

Nitrite benefits to mediate fatigability in older HFpEF patients

Daniel E. Forman, MD
Principal Investigator

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

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PROTOCOL SYNOPSIS

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Investigational Drugs: Nitrogen 14 Sodium Nitrite (¹⁴N Sodium Nitrite)

Trial Site: *University of Pittsburgh Single-Center Randomized Trial*

IND Sponsor: Mark T. Gladwin, MD
Professor & Chairman of the Department of Medicine
Director, Pittsburgh Heart, Lung, Blood and
Vascular Medicine Institute
Department of Medicine
1218 Scaife Hall
3550 Terrace St, Pittsburgh, PA 15261
412-648-9636

Investigator: Daniel E. Forman, MD
Professor of Medicine
Chair, Geriatric Cardiology Section
University of Pittsburgh
3471 Fifth Avenue, Suite 500
Pittsburgh, PA 15213

Sub-Investigators: Jessica Bon Field, MD
Mark T. Gladwin, MD
Nancy Glynn, PhD
Bret Goodpaster, PhD
John Gorcsan, MD
Hoby Hetherington, PhD, MS
Gavin Hickey, MD
Kara Hughan, MD, MHSc
Theodore Huppert, PhD
Manisha Jhamb, MD, MPH
Andrea Levine, MD
Anne Newman, MD, MPH
Subashan Perera, PhD
Michael Risbano, MD
Adam Santanasto, MPH, PhD
Sruti Shiva, PhD
Marc Simon, MD, MS
Frederico Toledo, MD

Study Monitor:

Mark T. Gladwin, MD
Chairman, Department of Medicine
University of Pittsburgh School of Medicine
3550 Terrace St. - 1218 Scaife Hall
Pittsburgh, PA 15261

Research Facilities:

UPMC Montefiore Hospital (UPMC Montefiore) Clinical
Translational Research Center (CTRC)
3459 Fifth Ave. 6 North
Pittsburgh, PA 15213

UPMC Montefiore Endocrinology and Metabolism Research
Center (EMRC)
3459 Fifth Ave. NW 823
Pittsburgh, PA 15213

UPMC Presbyterian Hospital (UPMC Presbyterian)
• Cardiac Catheterization Lab
• Magnetic Resonance Research Center
200 Lothrop St
Pittsburgh, PA 15213

University of Pittsburgh Pepper Center–SMART Center
(Senior Mobility Aging Research Training)
3471 Fifth Ave. Suite 1200
Pittsburgh PA 15213

University of Pittsburgh Vascular Medicine Institute
Biomedical Science Tower E1240
200 Lothrop St.
Pittsburgh, PA 15261

Clinical Laboratories:

UPMC Presbyterian Hospital
3477 Euler Way
Pittsburgh, PA 15213

Study Rationale:

Heart failure (HF) is epidemic with aging and prevalence of HF is steadily increasing¹ 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 prognosis², 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 dysfunction³ despite the fact that HF-outcomes are strongly related to functional decrements that are largely mediated by age⁴ and disease-related⁵ 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 implications⁶. Fatigability is rooted in HF with

preserved ejection fraction (HFpEF) pathophysiology, and while it often determines a patient's experience of his/her disease, it has not been addressed as a key part of management. We propose a prospective, randomized, controlled, double-blinded trial to study the utility of nitrite (NO₂) therapy in older (age ≥70 years) HFpEF patients. Subjects (N=18) will be assessed at baseline and after 4 weeks of NO₂ therapy. Thus we will study the utility of NO₂ therapy as a highly innovative means to modify fatigability. We hypothesize that NO₂ therapy will reduce perceived and performance fatigability indices and improve peak oxygen uptake (VO₂) in HFpEF patients and that decreased fatigability indices will correspond to increased daily activity as measured by actigraphy. We will also clarify the mechanisms by which NO₂ benefits are mediated. We also hypothesize that the primary mechanism by which NO₂ decreases fatigability is improved mitochondrial bioenergetics as measured by in vivo 31P Magnetic Resonance Spectroscopy (MRS) and in vitro mitochondrial respiration and Adenosine Triphosphate (ATP) generation. Thus, among HFpEF patients with pulmonary hypertension, NO₂ additionally mitigates fatigability by reducing pulmonary pressures and improving right ventricular-pulmonary artery coupling. Secondary aims will not only elucidate important NO₂ properties, but also catalyze broader focus on the principles of patient-centered care by aligning a mechanistic perspective regarding NO₂ benefits and targets for therapy with the patient-centered experience of fatigability.

Study Objectives:

Studies have demonstrated NO₂ therapy increases ATP synthesis in skeletal muscle mitochondria concomitant with reduced whole-body oxygen cost during steady state (SS) exercise⁷. Our own work has demonstrated safety and efficacy of a FDA-IND approved sodium nitrite (10 mg and 20 mg) capsule, and its utility to upregulate the NAD-dependent deacetylase sirtuin-3 adenosine monophosphate (SIRT3-AMP) pathway of skeletal muscle of younger HF patients⁸. It now seems exceptionally logical and opportune to:

- apply these insights to older HF patients
- delineate mechanisms of disease and aging that respond to NO₂ therapy
- delineate indices of perceived and performance fatigue.

Study Hypothesis:

1. We hypothesize that NO₂ therapy will reduce performance and perceived fatigability indices and improve peak VO₂ in HFpEF patients as measured by cardiopulmonary exercise testing (CPET).
2. We hypothesize that decreased fatigability indices will correspond to increased daily activity as measured by actigraphy.
3. We hypothesize that the primary mechanism by which NO₂ decreases fatigability is improved bioenergetics as

measured by in vivo ^{31}P MRS and in vitro mitochondrial respiration and ATP generation.

4. We hypothesize that among HFpEF patients with pulmonary hypertension, NO_2 additionally mitigates fatigability by reducing pulmonary pressures and improving right ventricular-pulmonary artery coupling.

Study Design:

This is a prospective, randomized, double blind controlled trial of oral sodium nitrite vs. placebo in adults ($n=18$), ≥ 70 years of age with HFpEF.

Subjects who meet the Inclusion Criteria and none of the Exclusion Criteria will receive oral sodium nitrite or placebo for 4 weeks. We will stratify dosing as below:

Drug:

a. oral formulation of sodium nitrite 40 mg/placebo three times daily (tid), once in the morning and again in the early afternoon and again in the evening, approximately 6 hours apart in subjects age 70 to <80 years with low risk for hypotension

b. oral formulation of sodium nitrite 20 mg/placebo three times daily (tid), once in the morning and again in the early afternoon and again in the evening, approximately 6 hours apart in subjects ≥ 80 years of age, or subjects age 70 to <80 years with risk factors for hypotension

Subjects will be evaluated as outpatients with the first dose of study drug/placebo. Blood pressure (BP), heart rate (HR), respiratory rate (RR), oxygen saturation (SpO_2), methemoglobin % (MetHb) and plasma NO_2 will be measured at baseline and during the first two hours after dosing. Frequent safety monitoring will occur during the time subjects are on drug as well as after completion of the post drug assessments, by phone and in clinic to assess adverse events (AE), interval histories, and medication compliance and to monitor NO_2 and methemoglobin levels.

Study Aims

Aim 1: To complete a randomized, placebo-controlled trial in HFpEF patients aged ≥ 70 years to assess benefits of 1 month of NO_2 vs. placebo therapy to reduce perceived and performance fatigability and to improve aerobic functional capacity.

Aim 2: To delineate factors which underlie fatigability and the utility of NO_2 to modify them.

Planned Sample Size:

18 subjects: 9 sodium nitrite and 9 placebo

- Duration of Treatment:** 4 weeks plus range 2-3 weeks for post drug outcome assessments
- Major Inclusion Criteria:**
- Age ≥ 70 years
 - Diagnosis of HFpEF [adapted from the 2016 European Society of Cardiology (ESC) Guidelines (<http://eurheartj.oxfordjournals.org/content/37/27/2129#sec-12>)] to include:
 1. Prior diagnosis of HF via one of these:
 - medical record diagnosis by attending cardiologist
 - verbal confirmation of HFpEF with attending cardiologist
 - PI review of medical record to confirm HFpEF
 - AND
 2. EF% ≥ 40
 - Clinically stable (euvolemic; baseline HR < 100 bpm) and without hospitalization or invasive cardiac procedure for 6 weeks
 - Patients using 81 milligram (mg) aspirin (ASA) will be eligible, but will be asked to hold the medication for 3 days 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 2 days 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 cardiologist.
- Major Exclusion Criteria:**
- Allergy to lidocaine
 - BP $> 180/95$ or $< 100/60$ mm Hg (either systolic or diastolic)
 - Anemia: Hgb < 11.0 (♂), 10.0 (♀) gm/dl
 - Dementia or inability to give informed consent
 - End-stage malignancy
 - Severe orthopedic exercise limitation
 - Use of chronic oral corticosteroids or other medications that affect muscle function.
 - Chronic alcohol 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
 - 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 uncorrected primary valvular heart disease (if valve replacement has been performed, patients will not be eligible for at least 12 months)
- Mechanical valve replacement requiring warfarin
- Severe peripheral or pulmonary artery disease
- Currently taking clopidogrel for a recent stent placement and/or a complex atherosclerotic lesion such that holding clopidogrel creates disproportionate risk.
- Current use of organic nitrates or phosphodiesterase type 5 inhibitors (PDE5s)
- Unable to hold warfarin or use bridging therapy, or to hold aspirin for 3 days (81 mg) or (325 mg), prior to muscle biopsy or thienopyridine medications for 5 days prior to muscle biopsy.
- Subjects with diabetes whose HgbA1c >10.0%
- Other chronic unstable disease such as active neoplasm, end stage chronic kidney, liver or other organ disease

Relative Exclusions

- Subjects with a non- 3T MRI compatible pacemaker or implantable cardio defibrillator or identified to have other metal in their body will be excluded from the Magnetic Resonance Spectroscopy study.
- Subject who use PDE5s for erectile dysfunction and are willing to withhold use 24 hours prior and during entire dosing period may be enrolled.
- Subjects may choose not to do the pre- and post- right heart catheterization exercise test.

Study Endpoints:

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

1. Measures of physical function as measured by non-invasive CPET (nCPET) aerobic capacity, SS fatigability assessment, 6 minute walk test (6MWT), near infrared spectroscopy (NIRS) assessments of blood flow, Short Physical Performance Battery (SPPB), handgrips, accelerometry and invasive CPET (iCPET):
 - a. gait speed
 - b. strength (endurance, power)
 - c. balance
 - d. pulmonary pressure and ventricular-pulmonary artery coupling
2. Skeletal muscle bioenergetics: spectrophotometric, mitochondrial respiration, polymerase chain reaction (PCR) including ribonucleic acid (RNA) isolation, protein isolation, electron microscopy
3. Serology: inflammatory peptides (interleukin (IL) 1, IL-6, IL-15, TNF α ; adipokines (leptin and adiponectin); vitamin D, glomerular filtration rate (GFR), brain natriuretic protein (BNP) or N-terminal *pro* b-type natriuretic peptide

(NT-proBNP), thyroid stimulating hormone (TSH), glycosylated hemoglobin (HgbA_{1c}) and platelet bioenergetics [using Seahorse Extracellular Flux (XF) analysis]⁹, i.e., including glycolytic as well as basal and maximal respiratory rates.

4. Additional secondary factors include Quality of life (QoL) and related indices: questionnaires that include measures of sleep, depression, pain, cognitive function, physical activity, co-morbidities, cardiac self-care efficacy, and fatigability, HF QoL, via the Kansas City Cardiomyopathy Questionnaire (KCCQ), nutrient intake, and physician assessment of frailty.

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

1.1 OBJECTIVE

Studies have demonstrated NO₂ vs. placebo therapy increases ATP synthesis in skeletal muscle mitochondria concomitant with reduced whole-body oxygen cost during SS exercise⁷. Our own work has demonstrated safety and efficacy of a FDA-IND approved sodium nitrite (10 and 20 mg) capsules, and its utility to upregulate the SIRT3-AMP pathway of skeletal muscle of younger HF patients⁸. 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 NO₂ therapy.

1.2 SPECIFIC AIMS

Aim 1: To complete a randomized, placebo-controlled trial in HFpEF patients aged ≥70 years to assess benefits of 1 month of NO₂ vs. placebo therapy to reduce perceived and performance fatigability and to improve aerobic functional capacity.

- *We hypothesize that NO₂ therapy will reduce perceived and performance fatigability indices (rated perceived exertion) and improve peak oxygen uptake (VO₂) in HFpEF patients*
- *We hypothesize that decreased fatigability indices will correspond to increased daily activity as measured by accelerometry.*

Aim 2: To delineate factors which underlie fatigability and the utility of NO₂ to modify them.

- *We hypothesize that the primary mechanism by which NO₂ decreases fatigability is improved bioenergetics as measured by in vivo ³¹P MRS and in vitro mitochondrial respiration and ATP generation.*
- *We hypothesize that among HFpEF patients with pulmonary hypertension, NO₂ additionally mitigates fatigability by reducing pulmonary pressures and improving right ventricular-pulmonary artery coupling.*

1.3 BACKGROUND AND RATIONALE

HFpEF: Today's large and rapidly growing population of old adults is intrinsically susceptible to age-related HFpEF¹⁰. There are no effective treatments for what is becoming an epidemic¹⁰⁻¹². High mortality and morbidity, exercise intolerance, fatigability, diminished QoL and spiraling healthcare costs are typical consequences¹²⁻¹³. Pharmacological interventions that targeted ventricular myocardial relaxation (lusitropic) abnormalities failed to show anticipated mortality and morbidity benefits¹⁴⁻¹⁵. More recent studies indicate that systemic inflammation¹⁶ and its downstream effects may be a more fundamental basis of HFpEF¹⁷. Thus, it stands out that

several small exercise training (ExT) trials for HFpEF demonstrate improved aerobic exercise capacity¹⁸⁻²⁰, as it infers that ExT may provide related therapeutic benefits^{21, 23}. Associated analyses show that the ExT functional gains were achieved primarily by improvements in peripheral oxygen utilization²⁰⁻²², and are substantiated by changes in skeletal muscle (i.e., increased proportions of type I relative to type II fibers)^{17, 22}, analogous to exercise-induced shifts in gene expression that have been demonstrated in HF with reduced ejection fraction (HFrEF)^{24, 25}.

Yet such compelling benefits of pilot ExT HFpEF trials were only demonstrated after months of moderate to high intensity exercise regimens, regimens that are unfeasible for most HFpEF patients. We propose there are intrinsic physiological components of HFpEF pathophysiology that predispose to “fatigability”, a concept we further delineate as “perceived fatigability” measured by rate of perceived exertion (RPE) during a steady-state walking stimulus and “performance fatigability” (deterioration in a self-selected walking speed over time)²⁶ during the 6 minute walk test (6MWT). Both perceived and performance fatigability indices are submaximal activity-based metrics, and are significantly more clear and reliable than “fatigue” assessed qualitatively. Overcoming fatigability is an important HFpEF therapeutic goal.

