly trained athletes.

- Short Physical Performance Battery (SPPB): a brief performance battery is used to assess how well older persons perform simple movements that represent the building blocks of daily activities that require good lower extremity function. It is based on timed 4 meter walk, repeated chair stands, and a set of balance tests.
- Fatigability will be assessed based on rate of perceived exertion (RPE 6-20 scale) reported after a steady state walking on a treadmill. Patients will walk 1.5 miles/hour (0.67 m/sec) for 5 minutes on a treadmill as per techniques validated by Simonsick, et al. (J Am Geriatr Soc. 2014;62:347-51). Those with RPE ≥ 10 will be categorized as highly fatigued. Our prior work in HF patients indicates this steady state walking provocation is feasible for the patients we anticipate we will enroll, and that it will (appropriately) generate a wide range of perceived fatigability responses for meaningful analyses of underlying mechanisms. Aerobic capacity will be assessed with gas-exchange (as noted above) during the steady state walk for VO_2 , VE/VCO_2 slope, and respiratory exchange ratio (RER).
- Hand Grip Strength Assessment will be performed to measure muscle strength. Participants will be asked to sit in a chair without arms and squeeze a device as hard as they can with one hand at a time, rest and switch to the other hand.
- The Kansas City Cardiomyopathy Questionnaire will be used to measure disease-specific health-related quality of life (QOL). This 23-item instrument produces the following scores: Physical Limitation, Symptoms, Symptom Stability, Social Limitation, Self-efficacy, Quality of Life, and two summary measures, and is widely used as a standard by which quality of life, self-efficacy, and other personal perspectives related to functional capacity at baseline and over time. Subjects in the healthy elderly control group without HF will be asked to complete the SF-12 which is a practical, reliable and valid measure of physical health.
- 24-hour dietary recall. The study coordinator will collect a 24-recall of all foods eaten the day before from subjects for analysis for calories, macronutrients, sodium, nitrates and nitrites.

Visit 3 (Second Baseline Assessment Visit)

This visit involves a percutaneous muscle biopsy, blood draw, first dose of study medication and dispensing of study medication. To prevent acute effects of exercise on muscle mitochondria function, this fasting visit will occur 2-7 days after Visit 2. This visit will take place at the MUH-CTRC and will last 4-5 hours. Subjects will be asked to observe 8 hours fasting prior to arrival. Upon arrival subjects will be weighed and then served a standardized breakfast [15% of 30 calories/kg current body weight (CBW) and 15% of 1.0 gm protein/kg CBW, low nitrite/nitrate] in

the CTRC. A dietitian will review and adjust subjects' food choices as needed to meet the standardized diet prescription.

Interval history and brief physical exam: temperature, blood pressure, pulse oximetry, breathing rate, heart rate and body weight. Blood pressures may be re-measured to allow for clinical variation. Prior to the biopsy, study staff will call indicated subjects to hold their anti-platelet and anti-coagulation medications for 1-5 days (depending on the different medications and relative half-lives). In each instance a subject is on an anti-platelet or anti-coagulation medication, the plan to hold the medication prior to the biopsy will first be cleared by the study physician with the subject's own primary care provider or cardiologist. Physician orders for CTRC will include confirmation with subject upon arrival.

- Blood sampling via IV line to include: research blood for inflammatory peptides (IL-1, IL-6, IL-15, TNF α), platelet sample and nitrite levels. Blood samples will be drawn into a 1) heparinized syringe (baseline and approximately 30 mins. and 2 hours post study drug administration) and 2) a cell preparation tube (CPT) (baseline and approximately 30 mins and 2 hours post study drug administration). Heparinized blood will be immediately centrifuged at bedside to separate plasma and the plasma and red cells separately flash frozen for later nitrite measurements (via reductive chemiluminescence to determine that nitrite levels were increased in the muscle as described. The second tube of blood (CPT tube) will be immediately subjected to differential centrifugation in order to isolate platelets. Platelets will then be subjected to Seahorse extracellular flux analysis in order to measure bioenergetic profile including basal, non-ATP producing, maximal and non-mitochondrial respiratory rates along with glycolytic rate. Extra aliquots will be stored for use in future studies. Two 10 ml purple top and 2 10 ml red top tubes will be used to collect blood for inflammatory peptides. Total volume of blood drawn will be 88 ml (6 tablespoons).

- Skeletal Muscle Biopsies: Patients will lie comfortably in the supine position for biopsy of the vastus lateralis muscle of the non-dominant leg. Biopsy sites will be prepped with betadine. 2% xylocaine (without epinephrine) will then be administered, superficially initially and then more deeply into the muscle, using a small gauge needle. Subjects who have report a history of poor pain control or low threshold will be offered the use of a topical anesthetic cream prior to the xylocaine. Ultrasound may be used to enhance visualization of the site for the needle insertion. A small superficial incision will next be completed using a #11 blade scalpel. A 5 mm Bergstrom muscle biopsy needle will be inserted through the skin incision and advanced into the muscle. Suction will be generated using a syringe attached to the outside portion of the needle, to thereby suck skeletal muscle into a hole on the side of the needle positioned in the muscle tissue; this draws a small piece of muscle tissue (about 150 mg of muscle tissue) into the hollow shaft of the needle, which is then cut with a cutting trochar that slides through the shaft to cut the tissue drawn within its core. After harvesting the first sample, the needle will be rotated 90 degrees, and a second sample extracted to maximize yield for analysis. Additional passes of the needle may be necessary for an adequate muscle sample. The wound site will be closed with steri-strips and a sterile pressure bandage. Muscle specimens will be immediately processed, and stored. One fragment will be placed in sterile cryotubes containing RNA preservation solution (RNAlater) and immediately frozen in liquid nitrogen for measurement of mRNA expression. A second fragment will have the muscle fibers aligned for microscopy, placed in OCT, then cooled in a thawing isopentane slurry and placed in a cryotube and frozen in liquid nitrogen for morphological analysis. One specimen will immediately be used for high resolution respirometry. Briefly, muscle fibers will be dissected and immediately placed in an Oroboros respirometry. Basal respiratory rate will be measured followed by respiration after the administration of oligomycin A to assess non-productive respiration and FCCP to measure maximal respiratory capacity. A separate muscle specimen will be saved for Western blots and to measure nitrite concentrations by reductive chemiluminescence.

Subjects will rest for approximately 30 minutes post biopsy while wound is dressed and prior to baseline vitals prior to dosing. Study staff will record a baseline level of symptoms, including shortness of breath, dizziness, lightheadedness for use during weekly phone calls, prior to dosing.

Following biopsy, subjects will receive their first dose of study drug as follows:

- 14N sodium nitrite, 20 or 40 mg per physician order
- Blood pressure (sitting and then standing after 3 minutes), HR, RR, pulse oximeter and methemoglobin will be measured to ensure they are at a steady state before taking the study drug (baseline) and then approximately every 15 minutes for the 2 hours post-drug administration. Any perturbations of concern will be addressed per the Dose Reduction protocol described in Section 2.2.6.
- AE assessment
- Provide a 42-day supply of study drug, pre-filled in 4 weekly pill boxes and an extra bottle and drug diary cards to assist with compliance and reduce burden or confusion. Study staff will review medication administration and use of the drug diary card with all subjects. Study staff or CTCR RN will review biopsy site care with subjects and if indicated, remind subjects to resume their normal anti-coagulation and anti-platelet medications the following day (as indicated).
- 24-hour dietary recall. The study coordinator will collect a second 24-recall of all foods eaten from subjects for analysis for calories, macronutrients, sodium, nitrates and nitrites.

Subjects will be reminded before departure to report promptly any changes in their medication, health or symptoms of concern, not to wait for the weekly call.

Visit 4 (First Completion Assessment visit to be scheduled 25-35±7 days following first dosing of 20-40 mg oral nitrite) Subjects who need more than 42 days' supply of drug due to scheduling later in window will be issued additional at visit 3.

This visit will take place in the EMRC and/or Pepper SMART Center and is identical to visit 2 (approximately 3 hours):

Interval history and brief physical exam: temperature, blood pressure, pulse oximetry for methemoglobin %, breathing rate, heart rate and body weight; additionally, AE and symptom assessment will be collected and medication compliance via drug diary cards and pill boxes will be assessed.

Physical Function Battery:

- Cardiopulmonary Stress Exercise Testing: Exercise testing in association with air-gas-exchange, an optimal gauge of aerobic capacity will be conducted by trained research exercise physiologists. Dr. Forman or another physician will be immediately available if needed. A computerized cycle ergometer using a ramp protocol will be used to generate a symptom limited exercise stimulus. A lightweight disposable pneumotach device face mask will be positioned over a subject's mouth and nose during the exercise for gas exchange assessments VO_2 , VE/VCO_2 slope, and respiratory exchange ratio (RER) will be measured as well as hemodynamics (max heart rate [HR] and blood pressure [BP]), time, and ECG waveforms). Any unexpected abnormalities will be reported to a patient's cardiologist or primary care physician; continued participation in the study will require physician clearance. Both the Borg Rate of Perceived Exertion (RPE) and the Modified Borg Scale for Perceived Dyspnea (Shortness of Breath) will be completed during the CPX. The EMRC has performed similar tests in more than 1,000 volunteers ranging from elderly women in their 80's to highly trained athletes.

- Short Physical Performance Battery (SPPB): a brief performance battery is used to assess how well older persons perform simple movements that represent the building blocks of daily activities that require good lower extremity function. It is based on a timed 4 meter walk, repeated chair stands, and a set of balance tests.
- Fatigability will be assessed based on rate of perceived exertion (RPE 6-20 scale) reported after a steady state walking on a treadmill. Patients will walk 1.5 miles/hour (0.67 m/sec) for 5 minutes on a treadmill as per techniques validated by Simonsick, et al. (J Am Geriatr Soc. 2014;62:347-51). Those with RPE ≥ 10 will be categorized as highly fatigued. Our prior work in HF patients indicates this steady state walking provocation is feasible for the patients we anticipate we will enroll, and that it will (appropriately) generate a wide range of perceived fatigability responses for meaningful analyses of underlying mechanisms.
- Hand Grip Strength Assessment will be performed to measure muscle strength. Participants will be asked to sit in a chair without arms and squeeze a device as hard as they can with one hand at a time, rest and switch to the other hand.
- The Kansas City Cardiomyopathy Questionnaire will be used to measure disease-specific health-related quality of life (QOL). This 24-item instrument produces the following scores: Physical Limitation, Symptoms, Symptom Stability, Social Limitation, Self-efficacy, Quality of Life, and two summary measures, and is widely used as a standard by which quality of life, self-efficacy, and other personal perspectives related to functional capacity at baseline and over time. Subjects in the healthy elderly control group will be asked to complete the SF-12.
- 24-hour Dietary recall. The study coordinator will collect a post 24-hour recall of all foods eaten from subjects for analysis for calories, macronutrients, sodium, nitrates and nitrites.

Visit 5 (Final Completion Visit - 2-7 \pm 7 days following Visit 4)

Subjects will be fasting and take their last dose of study drug at home on their usual schedule prior to scheduled testing time. Upon arrival to the CTRC, subjects will be served a standardized breakfast as described in visit 3, then interval history and brief physical exam; temperature, blood pressure, pulse oximetry for methemoglobin %, breathing rate, heart rate and body weight will be documented; additionally, AE and symptom assessment, medication compliance via drug diary cards and pill boxes, and confirmation of last dose will be performed prior to assessment procedures. Prior to the biopsy, study staff will call indicated subjects to hold their anti-platelet and anti-coagulation medications for 1-5 days (depending on the different medications and relative half-lives). This visit will last approximately 2 hours.

- Blood draw to include: research blood for inflammatory peptides (IL-1, IL-6, IL-15, TNF α), platelet sample and nitrite levels: Two blood samples will be drawn into a 1) heparinized syringe and 2) a cell preparation tube (CPT). Heparinized blood will be immediately centrifuged at bedside to separate plasma and the plasma and red cells separately flash frozen for later nitrite measurements (via reductive chemiluminescence to determine that nitrite levels were increased in the muscle as described). The second tube of blood (CPT tube) will be immediately subjected to differential centrifugation in order to isolate platelets. Platelets will then be subjected to Seahorse extracellular flux analysis in order to measure bioenergetic profile including basal, non-ATP producing, maximal and non-mitochondrial respiratory rates along with glycolytic rate. Extra aliquots will be stored for use in future studies. Total volume of blood drawn will be 56 ml (4 tablespoons)

• **Skeletal Muscle Biopsies:** Patients will lie comfortably in the supine position for biopsy of the vastus lateralis muscle of the non-dominant leg. Biopsy sites will be prepped with betadine. 2% xylocaine (without epinephrine) will then be administered, superficially initially and then more deeply into the muscle, using a small gauge needle. Subjects who have report a history of poor pain control or low threshold will be offered the use of a topical anesthetic cream prior to the xylocaine. Ultrasound may be used to enhance visualization of the site for the needle insertion. A small superficial incision will next be completed using a #11 blade scalpel. A 5 mm Bergstrom muscle biopsy needle will be inserted through the skin incision and advanced into the muscle. Suction will be generated using a syringe attached to the outside portion of the needle, to thereby suck skeletal muscle into a hole on the side of the needle positioned in the muscle tissue; this draws a small piece of muscle tissue (about 150 mg of muscle tissue) into the hollow shaft of the needle, which is then cut with a cutting trochar that slides through the shaft to cut the tissue drawn within its core. After harvesting the first sample, the needle will be rotated 90 degrees, and a second sample extracted to maximize yield for analysis. Additional passes of the needle may be necessary for an adequate muscle sample. The wound site will be closed with steri-strips and a sterile pressure bandage. Muscle specimens will be immediately processed, and stored. One fragment will be placed in sterile cryotubes containing RNA preservation solution (RNAlater) and immediately frozen in liquid nitrogen for measurement of mRNA expression. A second fragment will have the muscle fibers aligned for microscopy, placed in OCT, then cooled in a thawing isopentane slurry and placed in a cryotube and frozen in liquid nitrogen for morphological analysis. One specimen will immediately be used for high resolution respirometry. Briefly, muscle fibers will be dissected and immediately placed in an Oroboros respirometry. Basal respiratory rate will be measured followed by respiration after the administration of oligomycin A to assess non-productive respiration and FCCP to measure maximal respiratory capacity. A separate muscle specimen will be saved for Western blots and to measure nitrite concentrations by reductive chemiluminescence.