The large randomized trial Heart Failure (HF): A Controlled Trial Investigating Outcomes of Exercise Training (HF-ACTION) provides critical perspective regarding relevance of fatigability²⁶ in subjects with HFrEF. Despite multiple steps to bolster adherence, poor compliance and attrition were substantial. High fatigability was a common barrier to adherence and compliance^{27, 28}. Investigators of this proposal are at the forefront of the inorganic nitrate (NO₃) and NO₂ salts therapeutics field, and are pursuing pioneering studies to show safety and utility of oral NO₂^{29, 30}. Related studies have demonstrated acute benefits of NO₃ and NO₂ in respect to improving exercise efficiency; after ingesting NO₃, VO₂ lessens for an equivalent workload^{7, 31}. Our pilot work shows safety and efficacy of chronic NO₂ therapy. In a 1-year, randomized, controlled, double-blinded trial, we propose to study efficacy of 4 weeks of NO₂ capsules to reduce fatigability and increase VO₂ in older (≥70 years) HFpEF patients and to comprehensively delineate the mediating mechanisms by which these benefits are achieved.

This proposal is significant and innovative in multiple respects. First, it focuses on HFpEF, a disease which is endemic with aging and which constitutes one of today’s foremost healthcare challenges for the growing population of older adults. Second, it focuses on fatigability as a relevant dimension of HFpEF management. Fatigability is a particularly important therapeutic concept as it is rooted in HFpEF pathophysiology, and it often determines a patient’s experience of his/her disease. No matter how compelling the rationale for ExT, implementation is unlikely unless intrinsic fatigability is mitigated. Third, it assesses the novel concept of NO₂ therapy as a means to modify fatigability. The application of accelerometry to gauge changes in daily activity in relation to NO₂ is also novel and formative. Fourth, the proposal is significant as it aims to clarify mechanisms that mediate NO₂ benefits, including assessments of skeletal muscle as well as right and left heart vascular flow dynamics. Not only will such comprehensive methodology clarify NO₂ physiologic utility, but it advances the principles of patient-centered care by clarifying mechanisms that determine a patient’s day-to-day experiences of their disease. Systemic, cellular, and subcellular mechanisms of fatigability will be delineated, providing insights that will help reorient care towards what a patient feels.

1.4 SIGNIFICANCE

Fatigability as a novel clinical metric: While investigators have focused on HFpEF pathophysiology in terms of mortality, morbidity, and other traditional clinical endpoints, there has been negligible consideration of fatigability as an important clinical target. To some extent,

this reflects inherent ambiguity and unreliability of “fatigue” as a qualitative index. This study is innovative in utilizing clearer-cut performance-based metrics of perceived and performance “fatigability”^{32, 33}. This clarity of definition and assessment facilitates the application of fatigability as a benchmark with which to reliably assess physiological changes achieved using NO₂.

Eldadah³⁴ emphasized the importance of fatigability as a vital dimension of care. High fatigability predicts reduced physical activity (i.e., people curtail or stop exercise if it is too tiring)³⁵ as well as increased disability and death³⁶. High fatigability is also associated with slower walking speeds that entail relatively greater percentages of peak exercise capacity³⁷. Co-I Glynn advanced fatigability as a reliable metric in healthy adults^{32, 38}. In this study we apply these concepts to a disease state.

Fatigability and HFpEF: HFpEF is typically characterized in relation to cardiac, vascular, chronotropic abnormalities, and skeletal muscle abnormalities²¹. Fatigability may serve as an important integrative metric. Kitzman demonstrated HFpEF’s impact in skeletal muscle, including reduced type I aerobic fibers relative to type II³⁹. Age-related sarcopenia also entails significant atrophy of type II fibers⁴⁰ with aggregate effects of reduced muscle mass and abnormal muscle function⁴¹. Muscle contraction depends on adenosine triphosphate (ATP) generation from mitochondrial oxidative phosphorylation, a process that relies on nutrition and cellular respiratory capacity as well as oxygen (O₂) delivery (contingent on vascular flow and the microcirculation). When aerobic metabolism is insufficient to meet energy demands for physical activity, aerobic capacity diminishes (phosphocreatine [PCr] depletion, adenosine diphosphate [ADP] accumulation) and anaerobic glycolysis is activated (with lactic acid accumulation). Diminished PCr and increased lactic acid have both been linked directly with muscle performance fatigability⁴² and to increased minute ventilation (VE) as the means to disperse increased skeletal muscle carbon dioxide (CO₂) production. Fatigability may relate to both insufficient energy and/or the extra work of ventilation. Furthermore, fatty infiltration of muscle is also common among older healthy⁴² and HFpEF⁴³ populations, with associated increases in exercise intolerance and fatigability. Related studies in healthy elderly show age-related decreases in the capacity for oxidative phosphorylation⁴⁴⁻⁴⁷. This may have important functional consequences as mitochondria produce over 90% of ATP needed for movement and lower mitochondrial function is related to higher fatigability⁴⁷⁻⁵⁰.

Other HFpEF features compound fatigability risks. Central factors^{22, 51, 52} include insufficient contractile reserve, pulmonary hypertension (PH), chronotropic incompetence, and increased pulmonary dead space. Peripheral factors^{22, 53, 54} include endothelial dysfunction, reduced microvasculature, central vascular stiffening and ergoreceptor activation. Systemic factors include inflammation⁵⁵⁻⁵⁷, nutrition (diet and absorption), vitamin K, sleep (changes in sleep quality, increased nocturia and sleep apnea), and comorbidity (e.g., diabetes, chronic kidney disease, hypertension, anemia, depression). Relevant covariates are factored in this analysis.

Oral NO₂ salts as a novel therapy to treat HFpEF: This study is particularly innovative in applying inorganic NO₂ salt to treat HFpEF. Novel factors include: (i) Direct NO₂ benefits on skeletal muscle in HFpEF; (ii) NO₂ benefits on pulmonary and systemic vascular coupling and exercise responses in HFpEF; (iii) Utility of chronic NO₂ therapy in a capsule form (as compared to NO₃ ingested in foods, inhaled as gas, or infused).

Nitric oxide (NO) is known to regulate multiple physiological processes pertinent to exercise capacity and overall health^{29, 31, 58-60}. Nitric oxide synthase (NOS) is an endogenous enzymatic source of NO. Dietary NO₃ is an alternate source of NO. Leafy green vegetables and beetroot contain high content of NO₃. NO₃ is reduced to NO₂ by bacteria in the saliva, absorbed into the

blood, and then circulating NO₂ is reduced to NO, particularly in hypoxic conditions, and functions in multiple vascular and metabolic controlling roles⁶¹.

Studies in young healthy subjects have demonstrated benefits of NO₃ in respect to improving exercise efficiency^{31, 60}. VO₂ is reduced for an equivalent workload³¹. Larsen compared healthy men before and after dietary NO₃ supplements³¹. Gross efficiency increased from 19.7 ±1.6 to 21.1±1.3%, with no differences in HR, lactate, VE or the respiratory exchange ratio (RER) indicating this was mediated purely as an intrinsic skeletal muscle change. These analyses also clarify the role of enhanced mitochondrial efficiency with NO₂-mediated performance gains. Mechanistically, this may be due to decreased mitochondrial proton leak with the adenine nucleotide translocase being a potential protein target¹⁶. Gladwin has significantly advanced insights regarding NO₂'s therapeutic potential^{29-30, 61-62}, in studies of patients with metabolic syndrome and hypertension, oral NO₂ was well tolerated and upregulated skeletal muscle SIRT3, mitochondrial SIRT3 and AMP-activated protein kinase (AMPK) in HFpEF-PH⁸. Related benefits include improved glucose uptake (mediated by improved Glucose transporter type 4 [Glut4] expression)⁶⁸. Related literature highlights disproportionate benefits of dietary NO₃ in type II skeletal muscle fibers⁵⁸, implying the conceptual utility for older adults prone to sarcopenia-associated Type II fiber atrophy. Benefits are greater in those not already optimally fit (which seems well-suited to most older adults)⁵⁸.

Co-I Simon demonstrated utility of aerosolized NO₂ in 6 HFpEF patients (age 65±8). All patients tolerated NO₂ well. Acute hemodynamic effects of NO₂ included significant decreases in the right atrium (RA), right ventricle (RV), pulmonary artery (PA) and pulmonary capillary wedge pressures (PCWP).* (Presented at American Thoracic Society, 2016 Meeting). These findings reinforce Borlaug's recent study of infused NO₂ in HFpEF patients aged 69±6 years¹⁰³. Here too, NO₂ was well-tolerated and also showed significantly improved acute exercise hemodynamics. Borlaug concludes with a strong mandate to implement clinical trials incorporating chronic NO₂ therapy (such as we now propose).

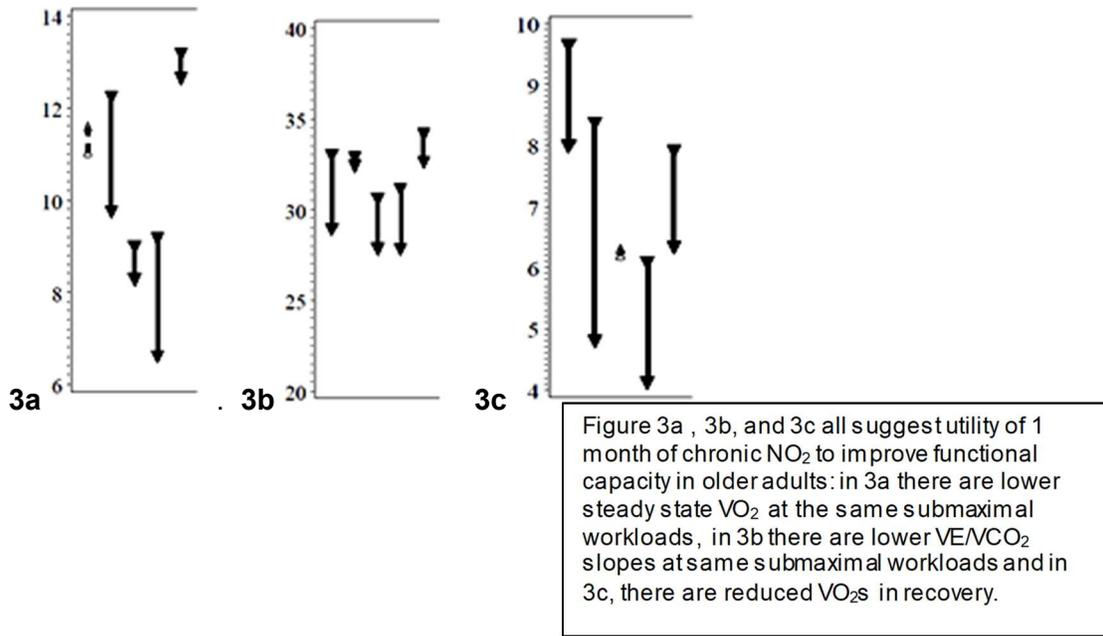
In order to clarify muscle vs. vascular-mediated NO₂ benefits, this protocol includes thorough muscle and vascular assessments. NO₂ effects on skeletal muscle mitochondrial bioenergetics will be measured using in vivo MRS and near infrared spectroscopy (NIRS) as well as ex vivo analysis of biopsy specimens using high-resolution respirometry and other analyses. In addition, a subset of the sample will undergo right and left heart vascular flow dynamics using invasive CPET (iCPET) and echocardiography.

To date, most published studies have utilized NO₃-rich beetroot juice, which provides short-acting effects, including acute changes in exercise duration, shorter time to achieve a pre-specified work level, and greater performance during intense intermittent exercise^{58, 64}. However, the challenge of developing and studying more prolonged NO₂ therapy remains. Co-I Hughan is leading formative pharmacokinetic (PK) studies to determine optimal safety and efficacy of NO₂ capsules, with studies of escalating dosing and chronic therapy⁶⁵. Her expertise in human NO₃/NO₂ metabolism and application of NO₂ therapy provides a critical dimension of innovation and rigor of the proposal. NO₂ capsules may also help overcome inherent limitations to dietary NO₃ in green vegetables, e.g., diet restrictions in the many older patients using warfarin, and/or abnormalities in mastication, salivation or achlorhydria that are also common in older adults.

The PI, Dr. Forman has an ongoing pilot study of oral NO₂ 40 mg tid with healthy controls, HFpEF and HFrEF subjects. Ten control subjects have completed the pilot without any significant product related adverse events. Mild and transient symptoms consistent with what other investigators have observed have been present in some of the other subjects.

Experience thus far in the pilot with heart failure subjects has suggested a tendency for a vasoactive response to nitrite not seen in the controls. Subjects with both HFrEF and HFpEF enrolled thus far have all required dose reductions to 20 mg tid to prevent hypotension. Thus we have adopted the stratified dosing as described for this protocol.

As the pilot is ongoing, data is incomplete. One preliminary analysis has been completed. In assessments of the 5 healthy controls, needle point data show 1 month of NO_2 at 40 mg tid achieved reduced VO_2 at the same submaximal workloads (Figure 3a), reduced VE/VCO_2 (Figure 3b) slopes at SS workloads, and significantly improved post-exercise recovery dynamics (Figure 3c)



2. RESEARCH DESIGN AND METHODS

2.1 CLASSIFICATION AND METHODOLOGICAL DESIGNS

The study is a prospective, double blind, single-center, randomized drug treatment study of 4 weeks of oral sodium NO_2 (20 or 40 mg tid) in elderly subjects, 70 years of age or older with HFpEF.

2.2 DETAILED DESCRIPTION OF STUDY DESIGN

This is a prospective, randomized, double blind controlled trial of oral sodium NO_2 vs. placebo in adults ($n=18$), ≥ 70 years of age with heart failure and preserved ejection fraction ("HFpEF"). Subjects who meet the Inclusion Criteria and none of the Exclusion Criteria will receive oral sodium NO_2 or placebo for 4 weeks.

We will stratify dosing as below:

Drug:

- a. oral formulation of sodium nitrite/placebo 40 mg three times daily (tid), once in the morning, in the early afternoon and again in the evening, approximately 6 hours apart in subjects age 70 to <80 years with low risk for hypotension
- b. oral formulation of sodium nitrite/placebo 20 mg tid, once in the morning, in the early afternoon and again in the evening, approximately 6 hours apart in subjects ≥ 80 years of age, and subjects age 70 to <80 years with risk factors for hypotension

Subjects will undergo a screening visit, then pre-drug testing protocol in 3-4 visits, and then repeat those tests after 4 weeks on drug in 3-4 visits (number of visits dependent upon whether subjects consent to undergo right heart catheterization testing pre- and post-drug).

On visit 4, subjects will be evaluated as outpatients with the first dose of study drug/placebo. BP, HR, RR, SpO₂, MetHb levels and plasma NO₂ will be measured at baseline and in the first two hours after dosing. Frequent safety monitoring will occur during the time subjects are on study drug/placebo by phone, through the post drug assessments in clinic to assess adverse events (AE), interval histories, medication compliance, symptoms and methemoglobin levels, and at the final visit, NO₂ levels.

Subjects will not be withdrawn from known effective therapy for the purposes of participating with the exception of:

1. holding of anti-coagulant medications prior to the biopsies
2. holding warfarin, dabigatran, rivaroxaban, apixaban or other novel anti-coagulants (NOACs) prior to right heart catheterization
3. holding or reducing dose of hypoglycemic medications on an individualized basis for Visits 2, 3B, 4, 5, 6B, 7.

Study Visits

Visit 1

The outpatient screening will take place in the UPMC Montefiore CTRC and will last approximately 2 hours. The following procedures and assessments will be completed:

1. Obtain written informed consent using standardized procedures. The consent will include the option to participate in or decline the iCPET at visits 3B and 6B.
2. Medical history obtained prior to the 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. History and physical examination. Dr. Forman or study physician will evaluate subjects 70-<80 years of age for risk of hypotension and need for dose reduction. The study physician will also complete the Canadian Clinical Frailty Scale. Subjects will be reminded to inform study staff as soon as possible of any health or medication changes while they are in the study.
3. Body weight and height. BP and other vital signs [temperature, pulse, respiratory rate (RR), HR, SpO₂]. BP may be re-measured once 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.
Screening evaluation will include a hemoglobin and hematocrit (H/H) by the UPMC Presbyterian 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 subjects have a recent hemoglobin value, the physician will determine if this blood draw can be deferred.
4. Subjects will be issued an ActiGraph GT3X+, non-invasive wrist monitoring device to be worn for a full 7 days during the first and approximately 9th weeks of the study to assess changes in daily activity, level/intensity and sleep. Subjects will receive instruction in the use of the

- ActiGraphs and complete a physical activity/sleep diary for days wearing the device. Subjects will be reminded to return their ActiGraph at Visit 2/3A.
5. Transthoracic Echocardiogram (performed by trained study staff). If subjects have had a recent echocardiogram, the records will be obtained prior to visit one and the test results will be reviewed and interpreted by a study physician in lieu of performing one. A trained sonographer will obtain the echocardiogram and a cardiology attending will be responsible for interpretation. The subject may be transported to a lab in the UPMC Presbyterian/Montefiore system for the echocardiogram.
 6. Subjects will be instructed in and provided forms to complete a three-day food record (3DFR) to observe subject's dietary intake.

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

Visit 2

Subjects will be scheduled for an outpatient EMRC and/or Pepper Center/SMART Center visit within 28 days of Visit 1. 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. For subjects who have diabetes, we will review their medication regimen and measures of diabetes control and advise subjects accordingly to prevent any exercise-induced hypoglycemia. In most cases, no adjustment will be needed for this non fasting visit. Subjects will undergo:

1. Interval history and brief physical exam. Measurement of body weight, BP and other vital signs (temperature, pulse, RR, HR, SpO₂)

Physical Function Battery:

2. Non-invasive Cardiopulmonary Exercise Testing (nCPET): A maximal graded exercise test 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 treadmill will be used to generate a symptom-limited exercise stimulus. ACSM criteria for starting and stopping will also be utilized. A lightweight mouthpiece or face mask will be positioned over the subject's mouth and nose during the exercise for gas exchange assessments. VO₂, VE/VCO₂ slope, and RER will be measured as well as hemodynamics (max HR and BP), time, and ECG waveforms). Any unexpected abnormalities will be reported to the patient's cardiologist; continued participation in the study will require physician clearance. Both the RPE and the Modified Borg Scale for Perceived Dyspnea (Shortness of Breath) will be completed during the nCPET. Dr. Forman has performed this test in more than 1,000 subjects ranging from elderly women greater than 80 years old to highly trained athletes.
3. Fatigability assessment: fatigability will be assessed based on RPE (6-20 scale) reported after a SS 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 al³². Those with RPE ≥10 will be categorized as highly fatigued. Our prior work in HF patients indicates this SS 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. Subjects who are unable to complete 5 minutes of walking will be considered highly fatigued.
4. A 6MWT will be performed. Subjects walk as quickly as possible on a 50 meter course⁶⁶. Total distance and segment gait speed are assessed. Performance fatigability is assessed

as the ratio of gait speeds in the second vs. next-to-last segments. Based on Murphy, et al., a $\geq 6.5\%$ decrease in speed is considered high performance fatigability¹⁰⁶.

5. Portable CPET during SS and 6MWT

Subjects will wear a portable CPET device during the SS and 6MWT. Standard breath-by-breath CPET ventilatory indices VO_2 , VE/VCO_2 , and RER are collected and used to characterize metabolic parameters in correlation with self-determined walking speeds over time⁶⁷. Average VO_2 per lap during steady-state (2 minutes of plateaued VO_2) is used to calculate the energy cost of walking by normalizing the VO_2 to lap gait speed (ml/kg/m). Using these data, performance fatigability can be assessed in proportion to the peak aerobic capacity (i.e., VO_2 during 6MWT relative to peak VO_2 assessed by nCPET-treadmill), as well as energy cost of walking (VO_2 during 6MWT relative to gait speed). Mechanisms underlying NO_2 benefit are also distinguished by comparing ventilatory vs. metabolic parameters, with the potential for metabolic performance to improve if fatigability is mitigated.

6. Participants will complete the “Short Physical Performance Battery” which is a series of 3 tests: 1. sitting and standing out of a chair; 2. walking over a 4 meter course (to measure walking speed); 3. standing with their feet in different positions to measure their balance.

7. Participants will also wear ActiGraphs during pre- and post-intervention nCPET, SS treadmill test, and 6MWT. These accelerometry data are used to establish individualized cut points that facilitate analysis of free-living accelerometry assessments. Volume and pattern metrics are used to analyze the raw data^{68,69}. We use the raw data and machine learning methods^{70,71} to explore patterns of activity that are most strongly associated with changes in fatigability.

8. Near Infrared Spectroscopy (NIRS)

NIRS will be performed during the nCEPT, SS and 6MWT. During these assessments, sensors will be placed on the skin of one leg to allow the non-ionizing, LED light source to assess tissue oxygenation and perfusion via measurements of the optical absorption of hemoglobin. Co-I Dr. Huppert will direct the training and data analyses of the NIRS measurements.

9. 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 with three trials.

10. Questionnaires

The following questionnaires will be administered:

Sleep	The Pittsburgh Sleep Quality Index ⁷²
Depression	PHQ-9: well-validated and responsive to change ⁷³
Self-efficacy	Sullivan, a five-item summative score ⁷⁴
Pain	McGill pain questionnaire-15 descriptors (11 sensory, 4 affective); pain rated on an intensity scale (0-3). Pain scores derived from the sum of the intensity rank values ⁷⁵
Comorbidity	The Charlson Comorbidity Index: provides a convenient and well-validated assessment of aggregate morbidities ⁷⁶
Frailty (done at Visit 1 & 7)	The Canadian Study of Health and Aging Clinical Frailty Scale is a 7 point frailty score that was developed for predictive sensitivity as well as its ease of use ⁷⁷

Cognition	Montreal Cognitive Assessment. A well validated tool to screen for dementia ⁷⁸
Quality of Life	Kansas City Cardiomyopathy Questionnaire (KCCQ). 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 quality of life measure for heart failure patients ⁷⁹
Physical Activity	CHAMPS (Community Healthy Activities Program for Seniors) Activities Questionnaire for Older Adults ⁸⁰
Fatigability	Pittsburgh Fatigability Scale ⁸¹

We will ask participants to complete them interspersed among the physical function tests so as to reduce fatigue of both assessments.

The 3DFR issued at Visit 1 will be reviewed with the subject for clarification and completion as well as the ActiGraph diary.

Prior to departing, we will remind subjects to resume their usual medication regimen if changes were made with regard to diabetes.

If study team or subject feel as though any of the study procedures at this visit are not safe and/or would be too burdensome to the subject, subject may not complete all of the procedures at this visit.

Visit 3A

Subjects will be scheduled for an outpatient visit at the University of Pittsburgh Magnetic Resonance Research Center (MRRC) in UPMC Presbyterian 5±2 days from Visit 2. This is a non-fasting visit (patients will be asked to eat a light meal) and will last approximately 2-3 hours. Subjects who are identified to have the presence of metal or non-MRI compatible device will not complete Visit 3A (or 6A).

Subjects will undergo:

1. Collection of interval history, measurement of BP and other vital signs (temperature, pulse, RR, HR, SpO₂) by study or MRRC staff.
Any concern for medical instability after vitals and interval history are collected will prompt contact with a study physician, and possible physical exam before proceeding.
2. The study coordinator will clear the subject as safe for MRI prior to the enrollment. Then the safety protocol and procedures of the MRRC will be followed as well as a double check for safety. The MRS is an in-vivo test to measure maximal mitochondrial capacity to generate ATP. It will be performed on the subject's leg as they complete a flexion-extension exercise within the bore of the magnet as described below.

Prior to the exercise, the technician will collect a fully-relaxed (FRS), high resolution ³¹P spectrum of the resting muscle. Then the exercise protocol will be performed: Participants lay supine with the non-dominant knee (unless contraindicated) elevated at ~30° and straps are placed over the legs. Participants perform voluntary isometric contractions (kicking) repeatedly as hard and as fast as they can for two bouts each followed by a rest period. The protocol is designed to deplete PCr stores by 33-66% to ensure high signal to noise defining PCr recovery without inducing acidosis (pH <6.8), which inhibits oxidative phosphorylation.

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3. Following completion of the MRS protocol, subjects will be provided parking and discharged.

Note: Visit 3B is an optional visit. Subjects who choose to do the right heart catheterization visits will need to have blood drawn for a basic metabolic panel, PT/INR, and CBC/diff/platelets, preceding Visit 3B by no longer than 30 days, per the UPMC Cath. Lab protocol. We will coordinate this draw at one of the study or if the subjects have bloodwork prescribed for their ongoing medical care. Subjects will be provided parking for the blood draw visit, if it requires a separate visit.

Visit 3B

This visit will be completed by the subjects in the recruited sample who have consented to invasive cardiopulmonary exercise testing (iCPET). Subjects may decline this portion of the testing at consent. This fasting visit will take place in the UPMC Presbyterian Cardiac Catheterization lab (Cath. Lab) within 5 ± 2 days from Visit 3A and last approximately 4-6 hours. Subjects with diabetes will receive advisement on issues related to blood sugar management per the standardized pre-catheterization protocol of the UPMC Cath. Lab. Subjects on warfarin or NOACs will be reminded by study staff to hold those medications 3 days prior and including the day of the catheterization.

Upon arrival in the Cath. Lab, subjects will undergo:

1. Collection of adverse events, interval history, measurement of BP and other vital signs (temperature, pulse, RR, HR, SpO₂), as well as completing a breathing test, called [spirometry](#), that assess subjects ability to breathe in and out. We will confirm appropriate medications were held per individual case.
2. Brief physical exam
3. Right heart catheterization

Dr. Risbano, Co-Investigator, or a qualified pulmonologist or cardiologist investigator will lead the UPMC protocol for right heart catheterization, with strict adherence for safety and sterile conditions as indicated, including the use of fluoroscopy to guide the catheter placement. PA (internal jugular approach) and radial artery catheters are placed. Systemic and PA pressures (PAP) are measured continuously during exercise provocation (described below). PCWP is measured at baseline and each minute thereafter. End-expiratory assessments of right atrial pressure (RAP), RV pressure, PAP, and PCWP are assessed.

Approximately 2 ml blood samples are drawn from the radial and PA catheters during rest and the last 15 seconds every other minute of exercise for measurement of blood gases (PA blood gas will be used for assessments of Qt and peripheral blood gas for assessment of systemic O₂ extraction i.e., PA oxygen (Ca) minus venous oxygen (vO₂), as well as for pH and lactate. Systemic and pulmonary arterial blood are analyzed at 37°C for partial pressures of O₂ and CO₂ (PO₂, PCO₂), pH, hemoglobin (Hb), and O₂ saturation. Blood O₂ content is calculated from Hb and O₂ saturation: $S(a)O_2 \times Hb \times 1.34$. The Fick principle is used to calculate Qt (i.e., $Qt = VO_2 / (Ca - vO_2)$) during exercise. Predicted cardiac output (Qtmax) is calculated from the predicted VO₂ max assuming a normal maximum exercise arterial-mixed venous O₂ content difference (Ca-vO₂) and Hb of 14 g/dL. The pulmonary vascular resistance (PVR) is calculated from $(PAP - PCWP) / Qt$. RPE and ratings of dyspnea are assessed during iCPET and analyzed in relation to simultaneously collected lactic acid levels as a further assessment of skeletal muscle metabolism underlying fatigability⁸⁴. Furthermore, these relationships will be assessed in relation to concurrent ventilatory dynamics (VO₂, VE/VCO₂,

and RER) as determined by concurrent CPET. The total blood volume will depend on the total time exercising but is likely not more than approximately 32 ml (2 ml at baseline and 2 ml per minute of exercise, maximum likely 15 minutes, from radial and pulmonary arteries)

Cardiopulmonary Stress Exercise Testing to be performed during the right heart catheterization (invasive CPET): Maximal graded exercise testing will be conducted in association with air-gas-exchange during the right heart catheterization. A qualified pulmonologist or cardiologist will be available throughout the exercise provocation. 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) are ventilatory parameters that will be measured during exercise. Simultaneously, intra-cardiac assessments of pulmonary wedge pressure, right heart pressures, and cardiac output will be determined by the pulmonary arterial catheter. HR and BP, time, and ECG waveforms will also be assessed. Any unexpected abnormalities will be reported to the patient's cardiologist or primary care physician; continued participation in the study will require physician clearance. Subjects will be asked for ratings of exertion and shortness of breath during the testing. ACSM criteria for starting and stopping will also be utilized.

iCPET may be completed in combination with echocardiography depending on the quality of baseline images. Echocardiographic images may be obtained at rest at baseline, and then every 2 minutes during exercise and immediately post-exercise. Quantitative echo measures of LV size, and systolic and diastolic function are made at rest and exercise. Lusitropy is assessed based on the calculation of left ventricular end-diastolic volume (LVEDV) divided by PCWP. The net effect on cardiac function is a Starling curve, i.e., $SV/LVEDV$ at rest and with exercise.⁸⁴

4. Following completion of the testing, subjects will have catheters removed and monitoring for about 2 hours post-test. They will be given any needed education for post catheterization, including site care and resuming anti-coagulant or anti-diabetic medications that were held for the procedure. Prior to discharge, they will be confirmed medically stable by a study physician. Subjects will be provided a meal.

Visit 4

Subjects will be scheduled for an outpatient visit at the University of Pittsburgh CTSC in UPMC Montefiore within 10 days \pm 4 days from Visit 3A for subjects who did not complete iCPET or within 5 days \pm 2 days from Visit 3B for those who did complete iCPET.

Subjects will be asked to observe 8 hours fasting prior to arrival. Upon arrival subjects will be served a limited standardized breakfast.

This visit will last approximately 4-5 hours. To prevent acute effects of exercise on muscle mitochondrial function, this visit will occur no earlier than 2 days after Visit 3A/3B. 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, as approved by their cardiologist). Subjects with diabetes who are on hypoglycemic medications will be advised if they need any adjustment to their usual regimen.

While the standardized breakfast is being prepared, subjects will undergo:

1. Interval history, measurement of BP and other vital signs (temperature, HR, body weight, RR, and SpO_2)

2. Staff will also confirm subject is fasting and that the anti-coagulation regimen was withheld as prescribed. Nursing will place an IV line.
3. A study physician will perform a brief physical exam.