• **Transthoracic Echocardiogram.** The subject may be transported to UPMC Presbyterian for the echocardiogram.

• **24-hour Dietary recall.** The study coordinator will collect a second post 24-recall of all foods eaten the day before from subjects for analysis for calories, macronutrients, sodium, nitrates and nitrites.

End of Trial and Follow-Up Period

Post Study Drug Telephone Assessment (about 1 week following Visit 5)

• Telephone assessment for interval histories, muscle biopsy follow up and AEs.

Table 1. Study Assessment and Procedures

Visit	Screening	Baseline		Phone call follow-ups during intervention time				Final		FU
	1	2	3	1	2	3	4	4	5	Phone call
				1	2	3	4			1
Week of Treatment	-	-4~-1	-4~-1	1	2	3	4	5	5	6
Day of Treatment	-			7	~14	~21	~28	~25-35	V4+2-7d	
Time Window		V1+not >4 wks.	V2+ 2-7d	±2d	±7d	±7d	±7	±7d		About 1 week following V5
Visit Type	OP	OP	OP	Phone	Phone	Phone	Phone	OP	OP	Phone
Informed Consent / Eligibility Criteria	x									
History & Physical Exam	x									
Body Weight, Height (V1 only)	x	x	x					x	x	
Vital signs	x	x	x					x	x	
Methemoglobin %			x					x	x	
Research Labs			x						x	
Two Hour Safety Blood Sampling			x							
Two Hour HR, BP (sitting and after standing 3 mins.), Methemoglobin Monitoring: baseline & q15 min.			x							
Hemoglobin & hematocrit	x									
Echocardiogram	x								x	
Muscle Biopsy			x						x	
Physical Function Testing*		x						x		
Questionnaire-KCCQ or SF-12		x						x		
Meal/snack			x ^s						x ^s	
AE Assessment			x	x	x	x	x	x	x	x
Medication Compliance Review				x	x	x	x	x	x	
Interval History Assessment		x	x	x	x	x	x	x	x	X
Brief Physical Exam		x	x					x	x	
Symptom review			x	x	x	x	x	x	x	x
24 hour dietary recall		x	x					x	x	

^sStandard kitchen meal/snack

*includes nCPET, Steady State Walking, SPPB and Hand-grip strength

2.4 STUDY ENDPOINTS

Overall, study endpoints pre- and post- nitrites will include:

1. Skeletal muscle bioenergetics: Mitochondrial function will be assessed using respirometry. Polymerase chain reaction (PCR) to assess pertinent gene expression (ubiquitin [MuRF, Atrogin1, FoxO], FNDC5, PGC1 α , Sirtuin 3).
2. Serology: plasma BNP, inflammatory markers, plasma nitrite and platelet bioenergetics (using Seahorse XF analysis) (40), i.e., including glycolytic as well as basal and maximal respiratory rates.
3. Measures of physical function will include CPX indices, gait speed, strength (endurance, power), and balance.
4. Quality of life: Kansas City Cardiomyopathy Questionnaire.

2.5 STATISTICAL ANALYSIS

Sample Size Justification: We believe our proposed sample size is adequate for successfully addressing the aims of this initial investigation. Numerous published pilot studies leading to subsequent successful larger trials have used similar sample sizes (47, 48). Our prior data indicate between-subject standard deviation of approximately 32 pmol O₂/min/10⁶ cells in platelet mitochondrial respiration rate change subsequent to administration of nitrite (49). Based on this estimate, with the proposed sample sizes, we will be able to detect statistical significance of a within-group change as small as 32 pmol O₂/min/10⁶ cells, and a between-group difference as small as 42 pmol O₂/min/10⁶ cells with 80% statistical power in two-tailed tests conducted at $\alpha=0.05$. These correspond to large effect sizes by Cohen's *d* criteria (50), and thus afford an adequate level of statistical sensitivity in our pilot investigation.

Data Analysis: Our analytic strategy will involve formal statistical analyses, but in our interpretation, we will also pay attention to descriptive statistics, graphical summarizations of individual participant data such as needle plots (51,52), and magnitudes of parameter estimates from modeling in addition to their statistical significance based on *p*-values and a strict criterion based on $\alpha=0.05$. Such an interpretive strategy is appropriate in an initial study such as ours, where the cost of a missed finding is far greater than a false finding. We will fit a series of linear mixed models using the SAS[®] MIXED procedure (SAS Institute, Inc., Cary, North Carolina) with each of the skeletal muscle, platelet, gene expression, inflammatory, oxygen uptake and physical function related measures as the dependent variable; participant group (HF_rEF/HF_pEF/control), time point (baseline/4-week) and their interaction as fixed effects of interest; and a participant random effect to account for multiple measurements from the same participants. We will appropriately create means contrasts to estimate (a) between time changes in each of the groups corresponding to periods of usual care and nitrite usage, and (b) compare the said changes across the three groups. We will use Pearson/Spearman correlations to quantify strength of associations between changes in measures of skeletal physiology and those of strength, gait speed and balance. Finally, in addition to drawing preliminary conclusions, we will use our descriptive statistics for formal sample size computations in planning a larger subsequent trial to more definitively examine the impact of nitrites.

3. HUMAN SUBJECTS

3.1 SUBJECT POPULATION

The anticipated age range for the study population will be ≥ 70 years of age based upon the target disease population. Thirty subjects with any ethnic background who have a confirmed diagnosis of HFrEF/HFpEF/healthy control will be eligible for enrollment. All subjects must provide written informed consent prior to participation.

3.1.1 Inclusion of Women and Minority

Women 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.

3.1.2 Inclusion of Children

This investigation will not enroll children based upon the target disease population and lack of safety data in adults.

3.2 INCLUSION CRITERIA

Inclusion Criteria HF Population

- NYHA class II or III for the previous three months despite a minimum of 6 weeks of treatment. Echo criteria will be confirmed as part of the initial study assessment.
- Age ≥ 70 years
- HFrEF patients left ventricular ejection fraction [LVEF] $< 40\%$
- HFpEF patients LVEF $\geq 40\%$, may also include E/E' > 8 , left atrial size > 40 mL/m²
- Optimal therapy according to AHA/ACC and HFSA HFrEF guidelines, including treatment with ACEI and beta-blocker therapy (for at least 6 weeks), or have documented reason for variation, including medication intolerance, contraindication, patient preference, or personal physician's judgment.
- Patients using aspirin (ASA) will be eligible, but asked to hold the medication for 48 hours prior to biopsy. This technique has previously been used with consistent safety. Patients will also be asked to avoid non-steroidal anti-inflammatory medications (NSAIDs) for 48 hours prior to the biopsy.
- Patients using anti-thrombin and anti-platelet therapy will plan to modify prior to muscle biopsies individually in coordination with the participant's primary care provider or cardiologist.
- Baseline BP $\leq 170/95$: subjects with either systolic or diastolic over stated level are ineligible.
- Subjects who report using PDE-5s will be asked to hold the medication 24 hours before initial nitrite dose and for the entire period on study drug.