4. Skeletal Muscle Biopsies

An important component of the protocol is the muscle biopsy. Subjects who refuse biopsy will not be enrolled. However, participants who complete the first biopsy and then state (prior to being given drug) that they will not complete a second biopsy at Visit 7 will be withdrawn from the study. Alternatively, patients who refuse the 2nd biopsy after they have completed the 4 weeks of NO₂/placebo therapy will still complete the other post drug assessments. If other unique subject situations arise, they will be evaluated by the PI and a decision will be made with regard to completing the other post assessments or not (e.g., completion of 2 weeks on drug, and then refusal to complete biopsy.)

For the biopsy, 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 chlorhexidine. Lidocaine will then be administered, superficially initially and then more deeply into the muscle, using a small gauge needle. Subjects who report a history of poor pain control or low threshold will be offered the use of a topical anesthetic cream prior to the lidocaine. 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 trocar 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. Muscle specimens are divided into portions. Fragment 1 is immediately frozen in liquid nitrogen in preparation for polymerase chain reaction (PCR) analysis for DNA /RNA isolation, protein homogenates used for enzymatic assays and Western analysis. Fragment 2 has 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 histochemistry. Fragment 3 is prepared for electron microscopy. Fragment 4 is processed in the CTCRC and EMRC immediately for bioenergetics analysis. Any extra tissue will be stored for future analyses.

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

5. Research bloods

After the standardized meal is completed, a blood sample via IV line will be drawn for inflammatory peptides (IL-1, IL-6, IL-15, TNF α), adipokines (leptin and adiponectin) vitamin D, GFR, BNP or NT-pro BNP, TSH, HbA1c. Samples for platelets and NO₂ levels will also be drawn at baseline into a 1) heparinized syringe and 2) a cell preparation tube (CPT). Additional collection of NO₂ levels will be drawn at 30 minutes, 60 minutes and 120 minutes.

Heparinized blood will be immediately centrifuged at bedside to separate plasma and red cells and then flash frozen for later NO₂ measurements (via reductive chemiluminescence to determine that NO₂ levels were increased in the muscle as described. The second tube of blood (CP tube) will be immediately subjected to differential centrifugation in order to isolate platelets. Platelet bioenergetics will then be measured via the Seahorse Extracellular Flux Analyzer including basal, non-ATP producing, maximal and non-mitochondrial respiratory rates along with glycolytic rate. If subjects have had an HgbA1c, TSH or vitamin D level within approximately 3 months, or a BNP or GFR within approximately 1 month, (all per physician discretion), we may defer this blood draw.

Extra aliquots will be stored for use in future studies. Total volume of blood drawn will be about 72 ml (about 4.9 tablespoons).

6. pK

CTRC nursing staff will measure BP, RR, HR, SpO₂ and MetHb to ensure they are at a SS before administering the study drug/placebo (baseline). Subjects who are 80 years of age or older or <80 with risk factors for hypotension will receive 20 mg drug/placebo; all others will receive 40 mg drug/placebo. Subjects will receive their first dose of study drug/placebo as follows: 14N sodium NO₂/placebo, from their day 1, AM dose, week 1 pill box, strength 20 or 40 mg dependent upon parameters described in section "Study Design", p. 6. Then, the following will be assessed: BP (sitting and then standing after 3 minutes), HR and MetHb, RR and SpO₂ every 15 minutes for the 2 hours post-drug administration period. If subjects experience high methemoglobin level, hypotension or symptoms, the PI will be informed and advise on plan for dosage or other management. See Dose Selection, subsection, Dose Reduction).

7. Drug distribution

Subjects will receive sufficient study drug/placebo supply to cover the period up to the next scheduled visit at Visit 5 and/or through Visit 7 as needed. We will instruct subjects to return all of the study drug packaging, including unused study drug and empty packaging at each study visit and/or at the time of discontinuation from treatment.

Study staff will review medication administration and use of the drug diary card with all subjects. Subjects will be asked to record the time they take the drug each day and any symptoms with severity rating and/or health events during the dosing period.

The study staff and/or CTRC RN will review biopsy site care with subjects and if indicated, remind subjects to resume their normal anti-coagulation/anti-platelet/anti-diabetic medications.

The subject will receive payment for visits 1-4 and can then be discharged.

Drug Dosing Period and Outpatient Monitoring

Subjects will then complete no less than 28 (-3) days on drug. Subjects will continue on drug/placebo through the post testing period; the duration will vary with the visit windows and if subjects are doing both 6A and 6B. During the 28-day period, subjects will be called on a weekly basis by study staff, beginning on the visit 4 day plus 7 days \pm 2 days, and continuing approximately weekly thereafter. Study staff will query subjects on any symptoms, home BP readings if applicable, health care, including medication changes, or adverse events, and compliance with study drug/placebo regimen. Any AEs or medical issues of concern will be promptly communicated to the PI or covering study physician by email or phone depending on urgency; and AEs will be documented in the study log which is reviewed with PI at least bi-

weekly. Subjects will be again reminded to communicate any medication changes prescribed by their health care team to study staff as soon as possible, not to wait for the weekly call. (Serious adverse events will be reported as described in Section 6.4.5.) Management of hypotension or methemoglobinemia related symptomatology will be as described in 2.3.3 Dose Selection, subsection Dose Reduction.

Post Assessments (continuing on NO₂/placebo tid)

Visit 5

This visit will be scheduled 28 ±3 days from Visit 4, take place in the UPMC Montefiore EMRC and/or Pepper SMART Center and include all of the Functional testing described in Visit 2.

1. Upon arrival subjects will have measurement of BP, temperature, RR, HR, SpO₂ and MetHb and body weight. Study staff will check for interval history, adverse events, and complete a symptom assessment. The drug diary cards and empty drug boxes for completed weeks on study drug/placebo will be collected. Medication compliance will be reviewed. A study physician will perform a brief physical exam.
Physical Function Battery
 - a. nCPET
 - b. Fatigability assessment on treadmill (SS).
 - c. Self-determined walking (6MWT)
 - d. NIRS will be collected during the nCPET, SS and 6MWT.
 - e. Short Physical Performance Battery (SPPB).
 - f. Participants again will be asked to wear the Actigraph during the post-intervention nCPET treadmill, 6MWT and steady-state treadmill. After testing, subjects will wear the ActiGraph for one week and complete a physical activity/sleep diary for days wearing the device.
 - g. Hand Grip Strength Assessment
2. The same questionnaires as at Visit 2 will be administered interspersed with functional testing.
3. Subjects will be asked to complete another 3DFR as per Visit 2.
4. Prior to discharge, subjects will be reminded to return the ActiGraph and 3DFR at visit 6A or 6B, depending on testing schedule.

If study team or subject feel as though any of the study procedures at this visit are not safe and/or would be too burdensome to the subject, subject may not complete all of the procedures at this visit.

Visit 6A

This visit will replicate the ³¹P Magnetic Resonance Spectroscopy protocol described at Visit 3A. The same safety precautions will be followed. This visit will be scheduled within 5 ±2 days of Visit 5. Following this visit, the subject will advance to either Visit 6B or 7 depending if consented to the iCPET. Pre- catheterization labs will be drawn as described per Visit 3A.

1. Upon arrival subjects will have interval history and measurement of BP, temperature, RR, HR, SpO₂, and MetHb. Study staff will check for interval history, adverse events, and complete a symptom assessment. The drug diary cards and empty drug boxes for completed weeks on study drug/placebo will be collected. Medication compliance will be reviewed.
2. Any concern for medical instability after vitals and interval history are collected will prompt contact with a study physician, and possible physical exam clearing to proceed.
3. If completion dates have passed, the 3DFR issued at Visit 5 will be reviewed with the subject for clarification and completion.

Visit 6B

This visit will replicate the iCPET testing described in visit 3B for those subjects who elected to participate. This visit will be scheduled within 5 ±2 days of Visit 6A. The same safety protocol and procedures will be followed as in 3B. This visit will be completed by the subjects in the recruited sample who have consented to iCPET, Visit 3A. This fasting visit will take place in the UPMC Presbyterian Cardiac Catheterization lab and last approximately 4-6 hours. Subjects with diabetes who are on hypoglycemic medications will be advised on medications to hold the day before and morning of visit.

iCPET

Upon arrival in the Cath. Lab, subjects will undergo:

1. Collection of adverse events, interval history, measurement of BP and other vital signs (temperature, pulse, RR, HR, SpO₂ and MetHb) and symptom assessment.
2. Brief physical exam
3. UPMC protocol for right heart catheterization will be followed, with strict adherence for safety and sterile conditions as indicated. PA (internal jugular approach) and radial artery catheters are placed as described in 3B. Volume of blood as noted approximately 32 ml, maximum.
4. Following catheterization, subjects will have catheters removed and be given any needed education for post catheterization site care. They will be confirmed medically stable by a physician, before leaving. Subjects will be provided a meal.

Subjects will return their Actigraph and diary at this visit, or Visit 7 if they do not complete Visit 6B with heart catheterization.

Visit 7 (Completion Visit to be scheduled 10 days ±4 days for subjects only doing visit 6A or 5±2 days following Visit 6B).

This visit will be at UPMC Montefiore CTSC and will replicate the blood draw and muscle biopsy procedures described in Visit 4. The same precautions and procedures will be followed including reminding, withholding, and confirming of the anti-coagulant/anti-diabetic regimen per subject. Subjects will take their last morning dose of study drug/placebo on the day of the visit. Subjects will be asked to observe 8 hours fasting prior to arrival. Upon arrival subjects will be served a limited standardized breakfast. Confirmation of last dose will be completed with subjects at the visit.. This visit will last approximately 2 hours.

1. While the standardized breakfast is being prepared, interval history/AEs and temperature, RR, BP, HR, SpO₂, MetHb, body weight, and symptom assessment will be collected. A brief physical exam will be completed. The Canadian Clinical Frailty Scale will be repeated.
2. Medication compliance via drug diary cards, empty pill box compartments, and subject report will be assessed. Unused capsules will be counted and returned to IDS following the visit.
3. The 3DFR issued at Visit 5 will be reviewed with the subject for clarification and completion.
4. ActiGraph and diary will be collected if not at previous visit
5. Blood draw to include: Inflammatory peptides (IL-1, IL-6, IL-15, TNFα), adipokines (leptin, adiponectin). GFR, BNP or NT-pro BNP, platelet sample and NO₂ levels. Additional NO₂ levels will be drawn at 30 minutes, 60 minutes and 120 minutes. Total volume of blood drawn will be about 64 ml (about 4.3 tablespoons). If subjects have had BNP or GFR within approximately the last month via their clinical care, the study physician can defer this blood draw.
6. Skeletal Muscle Biopsies: same as described in Visit 3.
7. Transthoracic Echocardiogram. The subject may be transported to a UPMC Presbyterian/Montefiore lab for an echocardiogram.

Following the echocardiogram subjects will be discharged. They will receive payment for visits 5-7 and study drug period.

End of Trial and Follow-Up Period

Post Study drug/placebo Telephone Assessment (about 7 days following Visit 7)

- Telephone assessment for interval histories, muscle biopsy follow up and AEs will be completed. Any remaining symptoms or AEs which are suspected to be related to the study drug/placebo will be evaluated by Dr. Forman and follow-up plan for care devised as needed.

Table.2.2.1 Study Visits and Assessments		
Phase	Visit	Assessments and Procedures
Screening	Visit 1	Informed consent and physical exam; Hgb* Home accelerometry; Echo†; issue 3DFR, frailty assessment
Pre- Assessment Series	Visit 2 (within 28 days of Visit 1)	Non-invasive functional assessment battery: Symptom-limited nCPET; Fatigability assessments (perceived [SS] and performance [6MWT]), NIRS during nCPET, SS, 6MWT, Accelerometry during nCPET, SS, 6MWT and 1 week at home with diary; Hand grip, SPPB, Questionnaires
	Visit 3A (5 days ±2 days from V2)	³¹ P MRS protocol; review 3DFR and return ActiGraph 3A or 3B per dates; pre-cath bloodwork if applicable
	Visit 3B (5 days ±2 days from V3A)	Symptom limited iCPET with associated exercise echocardiography
	Visit 4 (10 days ±4 days from V3A or 5 days ±2 days from V3B)	Research bloods**; Skeletal muscle biopsy; Administration of first dose of NO ₂ ; PK assessment and BP assessment; Drug distribution
Drug dosing	Weekly phone calls	Phone call monitoring of drug tolerance, symptom assessment, medication compliance, adverse events.
Post-Assessment Series	Visit 5 (28 ±3 days from Visit 4)	Non-invasive functional assessment battery: Symptom-limited nCPET; Fatigability assessments (perceived [SS] and performance [6MWT]), NIRS during nCPET, SS, 6MWT, accelerometry during nCPET, SS, 6MWT and 1 week at home with diary; Hand grip, SPPB, Questionnaires; issue 3DFR
	Visit 6A (5 days ±2 days from V5)	³¹ P MRS protocol; review 3DFR and return ActiGraph 6A or 6B per dates; pre-cath bloodwork if applicable
	Visit 6B (5 days ±2 days from V6A)	Symptom limited iCPET with associated exercise echocardiography
	Visit 7 (10 days ±4 days from V6A or 5 days ±2 days from V6B)	Frailty assessment, Skeletal muscle biopsy; Research bloods‡; echo after meal

*Unless Hgb within normal limits recently prior to Visit 1 and deferred by physician; †deferred if recent per physician discretion;

**Platelets, nitrite/nitrates (time 0), + nitrites/nitrates (time 30 mins, 60 mins, and 120 mins); inflammatory peptides (IL-1, IL-6, IL-15, TNFα); adipokines (leptin, adiponectin); vitamin D, GFR, BNP or NT-pro BNP, TSH, HgA1C (unless recently measured)

‡Platelets, nitrite/nitrates; inflammatory peptides (IL-1, IL-6, IL-15, TNFα); adipokines (leptin, adiponectin); BNP, GFR, unless recently measured

2.3 Study Drug (NITRITE)

The study drugs are oral formulation of sodium nitrite and matching placebo. Sodium nitrite is available in dose strengths of 20 mg and 40 mg. Sodium nitrite of both dose strengths and matching placebo will be supplied as capsules for oral administration. All capsule formulations will be identical in appearance (size, shape, color) and smell. The packaging and labeling will be designed to maintain blinding to the Investigator's team and to subjects.

2.3.1 Study Drug Preparation and Dispensing

The NO₂ and placebo capsules will be prepared at and obtained from Triangle Compounding Pharmacy, Inc., Cary, NC and the University of Pittsburgh Medical Center Investigational Drug Pharmacy Service (UPMC-IDS) will be utilized for dispensing of the study drug/placebo. The labeling and packaging will be conducted according to Good Clinical Practice and regulatory requirements. The study drug is administered only to subjects enrolled in the study and in accordance with the protocol.

Drug will be dispensed in four 7-day pill containers, with compartments for each dose, each labeled with subject name and directions for taking. Subjects will be given an additional capsule supply in a pill bottle as needed to maintain an adequate supply for the post-visit follow-up testing period.