Inclusion Criteria Age-Matched Control Population

- Age ≥ 70 years
- Absence of clinically significant chronic disease processes (including cardiovascular disease, diabetes, cancer, CKD, liver disease, COPD, and/or osteoarthritis). Non-significant cardiac rhythm abnormalities and stable hypertension are acceptable for inclusion.
- baseline BP $\leq 170/95$: subjects with either systolic or diastolic over stated level are ineligible.
- Patients using aspirin (ASA) or NSAIDs will be eligible, but asked to hold the medication for 48 hours prior to biopsy. This technique has previously been used with consistent safety. Patients

will also be asked to avoid non-steroidal anti-inflammatory medications (NSAIDs) for 48 hours prior to the biopsy.

- Patients using anti-thrombin and anti-platelet therapy will hold these medications prior to muscle biopsies in coordination with each subject's primary care provider or cardiologist.
- Subjects who report using PDE-5s will be asked to hold the medication 24 hours before initial nitrite dose and for the entire period on study drug.

3.3 EXCLUSION CRITERIA

Exclusion Criteria for All participants

- Allergy to lidocaine
- Anemia (Hgb <11.0 males, <10.0 females)
- Dementia
- End-stage malignancy
- Orthopedic exercise limitation
- Chronic use of oral corticosteroids or other medications that affect muscle function.
- Chronic ETOH or drug dependency.
- 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).
- Psychiatric hospitalization within the last 3 months
- Chronic nitrate use
- Baseline BP <100/60, either value
- Refusal to participate in muscle biopsy

Exclusion Criteria HF Population

- Major cardiovascular event or procedure within the prior 6 weeks.
- HF secondary to significant uncorrected primary valvular disease (except mitral regurgitation secondary to left ventricular dysfunction). If valve replacement has been performed, patient may not be enrolled for 12 months after this procedure.
- Severe valvular heart disease
- Mechanical valve replacement requiring warfarin
- Currently taking clopidogrel or other anti-platelet medication for a recent stent placement and/or a complex atherosclerotic lesion such that holding clopidogrel (or an equivalent medication) creates disproportionate risk. This decision will always be determined by the patient's own primary care provider or cardiologist.
- ICD device with heart rate limits that prohibit exercise assessments. Referring physicians will be provided with an opportunity to reprogram devices so that patients can participate.

4. RECRUITMENT AND INFORMED CONSENT PROCEDURES

4.1 RECRUITMENT METHODS

Heart failure patients can be identified by screening of the clinics of the Heart and Vascular Institute, Cardiology, and Geriatrics in which the PI provides clinical services, by their referral from UPMC cardiologists or by flyers placed in the clinic areas.

Dr. Forman will seek approval from physicians in those clinics to have the study coordinator pre-screen the clinic schedules for eligible candidates on an ongoing basis. On the day of the clinic visit of identified candidates, the study coordinator will reaffirm with the subject's physician that recruitment of the subject is medically appropriate and that she has permission to speak with the candidate. After speaking with the subject, if the potential participant is interested in the study, he/she will be asked if it is acceptable to review the medical records further and finalize eligibility. Recruitment and enrollment procedures will then follow, including apprising PCP or cardiologist of their patient's involvement, (and managing hold of anti-coagulants per protocol, if needed). If due to unforeseen circumstances, the coordinator is unable to meet with the subject on the day of clinic visit, he/she will contact the subject (phone, email, postal mail). This will only be done if the attending physician has approved contact; the contact will always be initiated by first informing the subject that their physician has approved the contact.

Subjects can also be recruited using flyers and advertisements including the use of the CTSI research registry and OCR study listing website. Potential subjects will be instructed to contact the investigators via a telephone number/email address that is provided in these advertisements. They will then be explained the study (with approved script) and with their permission, screened on the phone to make sure they comply with the eligibility criteria. Subjects' medical records will be accessed prior to visit 1 to confirm that they meet eligibility criteria (request for access for screening purposes only, without consent to be obtained). If the subjects are deemed eligible and are interested in the study, an outpatient screening visit (Visit 1) will be scheduled where the study, risks and potential benefits, and rights as a research subject will be described in detail and where eligibility will be confirmed. Screening data will be de-identified and stored in an on line document that is password protected and on a secure drive with access only by study PI and coordinator. Codes linking names and IDs will be on a separate document, also password protected on secure drive.

4.2 INFORMED CONSENT PROCEDURES

Subjects 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 investigator prior to performing any research study procedures.

5. POTENTIAL RISKS AND BENEFITS

5.1 POTENTIAL RISKS

5.1.1 Risk of Experimental Drug Intervention

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 blood pressure 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^{31,32}. 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).

Nitrite:

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 an 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 proposal, 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 subjects 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 disease³³⁻³⁵. 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 mmHg decrease in mean arterial pressure, a peak methemoglobin of less 3% and no other significant effects³⁴. Note this single dose is 1.9 times the single dose per time of day we plan to use in this trial.

In an open-label three-way crossover study, 9 healthy adult subjects received two single high dose oral sodium nitrite aqueous solutions (0.12 and 0.06 mmol NaNO₂/mmol Hb, equivalent to 290-380 mg and 140-190 mg sodium nitrite, respectively, depending on the total body hemoglobin level of the person) and one intravenous sodium nitrite dose (0.12 mmol NaNO₂/mmol Hb)¹⁸. Note that this is 1.2-3.2 times the daily dose we plan to use in this trial. There was a washout period of at least 7 days between each of the treatments. Mild headache occurred in 44-55% of subjects and was the most frequent complaint during each treatment session, which the authors ascribed to the sodium nitrite, not methemoglobinemia, as the percentage of methemoglobinemia stayed below clinically toxic levels (<15%). By report, up to 22% experienced nausea, which subsided within half an hour¹⁸. The pharmacokinetic analysis of this study indicated similar bioavailability of oral and IV delivery of nitrite, as well as similar side effect and safety profiles.

A recent study determined the safety and feasibility of prolonged intravenous nitrite infusion. Twelve adult volunteers received increasing starting doses of sodium nitrite, 4.2 to 533.8 µg/kg/hr for 48 hours. Dose limiting toxicity occurred at 445.7 µg/kg/hr (10.6 mg/kg/day) and was limited to asymptomatic transient decreases of arterial blood pressure of up to 20 mmHg and asymptomatic increases of methemoglobin levels above 5%. No tolerance or clinically significant rebound was observed³⁶. Note this is 8.2 times the daily dose we plan to use in this trial (based on an adult body weight of 95 kg).

For nitrite, two retrospective case-control studies have shown that high maternal dietary nitrite intake from cured meat or drinking water during pregnancy might be associated with risk of childhood brain tumors and possibly gastric and esophageal cancer. This evidence is only based on retrospective case-control studies; cohort studies have found no significantly increased risks²⁴.

In the 2001 National Toxicology Program (NTP) Report summarizing 2-year rodent drinking water studies, there was no evidence of carcinogenic activity of sodium nitrite 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*³⁷. Taken together, these findings suggest minimal carcinogenic nitrite-mediated risk.

In the current study, we will use much lower doses of nitrite than used safely in any of these studies. Our nitrite dose of 20-40 mg three times daily is lower than the dose used on the cardiovascular IND and <40% of the dose for cyanide poisoning.

Nine of 10 control subject enrollees into this pilot study have tolerated 40 mg po tid without incident; the one exception was a subject who had a decrease in systolic BP at pK but was asymptomatic. In the three heart failure subjects enrolled to date we have observed a vasoactive response at the pK with the 40 mg dose, such that low blood pressures or symptoms have been observed in the 2 hour monitoring period. This includes subjects with preserved and reduced ejection fraction. Therefore, we postulate the heart failure subjects appear to have a greater vasodilatory response to nitrite and could require a stratified dose based on age and baseline risk for hypotension. To further ameliorate risk, we will serve a standardized meal prior to drug dosing at Visit 3 so that lack of po intake is not a causative factor of hypotension.

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 subjects with heart failure
- Rare: flushing, tachycardia, hypotension in healthy control subjects

Neurologic

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

Respiratory

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

5.1.2 Risk of Study Procedures

The collection of the research data from history and physical examination such as body weight, height and vital signs carries minimal risk. Phlebotomy or intravenous blood sampling risks of bruising, infection, anemia and fainting are common.

The risks of percutaneous muscle biopsy are bleeding and infection (both rare), bruising (infrequent) and discomfort (likely). With the use of a local anesthetic, our subjects describe the discomfort of muscle biopsy as about twice that of intravenous cannulation. Some residual stiffness in the vastus lateralis can persist for 1-2 days (common). An elastic wrap and ice bag are applied post-biopsy to decrease the risk of bruising and for an anesthetic effect. In our experience using similar protocols, subjects have not experienced any adverse effects from these procedures other than a small amount of residual localized soreness at the biopsy site. Additional risk includes any unusual reaction to the elastic bandage wrap and ice, i.e. leg numbness which would indicate the elastic bandage had been applied too tightly or the ice left on too long; or any skin redness, irritation, and chafing from the applied steri-strips.

Use of anesthetic cream

There is a risk of reaction to the anesthetic cream (lidocaine and prilocaine). The use of anesthetic cream has a risk of methemoglobinemia when used with larger areas or times, or on certain meds including subjects taking nitrites. To prevent adverse reactions, the cream will only be used in subjects with a history of poor pain control. The PI will carefully screen subjects including medications with potential contraindications and evaluate the risk/benefit of use. In subjects who require it, use will be limited to no more than 2 applications of 2.5 grams as per package instructions, approximately 5-6 weeks apart to an area 2-4 cm on the thigh. The cream will be applied, and then biopsy will not proceed for an hour. Initial nitrite drug dosing occurs a minimum of 30 minutes after the biopsy. The subjects are then monitored for % methemoglobinemia for 2 hours. Screening of subject medication profiles, using the minimal required amount of the cream, spacing with drug dosing, and monitoring of methem% of subjects allows for safe use in those subjects for which it is indicated.

Risk of holding blood thinners, anti-clotting or anti-inflammatory medications

There is a risk of increased pain if the anti-inflammatory medication is taken for arthritis or other pain related diagnosis. There is a risk of cardiac event if the medication is taken for anti-clotting prophylaxis related to cardiovascular disease. The risk is managed by individually evaluating the

risk for subjects and working with their PCP or cardiologist, implementing bridging therapy if needed, and keeping the number of days the medication is held to the minimum necessary to prevent bleeding during the biopsy.

The exercise test (VO₂max) and subsequent exercise training sessions may cause muscle soreness or fatigue, but 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 obese 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.

American College of Sports Medicine (ACSM) criteria will be used to halt exercise testing. In addition to ACSM criteria, the exercise test will be stopped if the subject has either signs or symptoms of cardiovascular compensation, e.g. hypotensive response to exercise. These exercise tests will be interpreted by a physician at the University of Pittsburgh Medical Center. The staff conducting maximal exercise tests are ACLS certified. The Exercise Physiology Laboratory has a crash cart with emergency equipment (including a defibrillator-monitor, airway equipment, IV sets and fluids, syringes, needles, Lidocaine, Epinephrine, Nitroglycerine, etc.) and will be present during maximal exercise testing.

Heart rate monitoring may result in skin chafing or irritation from the EKG electrodes used during exercise testing.

The local anesthesia (buffered lidocaine) used for muscle biopsy may cause an anaphylactic reaction to 2% which could result in a severe allergic reaction that may be simple or severe enough to cause shortness of breath, swelling of the throat, inflammation of the skin, hypotension and death.

Echocardiograms present no direct risks, but subjects will need to remain quietly at supine rest as a small plastic probe is placed against their chest. A gel is also applied to increase transmission of sound waves for optimal imaging quality. In rare instances, some people may find this to be anxiety producing. Dr. Gorcsan, who has advanced training in echocardiography, will oversee trained study staff performing echocardiograms to assure these are reliably completed with a minimum of patient distress. Likewise the ultrasound which may be used to guide the muscle biopsy carries no direct risk and may improve experience for subject.

Patients will answer questionnaires as part of the study assessments, i.e., requiring responses about daily activities and quality of life 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.

There are no health risks of fasting for 8 hours but subjects may feel hungry, tired, or irritable until the standardized meal is provided in the CTRC upon arrival.

There is a potential risk of stress and anxiety while participating in this study. It is likely that at least mild anxiety will occur while the subject is hospitalized to participate in this study. There is a potential of developing an infection (viral or bacterial) while in the hospital because of exposure to sick patients. We will try to not pair participants to share a hospital room with a sick patient. Participants will not have tests that have more than a minimal risk of causing an infection. In our experience, the risk of developing an infection while at the CTRC is minimal and should be even

lower in the EMRC or Pepper Center where there are lower numbers of subjects seen and where only non-invasive procedures are performed.

5.2 ALTERNATIVE TREATMENTS

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

5.3 POTENTIAL BENEFITS

There may be no direct medical benefit to subjects in the study. It is hoped that researchers will learn more about the effectiveness of using oral nitrite 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 heart failure patients in the future.

5.4 DATA SAFETY MONITORING PLAN

5.4.1 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 PI. Both 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 consenting any participants. Moreover, the study will be reviewed on an annual basis 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 PIs. The PI will, per standardized procedures, report them to the IRB for their review. With regard to monitoring of data quality and protected health information, all required personnel proposed for this project will have the required human subjects 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 subjects, 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 PIs.