2.3.2 Drug Administration

Standard ¹⁴N Sodium NO₂ or placebo will be supplied as one capsule, three times daily oral administration at the dose strength of 20 or 40 mg (per subject as described in Study Design section), in four 7-day pillboxes for the first 28 days on drug, and then additional supplied for subjects to refill boxes dependent on their individual visit schedule.

2.3.3 Dose Selection

In the 2001 National Toxicology Program (NTP) Report summarizing 2-year rodent drinking water studies, there was no evidence of carcinogenic activity of NO₂ 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 NO₂ 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 NO₂ 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*⁸⁵. Taken together, these findings suggest minimal carcinogenic NO₂-mediated risk.

Multiple studies now demonstrate the predominant safety of NO₃ therapy in humans (most typically administered as beetroot juice)^{16,64}. At the University of Pittsburgh, several pilot studies show safety and efficacy of NO₂, the active metabolite of NO₃, and show strong physiological rationale for its use.

Kara Hughan is a co-investigator on this proposal. She has been studying utility of oral NO₂ (administered as capsules) to improve insulin sensitivity over 12 weeks in adults aged 18-60 years with metabolic syndrome in an NHLBI K23 funded study. Hughan's first subject (healthy) was started on NO₃ 1,000 mg once daily (with NO₃ supplying a more prolonged source of plasma NO₂ across the day via enterosalivary circulation until next dosing) in combination with NO₂ 20 mg once daily.

During the first subject's drug course, PK plasma NO_3 and NO_2 concentrations, MetHb and BP data were assessed every other week. Given that only minor increases in MetHb were detected (that remained well within the safety threshold) and that the plasma NO_2 rise after ingestion then fell to baseline after ~3-6 hours with NO_2 dosing, requests were made to the IRB to increase NO_2 dosing to 20 mg two times daily (bid).

At the same time that IRB approval was being pursued for NO_2 increases to 20 mg bid, a second subject was started on the original dosing regimen (NO_3 1,000 mg once daily and NO_2 20 mg once daily). At the 6-week time point, IRB approval was received to increase the NO_2 dose to 20 mg BID for the remaining 4 weeks. Thereafter Dr. Hughan recruited 3 subsequent subjects on sodium NO_3 1,000 mg once daily and NO_2 20 mg bid.

At these latter 4 subjects' biweekly safety visits, Hughan observed their morning NO_3/NO_2 dosing, measured BP and MetHb 30-40 minutes after dosing and plasma NO_3/NO_2 levels measured approximately 45-60 minutes after drug dosing. In all cases, none demonstrated 1) significant reductions in BP; 2) significant rise in MetHb (>5%); 3) excessive plasma NO_3 and NO_2 concentrations in comparison to the single dose PK profile⁶¹.

Given that there was only a brief and mild decrease in systolic BP with the single dose NO_3 dosing and no BP reduction when 12 weeks of NO_3 were combined with twice daily NO_2 , Hughan, Gladwin, and colleagues hypothesized that the moderate contribution of sodium in NO_3 might mitigate potential hypotensive effects.

In parallel, Mohler, ER⁸⁶ reported results of oral NO_2 40 mg bid vs. 80 mg bid vs. placebo for 10 weeks in 55 adults with peripheral arterial disease, most with diabetes. The high NO_2 doses were well-tolerated and were associated with relatively greater responses in flow mediated (endothelial) dilation. Given this report and the facts that 1.) Subjects at the University of Pittsburgh were similarly not approaching any safety stopping endpoints and, 2.) the NO_2 doses were well tolerated, another dose modification to the IRB/FDA was completed in which the NO_3 was removed from the regimen and NO_2 was increased in dose (from 20 to 40 mg) and frequency (from bid to tid).

Data below show results from 3 subjects (with metabolic syndrome and hypertension). 3 subjects (#6, 7, 8) were aged 51, 45, and 59 respectively; 2 were female (#6 and #8) and one was male (#7). NO_2 was initiated at 20 mg po tid and then after 2 weeks to 40 mg tid. Each subject was assessed serially over 12 weeks. Bi-weekly serum NO_2 levels (for individual subjects as well as the mean) are shown in Figure 1.

Fig. 1: Serum NO_2 levels

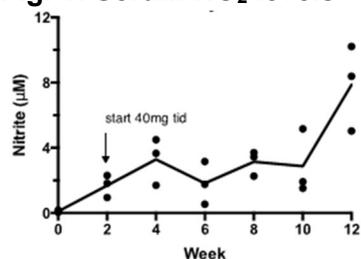
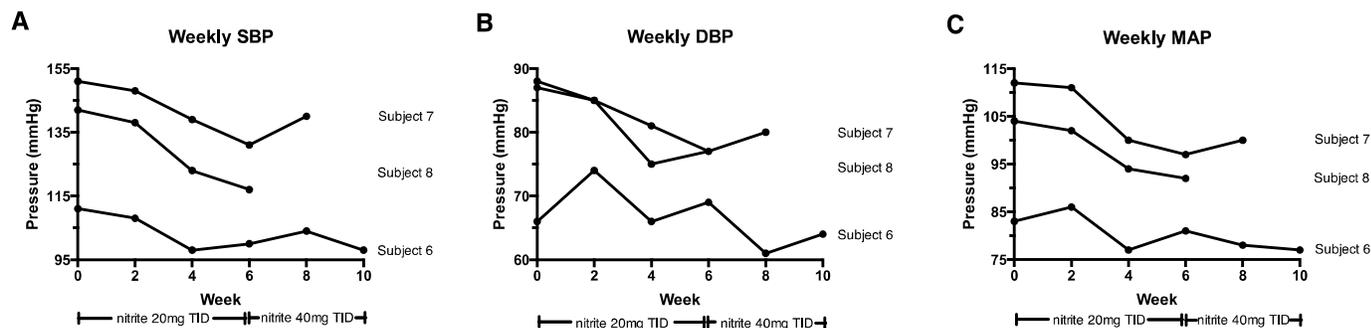


Figure 2: BP responses for 3 subjects

As figure 2 shows, there were mild drops in BP with 20 mg tid of NO₂ that in 2 of the 3 cases increased on the higher NO₂ dose thereafter. Subject #6 was also taking hydrochlorothiazide (HCTZ), subject #7 was also taking lisinopril, and subject #8 was also taking carvedilol. Most important, the NO₂ doses of 40 mg tid were well tolerated in all, both in relation to the hemodynamics and overall well-being. Consistently, MetHb never increased above 5%.

Dr. Hughan's trials also track other dosing strategies of exogenous NO₂ and NO₃ therapy and her data provide a broad profile of safety and excellent medication tolerance. In her trial, there have been no serious adverse events (SAE). The most frequently reported adverse events (AE) were headache, dry mouth and flushing. Other AEs reported in this patient population include low back pain or back spasm, nausea, anxiety, sleep improvement, joint/muscle pain, swelling in arms/legs, mild chest pain, shortness of breath, and cold symptoms. All AEs were mild to moderate in severity, and resolved spontaneously or with minor treatments.

Hughan's analyses of NO₂ capsules for patients with diabetes and hypertension is also linked to analyses of NO₂-mediated changes in skeletal muscle gene expression. Patients taking NO₂ capsules demonstrated upregulated skeletal muscle NAD-dependent deacetylase sirtuin-3 (SIRT3), mitochondrial SIRT3 and AMP-activated protein kinase (AMPK) in HFpEF-PH50. Related benefits include improved glucose uptake (mediated by improved Glucose transporter type 4 [Glut4] expression)^{8, 63}. This research is ongoing.

Co-Investigator Marc Simon has also been working on a NO₂ project as part of the University of Pittsburgh Translational Program Project Grant (TPPG). He studied acute efficacy of inhaled NO₂ in 27 subjects (ages 56-77 years, mean 65 ±8) with pulmonary hypertension (PH), including 6 subjects with PH associated with HFpEF. This was a prospective, open label safety and efficacy trial. The defined primary efficacy outcome was change in pulmonary vascular resistance. Safety endpoints included change in systemic BP and MetHb levels. All subjects underwent a standard right heart catheterization. Aerosolized NO₂ was administered with an initial dose of 45 mg and then increased to 90 mg if the initial 45 mg dose was tolerated (i.e., no decrease in systolic BP >40 mm Hg, no decrease in peripheral oxygen saturation >10%, no increase in MetHb level >5%, no severe bronchospasm or dyspnea). All patients tolerated NO₂ well and were asymptomatic. Only 1/27 patients did not receive the second dose of NO₂ due to a transient asymptomatic decrease in systemic systolic BP (that returned to baseline within 5 minutes without intervention). Serum NO₂ peaks at 15 min after inhalation of 45g was 7 μM and after 90 mg dose 15 μM. There were no significant decreases in peripheral oxygen saturation or increases in MetHb levels.

Hemodynamic effects of NO₂ in the HFpEF subjects included significant decreases in RA, RV systolic and diastolic, PA systolic/diastolic/mean, and PCWP. PCWP and MPAP decreased by 7.8 and 7.0 mm Hg, respectively (baseline median values 18 and 34 mm Hg, respectively). MetHb levels increased modestly but did not meet stopping criteria of the study. Dr. Simon presented his work at the American Thoracic Society Meeting in March, 2016. Overall, the data demonstrate feasibility and safety of aerosolized NO₂ in HFpEF, and in many respects corroborate the recently published analysis by Borlaug in a study of HFpEF patients aged 69 ±6 years that showed infused NO₂ benefits to acutely improve right heart hemodynamics during exercise¹. None of Borlaug's patients experienced hypotension or other adverse events. NO₂ levels were undetectable at baseline, but then increased to 8.39 ±1.88 μM. PCWP was substantially improved by NO₂ compared with placebo (p<0.0003). Likewise, cardiac output reserve improved with exercise (p<0.002) and an increase in cardiac output relative to O₂ consumption was normalized. NO₂ also improved pulmonary artery pressure flow relationships in HFpEF and increased left ventricular stroke work with exercise versus placebo, indicating an improvement in ventricular performance with stress.

As described in Section 1.4 Significance, the PI, Dr. Forman, has an ongoing pilot study of oral NO₂ 40 mg tid with healthy controls, HFpEF and HFrEF subjects. Ten control subjects have completed the pilot without any significant product related adverse events. Mild and transient symptoms consistent with what other investigators have observed have been present in some of the other subjects.

Experience thus far in the pilot with heart failure subjects has suggested a tendency for a vasoactive response to nitrite not seen in the controls. Subjects with both HFrEF and HFpEF enrolled thus far have all required dose reductions to 20 mg tid to prevent hypotension. Thus we have adopted the stratified dosing as described for this protocol.

Based on the combined efforts of Co-Is Hughan, Simon, and Gladwin, and PI Forman's ongoing pilot, as well as the published work by Borlaug, we have demonstrated feasibility, physiological utility, and safety for older HFpEF patients, and a dosing rationale of NO₂ 20-40 mg tid for this study, with a dosing range for subject safety. Our subjects will complete 28 ±3 days dosing period, followed by a range of 9 to 21 days depending upon scheduling needs, and whether subjects consent for the pre- and post- iCPET testing, resulting in a possible maximum days on drug/placebo of 52 days. The dosing period in PI Forman's NO₂ pilot ranged 25-49 days, when combining 28-day dosing with up to 21 day post-testing period. Subjects are followed closing during this post drug testing period. Because of the physical limitations of HFpEF patients and the multiple staff and facilities needed for the post testing period, a more chronologically compact post drug testing period is not feasible. All data to date suggest this does not pose undue risk to subjects.

Dose Reduction

There is a rare possibility that subjects will have a high methemoglobin level or low BP from the study drug dose during Visit 4 or while they are on study drug. If this happens, they may experience lightheadedness or dizziness.

High methemoglobin level

In CTRC

If this happens during visit 4 while they are in the CTRC, fluids and monitoring will be provided 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 CTRC as well as:

1. Determine if unblinding is needed (if methemoglobin % >5) and,
2. If subject is willing to do an interim visit with repeat drug dosing of 20 mg with follow up methemoglobin and BP pressure monitoring or
3. Subject should be withdrawn

During outpatient drug dosing period:

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

At this visit, methemoglobin will be assessed. If greater than 5%, the PI will be unblinded to drug or placebo assignment. If subjects are on nitrite, subjects will be asked to repeat drug dosing of 20 mg sodium nitrite with follow up methemoglobin and hemodynamic monitoring. A study physician or PI will evaluate data and make a determination regarding discharge on 20 mg tid. If discharged on 20 mg tid, 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% on lower dose, participation in the study for the subject will be discontinued.

Hypotension and related symptoms.

In CTRC

If this occurs while in the CTRC, 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 unblinding is indicated and if it is appropriate for the subject to proceed with study participation prior to discharge from CTRC. 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. The PI may indicate the need for a repeat pK with 20 mg dose in subjects who started on 40 mg study drug/placebo.

As outpatient:

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

- need for unblinding,
- possibility if subject was on sodium nitrite to lower dose to 20 mg tid or
- need to discontinue and drop from the trial.

If subjects proceed on lower dose they 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 who were initiated on the 20 mg dose at Visit 4 report symptoms as described above, they will be asked to complete an interim visit, methemoglobin and BP pressure assessment; the physician may have to withdraw the subject since a dose lower than 20 mg tid is not an option.

Other symptom

The PI, via subject phone call monitoring, AE reports or at follow-up visits, will evaluate any other clinical presentation, sign or symptom in subjects which may warrant consideration of dose reduction. The procedure described for high methemoglobin or hypotension will be followed.

2.3.4. Treatment Period

Subjects will undergo study treatment duration of 4 weeks plus up to approximately 3 weeks of sodium NO₂ or placebo three times daily. Three additional weeks are included to allow continuation of the study drug/placebo through the final outcome collection series of visits. This is considered appropriate to study benefits of oral NO₂ therapy to improve physical function in patients with HFpEF.

Randomization and Blinding

We will use the high quality pseudo-random deviate generator in SAS® (SAS Institute, Inc., Cary, North Carolina) to randomize participants to NO₂ or placebo in 1:1 ratio with a random block size. Block sizes will be revealed to the rest of the study team at the conclusion of the study to prevent educated guessing. Randomization will be stratified by the initial dose (20/40 mg) depending on whether age ≥80 or age 70-80 and hypotension risk to the extent possible within the small sample size. Study statistician will create schedules which pairwise link the randomization sequence number, participant identifier and treatment arm, and call in all prescriptions thereafter by the randomization sequence number. The independent research pharmacist at the institutional Investigational Drug Service will package all drugs/placebos to be similar in physical appearance. We have successfully employed the same process in other recently completed trials¹⁰⁵. Therefore, all participants and study personnel involved in assessments will be blind to treatment assignment.

2.3.5 Breaking the Blind

Should the PI (or designated physician investigator) suspect that an adverse event (namely, methemoglobinemia, see Section 6.4.7) is related to the investigational product, he/she will contact one of three sources who will have access to the code:

1. the UPMC IDS
2. Dr. Perera, Biostatistician-Investigator
3. Nydia Chien, MSN, RN, CCRC, Director of Regulatory Affairs, Translational Research
Division of Pulmonary, Allergy, and Critical Care Medicine, Heart, Lung and Blood
Vascular Medicine Institute

They will be able to break the blind for that subject. An *emergency* unblinding/treatment would only be anticipated in the case of accidental overdose. In this protocol, signs and symptoms of methemoglobinemia are being monitored such that intervention would likely take place before reaching an emergent level if taken as prescribed. However, the PI and Coordinator will maintain quick access numbers to unblind quickly, if needed. Other signs and symptoms such as lightheadedness will be evaluated by the PI for determination if unblinding is indicated.

2.3.6. Medication Compliance

At Visit 4, subjects will self-administer their first dose of study medication under the supervision of the physician investigator or RN in the outpatient CTRC (Clinical Translational Research

Center) following completion of all baseline clinic assessments. After the pK (and subject is stable after first dose), subjects will be dispensed their study medication for home use, in, 4 prefilled seven-day labelled pill boxes to help with compliance and to help reduce burden or confusion. Pill counts will not be used during the first 28 days (phone call monitoring period) as having subjects remove the pills from the pill boxes to count would defeat the purpose of the pillboxes. On the weekly phone calls, the study coordinator will review the daily diary card (study drug/placebo and symptoms), inquire about missed doses and confirm empty pill boxes each week. Subjects will return empty pill boxes when they return for Visit 5, withholding those as needed for drug dosing during the final weeks. Medication compliance will be reviewed. Subjects will return any remaining capsules at their Visit 7 for final accountability. In the event that the compliance rate is <80% at any time reported by the subject, 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. Compliance during Dr. Forman pilot study has been excellent with no subject approaching the 80% level of concern.

2.3.7. Medication Storage and Accountability

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

2.3.7 Concomitant Medications

No changes are needed to the subject's usual medication while taking sodium NO₂/placebo. Subjects will hold their anti-coagulation regimen prior to the biopsies to reduce the risk of excessive bleeding. The concomitant medication list will be retrieved from the patient's medical record, confirmed with the patient on the phone and at Visit 1 and then assessed for changes at study contacts thereafter. Any patient status or medication change during the study period advised by subjects' health care team will be reviewed with the PI.

2.3.8 Rescue Medications

Not applicable

2.4 STUDY ENDPOINTS

Overall, study endpoints pre- and post-NO₂ will include:

1. Measures of physical function will include CPET indices:
 - a. gait speed,
 - b. strength (endurance, power),
 - c. balance as measured by non-invasive CPET (nCPET) aerobic capacity, SS fatigability assessment, 6 minute walk test, non-infrared spectroscopy assessments of blood flow, Short Physical Performance Battery (SPPB), handgrips, and accelerometryAnd,
 - d. pulmonary pressure and ventricular-pulmonary artery coupling, as measured by invasive CPET (iCPET)
2. Skeletal muscle bioenergetics: spectrophotometric, mitochondrial respiration, ribonucleic acid (RNA) isolation, protein isolation, electron microscopy
Serology: inflammatory peptides (interleukin (IL) 1, IL-6, IL-15, TNF α ; adipokines (leptin and adiponectin); vitamin D, glomerular filtration rate (GFR), brain natriuretic protein (BNP) or N-terminal pro b-type natriuretic peptide (NT-proBNP), thyroid stimulating hormone (TSH), glycosylated hemoglobin (HgbA1c) and platelet bioenergetics [using Seahorse

Extracellular Flux (XF) analysis]⁹, i.e., including glycolytic as well as basal and maximal respiratory rates.

3. Additional secondary factors include Quality of life (QoL) and related indices: questionnaires that include measures of sleep, depression, pain, cognitive function, physical activity, co-morbidities, frailty, nutrient intake, cardiac self-care efficacy, fatigability and HF, via the Kansas City Cardiomyopathy Questionnaire (KCCQ).

2.5 STATISTICAL ANALYSIS

2.5.1 Sample Size Determination

Formal sample size justifications based on statistical power for detecting significant differences at a strict 0.05 level are not applicable for a pilot study such as ours. We will use the descriptive statistics obtained herein for formal sample size and power computations in planning the larger subsequent trial to obtain more definitive findings. We will base our sample size on preliminary data from the present project, published statistical methods for sample size and power for approaches as similar as possible to the proposed analyses, and commercially available specialized software (PASS 2012[®]; Number Cruncher Statistical Systems, LLC, Kaysville, Utah) in planning the future trial to be proposed under the R01 mechanism.

2.5.2 Study Conduct Analysis

We will clearly document participant flow from the number contacted to numbers screened, randomized and completed using a CONSORT diagram, with numbers and reasons for any exclusions, withdrawals, missed assessments and terminations at each stage. We will describe adherence to NO₂ therapy as measured by queries to subjects, self-report of empty compartments on weekly phone calls and pill counts at the post visits using summary statistics as well as any post-randomization adjustments. Insights gleaned from study conduct analyses will be used to adjust protocol in the subsequent larger trial to increase its feasibility.

2.5.3 Efficacy Analysis

Overview-We will use SAS[®] version 9.3 (SAS Institute, Inc., Cary, North Carolina) for all analyses. The main focus of the analyses of the present pilot study will be to establish feasibility and obtain preliminary findings, and descriptive statistics needed for formal sample size and power computations for a subsequent larger randomized trial to be proposed under the R01 mechanism to obtain more definitive conclusions. Therefore, we will pay greater attention to magnitudes of effects rather than statistical significance alone, and similar to published pilot studies in other settings, also employ graphical methods such as needle plots that focus on change at the individual level rather than on average^{87, 88}. First, we evaluate the distributional characteristics of the data set, the prevalence of missing values and general data quality using appropriate descriptive statistics and graphical summarizations for all variables for each arm for each time point, as well as baseline to follow-up change scores. Second, the baseline measures and participant characteristics will be compared between the two arms using differences in magnitudes of the descriptive statistics, and independent samples *t*-, Wilcoxon rank sum, Kruskal-Wallis, chi-square or Fisher's exact tests, as appropriate. Any variables found to be meaningfully different will be noted when interpreting the findings. Third, main analyses below will be performed.

Aim 1-We will conduct Aim 1 analysis on an intention-to-treat basis. We will fit a series of linear mixed models using the SAS[®] MIXED procedure with baseline to follow-up change in primary outcome RPE, secondary outcome peak VO₂, and each of the other secondary outcomes as the

dependent variable; and treatment (NO₂/placebo), follow-up time (4 weeks) and their interaction as main factors of interest; baseline value of the dependent variable as the sole and a catch-all covariate; and a subject random effect to account for multiple follow-up measurements from each participant. We will construct appropriate means contrasts to compare the treatment groups at each of the follow-up time points. The magnitudes of the estimated means contrasts and graphical displays of individual change will be considered to support Aim 1 hypotheses rather than and in addition to statistical significance at the 0.05 level.

Aim 2-We will use Pearson and Spearman rank correlation coefficients between outcomes (e.g., fatigability, daily activity) and potential mediators (e.g., bioenergetics, pulmonary pressures), both cross-sectionally and in terms of change from baseline, both combined and within arm, to simply quantify the influence of potential mediators on outcomes. We are aware of the formal methods for mediation analyses to elicit contributory role of NO₂ and potential mechanism such as Baron and Kenny framework⁸⁹, reduction in treatment effect of NO after additionally adjusting for potential mediators, and mediation models using the SAS[®] %SOBEL and %INDIRECT macros⁹⁰⁻⁹². However, our small sample size in the present pilot study does not afford the opportunity to reliably employ such methods. We plan to perform a rigorous mediation analysis using the said methods in the larger follow-up trial being planned with the results of the present study.

Exploratory/Secondary/Sensitivity Analyses: We have proposed a main analysis treating RPE as a continuous variable for greatest statistical sensitivity in a small sample, but are aware that a clinically meaningful threshold may be 10 indicating fatigue^{33, 49}. Therefore, we will consider additional descriptive summarizations based on the said dichotomous operational definition. We will also consider stratifying by or adjusting for (as a covariate) the initial dose (20/40mg).

2.5.4 Safety Analysis

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

2.5.5 Handling Missing Data

We will make every effort to prevent missing data by keeping the participants as engaged as possible with the study. The recommended approach for handling missing data in inferential analyses is multiple imputation⁹³⁻⁹⁵. We will construct m=5 analytic data sets after imputing each value with multiple guesses to represent inherent uncertainty in imputing missing values, analyze each data set as though complete, and finally combine the results from the analyses to obtain overall estimates. We will use SAS[®] MI and MIANALYZE procedures.

2.5.6 Data Management

The study forms will be paper-based in that the data will be first recorded on paper forms at the time of the interview. A relational database will be constructed on a local server with daily backups where only select research team members will have access to the database. The database will include data entry forms with the same appearance as the paper forms to facilitate accurate data entry, routine data edit checks for consistency both within and between forms, and provisions for double data entry. After data entry, the paper forms will be archived in secure file cabinets. Database development and maintenance will occur with Microsoft Access[®]. All study subjects will be assigned unique study identifiers that will appear on all data collection instruments, tapes,

documents, and files used in the statistical analysis and manuscript preparation. The case report forms with study IDs will be housed in the same chart as personal identifier documents are while the subject is active in the protocol. Personal information documents will be stored separately once each subject's participation is complete. No documents will contain both the study ID and personal information at the same time. Only limited team members will have access to charts and database. No personal information concerning study participants will be released without their written consent. Other data quality assurance measures will include verifying the data, out of range data checks, and repeated evaluation of the data process.

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. Eighteen subjects with any ethnic background who have a confirmed diagnosis of HFpEF 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.

3.1.3 Inclusion of Prisoners

Prisoners will not be recruited for this protocol given the lack of resources required to conduct study visits and manage logistics.

3.2 INCLUSION CRITERIA

Inclusion Criteria:

- Age ≥ 70 years
- Diagnosis of HFpEF adapted from the ESC Guidelines, to include:
 1. Prior diagnosis of HF via one of these:
 - medical record diagnosis by attending cardiologist
 - verbal confirmation of HFpEF with attending cardiologist
 - PI review of medical record to confirm HFpEF
 2. EF% ≥ 40
- Clinically stable (euvolemic; baseline HR < 100 bpm) and without hospitalization or invasive cardiac procedure for 6 weeks
- Patients using 81 mg aspirin (ASA) will be eligible, but will be asked to hold the medication for 3 days 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 2 days 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 cardiologist.

3.3 EXCLUSION CRITERIA

Exclusion Criteria:

- Allergy to lidocaine
- BP >180/95 or <100/60 mm Hg (either systolic or diastolic)
- 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 uncorrected primary valvular heart disease (if valve replacement has been performed, patients will not be eligible for at least 12 months)
- Mechanical valve replacement requiring warfarin
- Severe peripheral or pulmonary artery disease
- Currently taking clopidogrel for a recent stent placement and/or a complex atherosclerotic lesion such that holding clopidogrel creates disproportionate risk.
- Current use of organic nitrates or phosphodiesterase type 5 inhibitors (PDE5s)
- Unable to hold warfarin or use bridging therapy, or to hold (81 mg) aspirin for 3 days or (325 mg) aspirin for 3 days prior to muscle biopsy or thienopyridine medications for 5 days prior to muscle biopsy.
- Anemia: Hgb <11.0 (♂), 10.0 (♀) gm/dl
- Dementia or inability to give informed consent
- End-stage malignancy
- Severe Orthopedic exercise limitation
- Chronic use of oral corticosteroids or other medications that affect muscle function
- Chronic alcohol 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
- Subjects with diabetes whose HgbA1c >10.0%
- Other chronic unstable disease such as active neoplasm, end stage chronic kidney, liver or other organ disease

Relative Exclusions

- Subjects with a non-3T MRI compatible pacemaker, implantable cardio defibrillator, stent or other identified metal in their body will be excluded from the Magnetic Resonance Spectroscopy study.
- Subject who use PDE5s for erectile dysfunction and are willing to withhold use 24 hours prior and during entire dosing period may be enrolled.
- Subjects may opt not to do the pre- and post- right heart catheterization exercise test.

4. IRB APPROVAL AND FDA AMENDMENTS

The Investigator will obtain from the University of Pittsburgh HRPO, prospective approval of the clinical protocol and corresponding informed consent form(s); modifications to the clinical

protocol and corresponding informed consent forms, and advertisements (i.e., directed at potential research subjects) for study recruitment.

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

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

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

- for Phase 2 and 3 clinical studies: The Sponsor will submit (i.e., in advance of implementing the change) a Protocol Amendment to the IND describing any change to a Phase 2 or Phase 3 protocol that significantly affects the safety of subjects, the scope of the investigation, or the scientific quality of the study. Examples of Phase 2 and 3 clinical protocol changes requiring the submission of a Protocol Amendment include:
 - Any increase in drug dosage or duration of exposure of individual subjects to the investigational drug beyond that described in the current protocol, or any significant increase in the number of subjects under study.
 - Any significant change in the design of the protocol (such as the addition or deletion of a control group).
 - The addition of a new test or procedure that is intended to improve monitoring for, or reduce the risk of, a side effect or adverse event; or the dropping of a test intended to monitor the safety of the investigational drug.

5. RECRUITMENT AND INFORMED CONSENT PROCEDURES

5.1 RECRUITMENT METHODS

1. A variety of mechanisms will be used to recruit subjects for this protocol.

a. UPMC

Heart failure patients can be referred by their physician and/or cardiologist from the UPMC Advanced Heart Failure Center, General Cardiology clinic, Benedum Geriatric Center, the Comprehensive Pulmonary Hypertension (PH) Program or other UPMC or community facility. For UPMC Presbyterian/Montefiore-based clinics, we will obtain physician and HRPO approval for screening of subject medical records for identification of eligible candidates. Dr. Forman currently already has, and will confirm continued approval from the Advanced Heart Failure physician group to screen their patients. He will engage with additional cardiologists and geriatricians for permission to screen from those clinics. Once identified as preliminarily

eligible, the study coordinator/staff will reaffirm with the subject's physician on the clinic visit day that recruitment of the subject is medically appropriate. Staff will also request direction from the attending physician as to the timing of speaking to the subject, i.e., before or after the subject has seen the physician for their visit. This will be done on a case by case basis. Study staff will speak with candidates at clinic visits or by telephone call to assess interest and review medical history to assess eligibility.

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

Subjects can also be identified from the inpatient Cardiology service. The study coordinator will follow the same procedure as for outpatients by screening the inpatient service for subjects admitted with heart failure symptomatology, check medical history for heart failure diagnoses and engaging the attending cardiologist as to appropriateness for the study. Such subjects will need to be followed for 6 weeks disease stability before enrollment, but recruitment and ascertainment of interest can be initiated, with consideration of medical circumstances in each case. As noted, permission to speak with subject will be obtained from the Cardiology attending.

b. Research Registries

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

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

c. Advertisements

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

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

2. Once subjects have been determined to be eligible from medical record review and attending approval, recruitment and enrollment procedures will then follow, including:
 - a. Confirmation of eligibility by PI

- b. Updating subjects' cardiologist and confirming management of holding anti-coagulants medication pre-muscle biopsy and pre-catheterization per protocol, if needed.
- c. Setting dates for patient visits with subject and staff will also occur prior to screening visit to maintain timeliness of visits per protocol
- d. Scheduling outpatient screening visit (Visit 1) where study risks and potential benefits, and rights as a research subject will be described in detail, informed consent will be obtained and where final eligibility will be confirmed.