Data Monitoring Plan. Data will be collected using standardized forms and will only be identified using the participant's ID number (no names or identifying information will be on the forms). The codes that link the names of participants and their ID numbers will be kept confidential by the PI located on a password protected document on a secured drive. These data will only be accessible to the PI and staff working directly with data with the study. All data will be entered on-line, by the project coordinator. Data will be double entered in the computer independently by trained study staff, and data entry discrepancies will be monitored and corrected by the project coordinator, based on source documents. The quality of the data will be monitored on an ongoing basis. Data

quality will be monitored by inspection of the completed forms by the study coordinator and any problems detected will be discussed with the PIs.

In the proposed study we will use the following University of Pittsburgh IRB definition of reportable events for the reporting of adverse events.

1. Internal Adverse Events that are (i) Unexpected, (ii) Related or Possibly Related to the Research Intervention, and (iii) serious or otherwise suggests that the research places the subject or others at a greater risk of harm (including physical, psychological, economic or social harm) than was previously known or recognized.
2. External Adverse Events that are (i) Unexpected; (ii) Related to the Research Intervention and (iii) Serious or otherwise suggests that the research places subjects or others at greater risk than was previously recognized

Any event meeting #1 or #2 above will be reported immediately to the IRB in their required format. In the event that a participant either withdraws from the study or the PIs decide to discontinue a participant due to a SAE, the participant will be monitored by the co-PIs 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 will be regularly reported to the IRB. A summary of the SAEs that occurred during the previous year will be included in the annual progress report as well as in the annual IRB renewal. Suicidal ideation and AEs will be formally assessed immediately after treatment and referrals for further care will be made as needed. In the Informed Consent Form, we will provide specific information about emergency contacts. Participants are instructed to contact Dr. Forman during working hours. After hours participants will be provided the information on how to contact the cardiology fellow on call.

Stopping Rule:

For safety reasons, we propose to discontinue participation for any subject who experiences *any of the following*:

1. Methemoglobin greater than 5% after dose lowering
A nitrite dose reduction is proposed from 40 mg three times daily to 20 mg three times daily if this methemoglobin % is reached at any study interim visit, as described in section 2.2.6 (phone report of change in skin tone and dizziness or other symptom suggestive of methemoglobinemia). Follow up symptom assessment for 48 hours and repeat methemoglobin in 3 days will be implemented. If methemoglobin is greater than 5% on the lower nitrite dose, participation in the study for the subject will be discontinued.
2. Blood pressure drop after dose lowering
Blood pressure and related symptoms.

If subjects experience lightheadedness or dizziness after visit 3 while they are on the drug, we request that they call the study coordinator who will inventory their symptoms, compare to baseline (measured at pK) levels, and discuss need for interim visit with study physician. If a visit is indicated, we will measure blood pressure, RR, heart rate, and check methemoglobin level by a finger probe. A study physician will evaluate each subject, including:

- a. review of all clinical measures,
- b. subject history of symptoms,

c. blood pressures pre- and while on drug.

The physician will confirm a management plan including the possibility of lowering dose to 20 mg tid or need to discontinue and drop from the trial. Subjects will be reassessed via phone each day for the next 2 days thereafter to ensure that the patient is stable on the new dose. If stable, subjects will continue with the half dose. However if there are continued symptoms on lower doses, the physician investigator will reevaluate the patient's suitability for continued participation based on symptoms and/or hemodynamics.

If subjects initiated at Visit 3 on the 20 mg dose report symptoms as described above, the same process will be followed; however, the physician may have to withdraw the subject since a dose lower than 20 mg tid is not an option.

3. Biopsy refusal

Subjects who refuse the first biopsy will be excluded at screening. Participants who underwent the first biopsy at Visit 3 may refuse a second pass. (Participants may also refuse a second biopsy pass at Visit 5). However, participants who refuse to complete a second biopsy at Visit 5 (prior to starting sodium nitrite) will be withdrawn from the study. Alternatively, patients who refuse the 2nd biopsy after they have completed the 4 weeks of nitrite therapy will still be included.

4. Medication compliance

In the event that the medication compliance rate is <80%, subjects will be re-educated on medication compliance. If medication compliance repeatedly falls outside of the acceptable range, the study investigators will discuss subject eligibility for continued participation in the study.

5.4.3 Parameters to be monitored

The following progress will be monitored throughout the course of the research to ensure the safety of subjects 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, 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 subjects and the confidentiality of their research data.

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.

5.4.4 Frequency of Monitoring

The Investigator will review subject safety data as it is generated. The Investigator, sub-investigators, and the research staff will meet on a two week interval to re-evaluate study goals, subject recruitment, data coding and retention, documentation and identification of adverse events, complaints and confidentiality of subjects. There will be an evaluation of the progress of the research study, including assessments of data quality, time lines, participant recruitment, accrual, and retention. The Investigator 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.

5.4.5 Reportable Adverse Events

For this study, a serious adverse event is any untoward clinical event that is thought by the investigator to be study-related, that is also:

1. Fatal or immediately life threatening
2. Permanently disabling, or severely incapacitating.
3. Requires or prolongs inpatient hospitalization.
4. Important medical events that may not result in death, be life threatening, or require hospitalization may be considered serious adverse events 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.

5.4.6 Adverse Events Reporting Timeline

Life-threatening or fatal unexpected adverse events associated with the use of the study drug or procedures must be reported to the IRB 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 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.

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

5.5 RISKS MANAGEMENT PROCEDURES

5.5.1 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 subjects 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 on an electronic password-guarded study database under the supervision of the Investigator for this protocol. The electronic database that is being used for the purpose of this study has not been fully validated to be in compliance with the FDA regulations at 21 CFR Part 11; i.e. taking into account the limited scope of this clinical investigation. The data will be stripped of individual identifiers and stored anonymously with a subject number. Information linking subject identifiers with the coded subject number will be stored under password protection on computers in locked areas, with access only to the database manager. Maintaining records in locked files in locked offices will protect confidentiality of subjects. All staff will sign confidentiality statements. Access to the database will be limited to the data manager and staff under the supervision of the Investigator.

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. The Investigator may continue to use and disclose subjects' 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.

5.5.2 Protection Against Potential Risks of Experimental Intervention

- Involvement by trained staff / investigators with experience in the administration of the study drug
- Continuous monitoring by the Data and Safety Monitoring Board
- Required Education in the Protection of Human Research Participants

The Investigator and all sub-investigators listed on University of Pittsburgh Institutional Review Board approved protocol are required to participate in a course entitled The Education and Certification Program in Research & Practice Fundamentals (RPF). This web based tutorial is a requirement of the IRB for protocol submission.

6. COSTS AND PAYMENTS

6.1 COSTS

Study drug 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 subjects and/or their health insurance company.

6.2 PAYMENTS

Subjects will be reimbursed \$200 for the baseline study visits after completion of Visit 3 [Visits 1 (\$50), Visit 2 (\$75) and Visit 3 (\$75)]. Subjects will be reimbursed an additional \$370 following the completion of Visit 5 [Visit 4 (\$125), Visit 5 (\$145) and Study drug compensation (\$100)]. The total compensation will be \$570.00 and will be paid via the University's WePay debit card system. Subjects will also be provided a voucher for outpatient UPMC Montefiore parking at all visits. In the event that a subject drops out due to an AE, the subject will be reimbursed for the individual study visits that they completed to date per the above reimbursement schedule.