5.2 INFORMED CONSENT PROCEDURES

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 physician investigator prior to performing any research study procedures.

6. POTENTIAL RISKS AND BENEFITS

6.1 POTENTIAL RISKS

6.1.1 Risk of Experimental Drug Intervention

NO₂:

Numerous studies have evaluated acute, subacute and chronic drinking water exposures of NO₃ and NO₂ in laboratory animals and drinking water and dietary exposures in humans. Recent studies are available using high doses of NO₂ by oral route in the form of beet root juice. Recent studies have evaluated acute exposures of oral preparations of NO₂ and NO₃ on PK and BP and are characterized below. More extensive human data is available on parenteral sodium NO₂ as it is currently available and approved by the FDA for use in the emergency treatment of cyanide poisoning^{96, 97}. It is also notable that nutraceutical preparations are currently being sold with levels of NO₂ (12.7 mg per tablet) and NO₃ (3.9 mg per tablet).

Sodium NO₂ 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 NO₂ has been inhaled or ingested as a euphoric stimulant. NO₂ 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 NO₂ (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 NO₂ 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 NO₂ 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 NO₂ (IND # 70,411) for cardiovascular applications and currently has an approved IND for the use of sodium NO₂ for lung transplant recipients (IND # 111,643). The cardiovascular IND involved the administration of sodium NO₂ 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 NO₂ used in these investigational treatment regimens – 60-120 mg daily or 20-40% of the dose (300 mg) used in the emergency treatment of cyanide poisoning—do not produce methemoglobin levels greater than 3% and have not been associated with clinically significant hypotension. There have been no adverse events noted in the 80 treated normal human volunteers and patients with sickle cell disease^{96, 98, 99}. In another study by Gladwin et al., 18 healthy adults received an infusion of sodium NO₂ totaling 75 mg (15 minutes each x 2 infusions). This was associated with a 7 mm Hg decrease in mean arterial pressure, a peak methemoglobin of less 3% and no other significant effects⁹⁶. Note this single dose is 1.9 times the larger single dose (tid) 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 NO₂ aqueous solutions (0.12 and 0.06 mmol NaNO₂/mmol Hb, equivalent to 290-380 mg and 140-190 mg sodium NO₂, respectively, depending on the total body hemoglobin level of the person) and one intravenous sodium NO₂ dose (0.12 mmol NaNO₂/mmol Hb¹⁰⁰). 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 NO₂, not methemoglobinemia, as the percentage of methemoglobinemia stayed below clinically toxic levels (<15%). By report, up to 22% experienced nausea, which subsided within 30 minutes. The pharmacokinetic analysis of this study indicated similar bioavailability of oral and IV delivery of NO₂, as well as similar side effect and safety profiles.

A recent study determined the safety and feasibility of prolonged intravenous NO₂ infusion. Twelve adult volunteers received increasing starting doses of sodium NO₂, 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 BP of up to 20 mmHg and asymptomatic increases of methemoglobin levels above 5%. No tolerance or clinically significant rebound was observed¹⁰¹. 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 NO₂, two retrospective case-control studies have shown that high maternal dietary NO₂ 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 risks¹⁰². Note that in this protocol, no subjects will be of childbearing age.

In the 2001 National Toxicology Program (NTP) Report summarizing 2-year rodent drinking water studies, there was no evidence of carcinogenic activity of sodium NO₂ 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 NO₂ 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 NO₂ 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*¹⁰³. Taken together, these findings suggest minimal carcinogenic NO₂-mediated risk.

In PI Forman's current pilot study, we are using 120 mg tid, lower or similar to doses of NO₂ cited and used safely in any of these studies. Our higher NO₂ dose of 40 mg three times daily is <40% of the dose for cyanide poisoning.

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 heart failure subjects on higher nitrite doses
- Rare: flushing, tachycardia, hypotension in healthy controls

Neurologic

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

Respiratory

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

Renal

- Nocturia: infrequent

6.1.2 Risk of Study Procedures

Cardiopulmonary and Functional Assessments:

Both non-invasive CPET (nCPET) and invasive CPET (iCPET) entail symptom limited (maximal) exercise provocation. The exercise stimulus is associated with a 1 in 10,000 chance of significant untoward outcome (e.g., myocardial infarction, arrhythmia), including the possibility of death. However, all those enrolled will have a physical exam immediately before the test to best insure they are stable; the nCPET will have the cardiologist or physician present or immediately available; the iCPET will have the study physician doing the right heart catheterization procedure immediately available, and who will ensure they are maximally safe and well-cared for if any problems develop. All physicians and study exercise testing personnel are all ACLS trained and

a code cart is in the immediate vicinity. In addition, patients' cardiologists will be notified before patients are enrolled, and will be asked to approve only those patients they deem to be stable.

During the other physical activity assessments (e.g., 6MWT, 5 minute steady-state treadmill walk, SPPB, and hand grip) there are also inherent exercise-associated risks, yet since nCPET will be completed first, that initial assessment provides some certainty that subjects are stable for the walking based assessments thereafter.

- Other problems that might also develop from a CPET include skin reactions to the electrode leads. Men may also need to have parts of their chest hair shaved in order to attach electrodes; this hair grows back over the next few weeks.
- iCPET facilitates delineation of the central vs. peripheral mechanisms underlying exercise performance and fatigability. As an exercise provocation, iCPET entails the same (nominal) exercise-related risk as nCPET. In addition, iCPET entails risks related to right heart and arterial line catheterizations with added risks.
 - Right heart and A-line catheter placement entail risks of infection, bleeding, and local pain. Therefore, meticulous efforts to keep the catheter site free of germs help to minimize these risks. Xylocaine numbing medicine is used to reduce any possibility of pain, but sometimes the Xylocaine itself produces a brief burning feeling as it enters through the skin.
 - For right heart catheterization, there are added risks of arrhythmia and perforation associated with intra-cardiac central line placement. The catheter is placed in the right heart through an internal jugular vein. This procedure is completed in the heart catheterization suite, providing state-of-the art fluoroscopy guidance as well as benefits of a top nursing staff, ACLS code teams, and optimal standards of sterility. Serious risks with right heart catheterization (arrhythmia, bleeding, puncture, mortality) are <1% in the literature, and <.01% at UPMC. Of note, right heart catheter is only left in place during the procedure and removed immediately thereafter, thereby minimizing risks (infection, migration, thrombosis) that are associated with prolonged right heart catheterizations¹²⁵. Less serious risks (skin infection and/or bleeding) are also <1%.
 - Fluoroscopy also entails risks associated with cumulative radiation exposure, but since average fluoroscopic imaging time for a right heart catheterization is less than 30 seconds, radiation dose is minuscule. In pregnant women, exposure to radiation can cause birth defects, however pregnant patients are not eligible for this study.
 - Risks associated with radial line placement are primarily oriented to the possibility of residual occlusion¹⁰⁴. However, this has occurred in <.05% of cases at UPMC, and all have been temporary, resolving with conservative management. Other risks include pseudo-aneurysm, infection, and hematoma, which are <.05% cumulatively. In this protocol, radial artery catheterization will be completed in the hospital's catheterization suite, which will help minimize risks overall, as compared to hospitals where A-lines are placed at bedside with much less controlled environmental circumstances.
- Subjects are asked to hold anti thrombin agents (warfarin, NOACs) prior to right heart catheterization. Holding the anti-thrombin regimen s theoretically increases the risk of formation of thrombus and cardiovascular events, albeit a very small amount. Subjects will be reminded to resume their regimen post procedure
- Echocardiograms present no direct risks. 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 staff performing echocardiograms to assure these are reliably completed with a minimum of patient distress.

Skeletal Muscle biopsy

- Bruising. Muscle biopsies are associated with a chance of bruising (about 1 in 100). Subjects are instructed in what signs or symptoms at the biopsy site warrant contacting the study coordinator post biopsy.
- Muscle biopsies are also associated with a small chance of infection (less than one in 1000) and pain.
 - Infection. Careful steps to keep the area free of germs will minimize infection risks. At the biopsy visit, subjects will be given instructions for care and signs or symptoms of infection for which they should contact study staff.
 - Pain. Xylocaine numbing medicine is used to reduce any possibility of pain, but sometimes the Xylocaine itself produces a brief burning feeling as it enters through the skin. Additional numbing cream may be used in subjects who report lower pain thresholds or inadequate response to numbing in the past. The physician will evaluate the need and safety for this in select cases as needed.
- Holding of anti-coagulant regimen. With the permission of the attending cardiologist, blood-thinning medicines such as aspirin (81 mg - 3 days) as well as warfarin (3 days) or others will be held before the muscle biopsy, and restarted post muscle biopsy. However, if the primary cardiologist prefers, bridging therapy using a different blood thinning medication (enoxaparin) will be used while the aspirin and warfarin are on hold. Holding the anti-coagulant regimen which is in place for prevention of cardiovascular events theoretically increases the risk of cardiovascular events, albeit a very small amount.

Risks of withholding anti-diabetic medication.

Subjects may be asked to hold their anti-diabetic medication before or on the day of the exercise tests, biopsies and right heart catheterization visits. It will depend on the type of medication (and for the exercise test, current glycemic control), anticipated time of test and next meal. This may result in a transient increase in blood glucose which will self-resolve and does not carry long term risk. We will use Dr. Risbano and the UPMC Cardiac Catheterization lab care standard for guidance on this.

Genetic testing

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

Magnetic resonance spectroscopy

- The MR Research Center (MRRRC) has extensive experience with MRS studies in diverse populations and is well-trained to reassure subjects. During these MRS sessions, even with previously scanned subjects, measures are taken to insure subject comfort. If any participant exhibits any significant clinical findings on any of the exams (e.g., tumor on MRI), they will be referred to appropriate clinicians. In the case of an emergency during participation in the study, the staff will initiate the appropriate emergency procedures as per standard operating

procedure at UPMC. All staff at UPMC involved in MRI/MRS scanning are trained yearly in safety and emergency protocols.

- MRS is not associated with any known adverse effect except for people with metal or magnetic implants (such as metallic clips in the brain or cardiac pacemakers).
 - Metal objects within the body can heat to potentially dangerous temperatures or possibly move in the patient's body.
 - Some types of tattoos (home-made) can also heat and cause discomfort. Any subjects with the possibility of these risk factors will not be tested in the MRI/MRS studies.
 - Metal objects can also become projectile when placed near the magnetic field. This has been reported, but it is a very rare occurrence. Protection from magnetic objects can be safeguarded by the usual safety techniques that are practiced in MRI/MRS sessions, including having subjects and researchers take all metal objects off of the subject before entering the environment.

Thus any candidate who meets inclusion/exclusion criteria with the exception of a metal or possibility of metal in their body will be invited to participate, but will forego the MRI testing.

Double screening of participants, by the study coordinator and again on the day of testing will be completed for thorough assurance that the subject is safe for MRI.

- Another potential risk is claustrophobia caused by being in the enclosed space of the MRI/MRS scanners. Subjects who may become anxious or uncomfortable during any part of the procedure will be immediately taken out of the scanner and not tested. Subjects are instructed to talk to the experimenter if they feel uncomfortable for any reason during the testing procedures. If subjects feel uncomfortable they are immediately taken out of the scanner.
- Noise levels in the magnet can be uncomfortable for subjects and they will be wearing earplugs through all MRI/MRS procedures. There is also microphone in the bore of the magnet. As above, subjects who feel uncomfortable are taken out of the scanner.

NIRS

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

Actigraphy

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

Questionnaires

- Patients will answer questionnaires as part of the study assessments which require responses about daily activities, diet, quality of life, medication, health history, cognitive

ability and sleep which in some cases may be a source of emotional distress. Efforts will be made to keep the environment and support by staff to be reassuring and pleasant.

Peripheral Blood sample

- Blood samples for NO₂ monitoring as well as serological assessments for the study entail needle sticks and/or IV placement.. There is a small chance of infection and pain. Careful steps to keep the area free of germs will minimize infection risks. Furthermore, since only small gauge needles are used, pain is minimal.
- A risk of drawing blood is anemia, from a lower hemoglobin level. A common symptom of anemia is fatigue (feeling tired or weak). The amount of blood to be drawn over the course of this investigation is an approximate *maximum* of 224 ml over approximately 12 weeks, if subjects complete both pre- and post- right heart catheterizations (using an *overestimation* of HFpEF candidates cycling for 15 minutes during each catheterization.) This is equivalent to about 1 cup. For subjects who choose not to complete the right heart catheterization visits, the maximum volume for the study is about 168 ml, or about ¾ cup. Neither of these amounts are likely to cause anemia, especially with blood draws extended over 6-8 weeks. Also note that we are screening and excluding for anemia at Visit 1.

Risks of fasting

8 hours for visits 4 and 7

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

For visits 3B and 6B

These visits occur after an overnight fast and subjects will receive a meal after the testing, which may last 4-6 hours. Although there is no risk to a longer fast, subjects may feel tired, hungry or irritable until then.

Overall subject burden

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

6.2 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.

6.3 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 NO₂ to improve strength, gait speed and balance. Oral NO₂ 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.

6.4 DATA SAFETY MONITORING

6.4.1 Data Safety Monitoring Board (Safety Officer)

In lieu of a Data Safety Monitoring Board, the funding agency, the National Institute on Aging, (NIA) has approved the use of a Safety Officer for this small sample, Phase II trial. Dr. Forman has appointed Dr. Saul Silver, UPMC Shadyside-Cardiology as the Safety Officer. The NIA has approved the appointment. The PI will provide Dr. Silver a detailed written summary of all adverse events on a monthly basis; any that are serious, unexpected, and potentially treatment-related adverse events (SAEs) will be immediately (within 24 hours) reported to the Human Research Protection Office, the Safety Officer, NIA Program Officer, and the Sponsor. In the monthly SO report, we will compare and contrast any SAE with prior events (or more frequently as indicated). Dr. Silver will review accumulating data from research activities to assure the continued safety of human subjects, relevance and appropriateness of the study, and the integrity of research data.

See also sections 6.4.5 and 6.4.6 regarding reporting of SAEs and/or fatalities.

To remain objective, Dr. Silver will maintain independent from the study. Accordingly, he will not be directly involved in the conduct of the study and will not have scientific, proprietary, financial or other interests that may affect independent decision-making. Signed documents confirming no conflict of interest will be provided with the application for approval to the NIA and maintained in the study regulatory binder.

In addition, the report to Dr. Silver will address the following information as it is submitted to the University Of Pittsburgh Human Research Protection Office (HRPO) at the time of annual review or more often as required:

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

participation and final recommendations related to continuing, changing, or terminating the study.

6.4.2 Data Safety Monitoring Plan

Monitoring of safety and data quality in the proposed study will be the responsibility of all personnel on the project, with primary responsibility and supervision by the PI. The University of Pittsburgh HRPO will approve the informed consent document for the study and provide institutional oversight of data and safety issues. The study protocol will be approved prior to recruiting or obtaining consent from any participants. Moreover, the study will be reviewed at a minimum of an annual basis by the HRPO committee. Each participant will sign the informed consent document 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 physician investigators or PI. The PI will, per standardized procedures, report serious adverse events to the HRPO, Safety Officer, FDA, and NIA Program Officer for their review and to the sponsor as indicated. 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 double locked files and facilities for storing research charts, 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 by the PI.