7. QUALIFICATIONS AND SOURCE OF SUPPORT

7.1 QUALIFICATIONS OF THE INVESTIGATORS

Sponsor:

Mark Gladwin, MD, is Professor of Medicine, University of Pittsburgh. Dr. Gladwin is the Chairman of the Department of Medicine and Director of the Vascular Medicine Institute at the University of Pittsburgh. 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.

Investigator:

Daniel Forman, MD, is a Professor of Medicine at UPMC 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 heart failure (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. Dr. Forman's effort will be supported by the Department of Medicine.

Sub-Investigators:

Mark Gladwin, M.D., is Professor and Chairman of Medicine, University of Pittsburgh School of Medicine and Director of the Vascular Medicine Institute at the University of Pittsburgh. 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.

Sruti Shiva, Ph.D., is an Associate Professor in the Department of Pharmacology and Chemical Biology and Vascular Medicine Institute (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.

John Gorcsan, III, MD is a Professor of Medicine and Director of Echocardiography Laboratory. He will provide his expertise and two-decade experience in quantification of left ventricular function by echocardiographic methods. He has extensive experience in whole organ ventricular physiology and quantitative echocardiography in patients with both depressed and preserved ejection fraction, having directed echocardiography core labs for multi-center studies in the past.

Marc Simon, MD is an Assistant Professor of Medicine at the University of Pittsburgh Vascular Medicine Institute-Medicine, Bioengineering, and Clinical Translational Science including Director, Heart Failure Research / Clinical Hemodynamics Core Facility and Director, Vascular Clinical and Translational Research Center. He brings expertise in elucidating the mechanisms by which mitochondrial function is regulated in cardiovascular physiology and pathology, and thus evaluation of the data from this study concerning the mitochondrial analysis. As a cardiologist who specializes in heart failure, he brings added expertise to the protocol and can serve to assist Dr. Forman in evaluating, consenting and monitoring subjects.

Ana Mora, MD, is an Assistant Professor of Medicine, Division of Pulmonary, Allergy and Critical Care Medicine, UPMC. Dr. Mora will participate in data analysis and publications. Her effort will be supported by the UPMC Heart and Vascular Institute.

Nicole Helbling, MS RN, is a registered nurse with extensive clinical and translational research experience in the EMRC. She has been a research coordinator within the EMRC and is well trained to review study consent, obtain medical histories, assist with muscle biopsy procedures and exercise testing and day to day clinical trial execution.

Manisha Jhamb, MD is an Assistant Professor at the University Of Pittsburgh School Of Medicine, Department of Medicine, Renal-Electrolyte Division and a clinician at UPMC Presbyterian and Magee Women's Hospital. She has clinical trial experience in the renal population including in the areas of quality of life and fatigue, outcome parameters in this pilot. She is being added to the protocol to support Dr. Forman in evaluating subjects for enrollment, consenting, performing physicals, evaluating adverse events and monitoring subjects.

7.2 SOURCES OF SUPPORT

2014 Mitochondria, Aging and Metabolism/Basic Biology Aging Pilot Project Program

8. REFERENCES

References

1. Roger VL, Weston SA, Redfield MM, et al. Trends in HF incidence and survival in a community-based population. *JAMA*. 2004; 292:344-50.
2. Forman DE, Ahmed A, Fleg J. Heart failure in very old adults. *Curr Heart Fail Rept*. 2013;10:387-400.
3. Yancy CW, Jessup M, Bozkurt B, et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. *Circulation*. 2013;128:e240-327
4. Clark AL, Poole-Wilson PA, Coats AJ. Exercise limitation in chronic heart failure: central role of the periphery. *J Am Coll Cardiol*. 1996;28:1092-102.
5. Daniels K, Arena R, Lavie CJ, Cahalin LP, Forman DE. Exercise: a vital means to moderate cardiovascular aging. *Aging Health*. 2013. In press.
6. Gielen S, Adams V, Niebauer J, Schuler G, Hambrecht R. Aging and heart failure--similar syndromes of exercise intolerance? Implications for exercise-based interventions. *Heart Fail Monit*. 2005;4:130-6.
7. Duscha BD, Schulze PC, Robbins JL, Forman DE. Implications of chronic heart failure on peripheral vasculature and skeletal muscle before and after exercise training. *Heart Fail Rev*. 2008;13:21-37
8. Pu CT, Johnson MT, Forman DE, Hausdorff JM, Roubenoff R, Foldvari M, Fielding RA, Singh MA. Randomized trial of progressive resistance training to counteract the myopathy of chronic heart failure. *J Appl Physiol*. 2001;90:2341-50.
9. Kitzman DW, Nicklas B, Kraus WE, et al. Skeletal muscle abnormalities and exercise intolerance in older patients with heart failure and preserved ejection fraction. *Am J Physiol Heart Circ Physiol*. 2014;306:H1364-70
10. Wang Y, Pessin JE. Mechanisms for fiber-type specificity of skeletal muscle atrophy. *Curr Opin Clin Nutr Metab Care*. 2013;16:243-50.
11. Roubenoff R. Sarcopenia and its implications for the elderly. *Eur J Clin Nutr*. 2000 Jun;54 Suppl 3:S40-7
12. Forbes SC, Little JP, Candow DG. Exercise and nutritional interventions for improving aging muscle health. *Endocrine*. 2012;42:29-38
13. Forman DE, Rich MW, Alexander KP, Zieman S, Maurer MS, Najjar SS, Cleveland JC, Krumholz HM, Wenger NK. White Paper: Current care for older adults: Time for a new paradigm. *J Am Coll Cardiol*. 2011;57:1801-1810.
14. O'Connor CM, Whellan DJ, Lee KL, et al. Efficacy and safety of exercise training in patients with chronic heart failure: HF-ACTION randomized controlled trial. *JAMA*. 2009;301:1439-50.
15. Larsen FJ, Schiffer TA, Borniquel S, et al. Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab* 2011;13:149-59.
16. Lai YC, Tabima DM, Dube J, et al. SIRT3-AMPK activation by nitrite and metformin improves hyperglycemia and normalizes pulmonary hypertension in heart failure with

- preserved ejection fraction. To be presented at the American Heart Association Scientific Session, 2014. Manuscript in submission.
17. Lakatta EG, Levy D. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part II: the aging heart in health: links to heart disease. *Circulation*. 2003;107:346-54
 18. Roger VL, Weston SA, Redfield MM, et al. Trends in HF incidence and survival in a community-based population. *JAMA*. 2004; 292:344-50.
 19. Landi F, Cruz-Jentoft AJ, Liperoti R, Russo A, Giovannini S, Tosato M, Capoluongo E, Bernabei R, Onder G. Sarcopenia and mortality risk in frail older persons aged 80 years and older: results from the SIRENTE study. *Age Ageing*. 2013;42:203-9
 20. Goodpaster BH, Chomentowski P, Ward BK, et al. Effects of physical activity on strength and skeletal muscle fat infiltration in older adults: a randomized controlled trial. *J Appl Physiol* (1985). 2008;105:1498-503.
 21. Haykowsky MJ, Kouba EJ, Brubaker PH, Nicklas BJ, Eggebeen J, Kitzman DW. Skeletal muscle composition and its relation to exercise intolerance in older patients with heart failure and preserved ejection fraction. *Am J Cardiol*. 2014;113:1211-6.
 22. Adams V, Nehrhoff B, Späte U, Linke A, Schulze PC, Baur A, Gielen S, Hambrecht R, Schuler G. Induction of iNOS expression in skeletal muscle by IL-1beta and NFkappaB activation: an in vitro and in vivo study. *Cardiovasc Res*. 2002;54:95-104.
 23. Lenk K, Erbs S, Höllriegel R, et al. Exercise training leads to a reduction of elevated myostatin levels in patients with chronic heart failure. *Eur J Prev Cardiol*. 2012;19:404-11.
 24. Brum PC, Bacurau AV, Cunha TF, Bechara LR, Moreira JB. Skeletal myopathy in heart failure: effects of aerobic exercise training. *Exp Physiol*. 2014;99:616-20.
 25. Haykowsky MJ, Kitzman DW. Exercise physiology in heart failure and preserved ejection fraction. *Heart Fail Clin*. 2014;10:445-52
 26. Shiva S, Sack MN, Greer JJ, et al. Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. *J Exp Med*. 2007;204:2089-102
 27. Curtis E, Hsu LL, Noguchi AC, Geary L, Shiva S. Oxygen regulates tissue nitrite metabolism. *Antioxid Redox Signal*. 2012;17:951-61.
 28. Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free Radic Biol Med*. 2010;48(2):342-7.
 29. Bailey SJ, Fulford J, Vanhatalo A, et al. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol* 2010;109:135-48
 30. Larsen FJ, Weitzberg E, Lundberg JO, et al. Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol*. 2007;191:59-66.
 31. Cosby K, Partovi KS, Crawford JH, et al. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* 2003;9:1498-505.
 32. Gladwin MT, Shelhamer JH, Ognibene FP, et al. Nitric oxide donor properties of hydroxyurea in patients with sickle cell disease. *Br J Haematol*. 2002;116:436-44.

33. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nature reviews Drug discovery* 2008;7:156-67.
34. Santanasto AJ, Glynn NW, Jubrias SA, et al. Skeletal Muscle Mitochondrial Function and Fatigability in Older Adults. *J Gerontol A Biol Sci Med Sci*. 2014 Aug 28. pii: glu134. [Epub ahead of print]
35. Forman DE, Fleg JL, Kitzman DW, Brawner CA, Swank A, McKelvie R, Clare R, Ellis SJ, Dunlap ME, Bittner V. 6 Minute Walk Test Provides Prognostic Utility Comparable to Cardiopulmonary Exercise Testing in Ambulatory Outpatients with Systolic Heart Failure. *J Am Coll Cardiol*. 2012;60:2653-61.
36. Forman DE, Daniels K, Cahalin LP, Zavin A, Allsup K, Cao P, Santhanam M, Joseph J, Arena R, Lazzari A, Schulze C, Lecker S. Analysis of Skeletal Muscle Gene Expression Patterns and the Impact of Functional Capacity in Patients with Systolic Heart Failure. *J Card Fail*. 2014;20:422-30
37. Zavin A, Daniels K, Arena R, Allsup K, Lazzari A, Joseph J, Schulze PC, Lecker SH, Forman DE. Adiposity facilitates increased strength capacity in heart failure patients with reduced ejection fraction. *Int J Cardiol*. 2013;167:2468-71.
38. Jiang H, Torregrossa AC, Potts A, et al Dietary nitrite improves insulin signaling through GLUT4 translocation. *Free Radic Biol Med*. 2014;67:51-7.
39. Shiva S, Brookes PS, Darley-Usmar VM. Methods for measuring the regulation of respiration by nitric oxide. *Methods Cell Biol*. 2007;80:395-416.
40. Cardenes N, Corey C, Geary L, Jain S, Zharikov S, Barge S, Novelli EM, Shiva S. Platelet bioenergetic screen in sickle cell patients reveals mitochondrial complex V inhibition, which contributes to platelet activation. *Blood*. 2014;123:2864-72
41. Frazier EP, Isenberg JS, Shiva S, et al Age-dependent regulation of skeletal muscle mitochondria by the thrombospondin-1 receptor CD47. *Matrix Biol*. 2011;30:154-61
42. MacArthur PH¹, Shiva S, Gladwin MT. Measurement of circulating nitrite and S-nitrosothiols by reductive chemiluminescence. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007;851:93-105
43. Balady GJ, Arena R, Sietsema K, et al. Clinician's guide to cardiopulmonary exercise testing in adults: a scientific statement from the American Heart Association. *Circulation*. 2010;122:191–225.
44. Borg, G. A. V., Noble, B. J. Perceived exertion. *Exercise and Sport Sciences Reviews in New York: Academic Press* 1974:131-153
45. Sayers SP, Jette AM, Haley SM, Heeren TC, Guralnik JM, Fielding RA. Validation of the Late-Life Function and Disability Instrument. *J Am Geriatr Soc*. 2004;52:1554-9.
46. Green CP, Porter CB, Bresnahan DR, Spertus JA. Development and evaluation of the Kansas City Cardiomyopathy Questionnaire: a new health status measure for heart failure. *J Am Coll Cardiol*. 2000;35:1245-55
47. Duncan P, Richards L, Wallace D, Stoker-Yates J, Pohl P, Luchies C, Ogle A, Studenski S. A randomized, controlled pilot study of a home-based exercise program for individuals with mild and moderate stroke. *Stroke*. 1998;29:2055-60.
48. Duncan PW, Studenski S, Richards L, et al. Randomized clinical trial of therapeutic exercise in subacute stroke. *Stroke* 2003; 34:2173-80.
49. Personal communication Sruti Shiva (11/13/2014)
50. Cohen, J. (1977). *Statistical Power Analysis for the Behavioral Sciences*. New York: Academic Press.

-
51. VanSwearingen JM, Perera S, Brach JS, Cham R, Rosano C, Studenski SA. A randomized trial of two forms of therapeutic activity to improve walking: effect on the energy cost of walking. *Journals of Gerontology-Medical Science* 2009;64A:1190-8.
 52. Resnick JL, Gupta N, Wagner J, et al. Skeletal integrity and visceral transplantation. *American Journal of Transplantation* 2010;10:2331-40. Mohler III ER, Hiatt WR, Gornik HL, Kevil CG, Quyyumi A, Haynes WG, Annex BH. Sodium nitrite in patients with peripheral artery disease and diabetes mellitus: safety, walking distance and endothelial function. *Vascular Medicine* 2014; 19(1):10-17.
 53. Mohler III ER, Hiatt WR, Gornik HL, Kevil CG, Quyyumi A, Haynes WG, Annex BH. Sodium nitrite in patients with peripheral artery disease and diabetes mellitus: safety, walking distance and endothelial function. *Vascular Medicine* 2014; 19(1):10-17.