Data Monitoring Plan. The proposed study will use the FDA definition of adverse events (AE) and serious adverse events (SAE). Any SAE, which is unexpected and related to study intervention, will be reported immediately to the HRPO, Safety Officer, FDA, and NIA Program Officer and will be followed by an additional letter detailing the nature of the SAE. In the event that a participant either withdraws from the study or the investigators decide to discontinue a participant due to a SAE, the participant will be monitored by the PI physician investigator until (a) a resolution is reached (e.g., the problem has resolved or stabilized with no further change expected), (b) the SAE is determined to be clearly unrelated to the study intervention, or (c) the SAE results in death. Outcomes of SAEs will be regularly reported to the HRPO and the sponsor. 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 HRPO renewal.

6.4.3 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.

6.4.4 Frequency of Monitoring

Dr. Forman will review subject safety data as it is generated. Dr. Forman and the research staff will meet at least bi-weekly 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. Dr. Forman will also review the outcome and adverse event data to determine whether there is any change to the anticipated benefit-to-risk ratio of study participation and whether the study should continue as originally designed or should it be re-evaluated and changed. Dr. Forman will meet with the entire investigatory team at regular intervals for broader discussion of relevant details. Simultaneously, the Safety Officer will receive monthly reports of all AEs as described in section 6.4.1.

6.4.5 Reportable Adverse Event

For this study, a serious adverse event is any untoward clinical event that is thought by Dr. Forman, PI, to be study-related, and results in any of the following outcomes:

1. Death
2. A life threatening adverse event
3. Inpatient hospitalization or prolongation of an existing hospitalization

4. A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
5. A congenital anomaly or birth defect
6. Important medical events (that may not result in death, be life threatening, or require hospitalization) may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient, or subject, and may require medical, or surgical intervention to prevent one of the serious outcomes listed above.

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

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

Serious and unexpected adverse events associated with the use of the study drug or procedures must be reported to the HRPO with subsequent follow-up submission of a detailed written report in accordance with the respective policies and procedures of the HRPO. Written IND Safety Reports will be submitted to the FDA as soon as possible and, in no event, later than 15 calendar days following the investigator-sponsor's receipt of the respective adverse event information.

All reports noted in section 6.4.6 will also be provided to the NIA Program Officer and the protocol Safety Officer.

6.4.7. Withdrawal of Subjects and Stopping Criteria

Withdrawal of Subjects due to Adverse Events:

As described in Section 2.3.3. Dose Selection, sub-section Dose Reduction, we propose to discontinue participation for any subject who experiences any of the following:

- a. Continued MetHB level >5% or continued lightheadedness and/or dizziness after protocol described in Section 2.3.3 for dose reduction is followed.

Other symptoms
- b. The PI, via subject phone call monitoring, AE reports or at follow-up visits, will evaluate any other clinical presentation, sign or symptom in subjects which may warrant consideration of interim visit and dose reduction. The procedure described in Section 2.3.3. Dose Selection, sub-section Dose Reduction (regarding drug discontinuation and determination or need to withdraw the subject) will be followed.

Other Reasons for Withdrawal

Medication compliance

- c. 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.

- d. Participants who complete the first biopsy and then state (prior to being given drug) that they will not complete a second biopsy at Visit 7 will be withdrawn from the study. Alternatively, patients who refuse the 2nd biopsy after they have completed the 4 weeks of NO₂/placebo therapy will still complete the other post drug assessments. If other unique subject situations arise, they will be evaluated by the PI and a decision will be made with regard to completing the other post assessments or not (e.g., completion of 2 weeks on drug, and then refusal to complete biopsy.)
- e. The PI will evaluate any other clinical presentation, sign, symptom or behavior in subjects which may warrant consideration of withdrawal.

Discontinuation of the Clinical Trial

Stopping Rule:

Extensive published literature and considerable local experience at University of Pittsburgh indicates NO₂ is not inherently dangerous at the doses targeted in this trial. Certain side effects of NO₂ are idiosyncratic in older HFpEF patients and it is anticipated that there will be subjects who do not tolerate the treatment who may need to be withdrawn from the study as described for symptoms in 6.4.7. Nonetheless, if a subject enrolled in the study experiences a fatal event that is directly attributable to the NO₂, the overall study treatment will be discontinued.

6.5 RISKS MANAGEMENT PROCEDURES

6.5.1 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 during recruitment 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., due to the limited scope of this clinical investigation. The case report forms 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. Minimal identifying information will be stored in the subject research chart while active in the protocol. No research chart will contain documents with personal identifiers and their study subject number. Hard copy charts are stored in locked files in a locked office.

Research blood and muscle specimens will be collected in vials with only 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 samples to only those investigators with prior HRPO approval for their studies.

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

The Investigator will retain the data for the entire period of this study and will retain the specified records and reports for up to two years after the marketing application is approved for the investigational drug; or, if a marketing application is not submitted or approved for the investigational drug, until two years after investigations under the IND have been discontinued and the FDA so notified. The Investigator may continue to use and disclose 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. Subject names or other directly identifiable information will not appear on any reports, publications, or other disclosures of clinical study outcomes.

Source data to be included in the clinical study data, either on the original form or recorded by staff include:

- a. Information taken from or copies of electronic medical records to confirm inclusion/exclusion criteria
- b. Echocardiogram testing and interpretation
- c. All progress notes
- d. Communications from referring physicians
- e. De-identified emails communicating subject clinical information to PI or study physician
- f. Assessments of vitals, body weight at all visits
- g. Data generated from the nCPET, iCPET, MRS and all functional assessments,
- h. Questionnaires completed by subjects
- i. CTSC records of patient visits
- j. Analyses of screening and outcome assessments of blood and muscle tissue from internal and external investigator lab analyses
- k. Data collected from subjects' drug and diary cards

Missing data points that are *anticipated* will be documented in the progress notes and evaluated with HRPO input regarding the need for an Exception Report, i.e., unanticipated absence of staff or investigator to perform test, unanticipated absence of subject. *Unintentional* missing data due to subject inability will be noted. These will be reported to the PI for evaluation and corrective action plan implemented as needed.

All staff performing assessments that are witness to data collection which may be spurious are advised to communicate to the PI. As most of the measurements are objective, this is not anticipated to be an issue.

6.5.2 Protection Against Potential Risks of Experimental Intervention

- Involvement by trained staff/investigators with experience in the administration of the study drug:
Dr. Forman, PI, and physician Co-Is, Drs. Hughan and Simon are principally responsible for monitoring and protecting the safety of subjects' use of sodium NO₂. Their combined

experience in the use of the study drug is adequate to protect against (the relatively low) risks.

- Continuous monitoring by the Data and Safety Monitoring Board:

A Safety Officer, as noted in section 6.4.1, will function in lieu of a DSMB for this protocol. The monitoring plan is described there as well.

- Required Education in the Protection of Human Research Participants:

The Principal Investigator and all sub-investigators listed on the University of Pittsburgh HRPO approved protocol are required to complete the Collaborative Institutional Training Initiative (CITI) courses in research fundamentals, including Human Subjects Research and Responsible Conduct of Research. Staff will complete these courses as well, prior to any subject contact. Investigators and staff with subject contact will also complete the Good Clinical Practice module. Investigators with an identified Conflict of Interest (COI) will complete the COI module. These web-based tutorials are a requirement of the HRPO for investigators prior to protocol submission.

7. COSTS AND PAYMENTS

7.1 COSTS

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

7.2 PAYMENTS

Subjects will be reimbursed \$325 or \$475 for the baseline study visits after completion of Visit 4 [Visit 1 (\$75), Visit 2 (\$75), Visit 3A (\$75), Visit 3B (\$150)-optional, Visit 4, (\$100)]. Subjects will be reimbursed an additional \$350 or \$500 following the completion of Visit 7 [Visit 5, (\$75), Visit 6A (\$75), Visit 6B (\$150)-optional and Visit 7 (\$100) and study drug/placebo compensation (\$100)]. The total compensation will be \$675 or \$975 paid. Subjects will also be provided a ticket for outpatient parking costs at all visits. In the event that a subject must be withdrawn for any reason, they will be reimbursed for the individual study visits that were completed to date per the above reimbursement schedule.

	No heart cath visits	Doing heart cath visits
Participant Reimbursement V1 (Informed consent, physical, Hemoglobin, 3DFR, echo)	\$75	\$75
Participant Reimbursement V2 (nCPET, SS, 6MWT, NIRS, SPPB, HG, Actigraphy, Questionnaires)	\$75	\$75
Participant Reimbursement V3A (MRS)	\$75	\$75
Participant Reimbursement V3B (iCPET, w/echo)		\$150
Participant Reimbursement V4 (Biopsy, research bloods, pK, Drug)	\$100	\$100
Payment #1	\$325	\$475
Participant Reimbursement V5 - Post (nCPET, SS, 6MWT, NIRS, SPPB, HG, Actigraphy, Questionnaires), 3DFR	\$75	\$75
Participant Reimbursement V6A- Post (MRS)	\$75	\$75
Participant Reimbursement V6B- Post (iCPET w/ echo)		\$150
Participant Reimbursement V7- Post (biopsy and echo)	\$100	\$100
Study drug/placebo	\$100	\$100
Payment #2	\$350	\$500
TOTAL:	\$675	\$975

Subjects who require an interim visit will be compensated \$40 and provided parking.

8. QUALIFICATIONS AND SOURCE OF SUPPORT

8.1 QUALIFICATIONS OF THE INVESTIGATORS

Sponsor:

Mark Gladwin, MD, is Professor of Medicine, University of Pittsburgh School of Medicine. Dr. Gladwin is the Chairman of the Department of Medicine and Director of the Vascular Medicine Institute (VMI) at the University Of Pittsburgh School Of Medicine. He is an internationally recognized authority in the field of sodium NO₂ 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 NO₂ in lung transplant.

Investigators:

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

Sub-Investigators:

Mark Gladwin, MD is Professor of Medicine, University of Pittsburgh. Dr. Gladwin is the Chairman of Medicine, Department of Medicine, former Chief, Pulmonary, Allergy, and Critical Care Medicine and Director of the VMI at the University of Pittsburgh. He is an internationally recognized authority in the field of sodium NO₂ 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 NO₂ in lung transplant. He will be responsible for data interpretation.

Sruti Shiva, PhD is an Associate Professor in the Department of Pharmacology and Chemical Biology and VMI at the University of Pittsburgh. Her research lab focuses on the mechanisms by which reactive nitrogen species (particularly NO₂ and NO) 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.

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

Bret Goodpaster, PhD is Director of the Exercise Metabolism Core and Professor at the Sanford Burnham Medical Research Institute, Orlando, Florida. In his prior work at the University of

Pittsburgh he developed expertise regarding in vivo assessment of skeletal muscle bioenergetics with ^{31}P magnetic resonance spectroscopy as a means to assess oxidative ATP synthesis. He will oversee the processes used to analyze muscle biopsy samples and assist with interpretation of de-identified data.

Hoby Hetherington, PhD, MS is Director of the Magnetic Resonance Research Center at UPMC Presbyterian and Professor, Department of Radiology, University of Pittsburgh School of Medicine. He will facilitate acquisition of MRS spectroscopy as well as quality assessment of all data collected. Dr. Hetherington will also work directly with Drs. Goodpaster and Santanasto to facilitate analysis and interpretation of de-identified MRS data as part of an ongoing series of protocols that include multiple study cohorts.

Subashan Perera, PhD is an Associate Professor, Division of Geriatric Medicine and Department of Biostatistics at the University of Pittsburgh and has over 14 years of experience in providing statistical support to health science research at major academic medical institutions. He is a Senior Statistician and Co-Leader of Data Management, Analysis and Informatics Core of the NIA-funded Pittsburgh Claude D. Pepper Older Americans Independence Center. In addition, he is the principal source of statistical support to numerous clinical trials and intervention studies funded by NIH, PCORI, AHRQ, CMS and VA. Dr. Perera will play a key role in randomization, data analyses and publications.

Kara Hughan, MD is an Assistant Professor in the University of Pittsburgh School of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology and Diabetes. Dr. Hughan has designed and led the Phase I and early Phase II human subject trials with our IND-approved oral NO_2 at the University of Pittsburgh. She has extensive experience with evaluation of pK/pD, safety and efficacy of oral NO_2 . As Co-Investigator, she will play a role in data collected on oral NO_2 drug metabolism (plasma NO_3/NO_2 concentrations, RBC NO concentrations), safety (BP, MetHb) and efficacy (platelet and muscle mitochondrial function) and perform related statistical analyses on the data gained on these outcomes. Dr. Hughan will also assist with related trial regulatory/DSMB reporting. Dr. Hughan will work with Dr. Forman and his team on implementing the NO_2 therapy in this study. Dr. Hughan will provide leadership in protocol refinement, manuscript writing and publications.

Marc Simon, MD, MS is an Associate Professor of Medicine in the Department of Medicine-Cardiology and Director, Heart Failure Research/Clinical Hemodynamics Core Facility of the VMI who will oversee the iCPET cardiac catheterization. His work brings added insights regarding RV adaptation to elevated PA pressures. Using right heart catheterization and imaging techniques during iCPET, he has developed novel techniques to characterize RV contractility (end-systolic elastance, [Ees]) and its relationship to pulmonary artery (PA)-dependent afterload (arterial elastance, [Ea]). He will be available for call coverage for 24 hour physician investigator availability to subjects.

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

Theodore Huppert, PhD is an Associate Professor in the Department of Radiology and has extensive experience in the area of non-invasive optical imaging (NIRS) both within the area of

the human brain as well as muscle. He is the director of the NIRS Brain Imaging Laboratory at University of Pittsburgh. Dr. Huppert is a biomedical engineer and will work with Dr. Forman and his team on implementing the NIRS measures in this study. Dr. Huppert will provide leadership in protocol refinement, manuscript writing and publications, and will participate on other study committees as needed. Dr. Huppert will also work closely with Dr. Forman on the collection and novel examination of NIRS data in association to fatigability.

Adam Santanasto, MPH, PhD is a Research Assistant in the Department of Epidemiology with particular expertise in the conduct of MRS spectroscopy in older adults. Dr. Santanasto will administer the MRI testing protocol. Dr. Santanasto will also participate in data analyses and manuscript preparation.

Frederico Toledo, MD is an Endocrinologist and Associate Professor of Medicine at the University of Pittsburgh and the Director of the EMRC. He has specific expertise in evaluation of the relationship of mitochondria impact on the human body. As part of this project, Dr. Toledo will advise on withholding of hypoglycemic medications, provide backup for performing muscle biopsies and overseeing exercise tests as needed and provide insight on data analyses and publications.

Michael G. Risbano, MD is an Assistant Professor of Medicine in Division of Pulmonary Allergy and Critical Care Medicine in the Department of Medicine at the University of Pittsburgh. He is an attending physician in the Comprehensive Pulmonary Hypertension Program and performs right heart catheterizations to diagnose and follow the hemodynamic progression of patients with pulmonary hypertension. He will perform the right heart catheterizations of the subset of subjects who choose to undergo the procedure and contribute to the data analysis.

Manisha Jhamb, MD, MPH is an Assistant Professor in the Department of Medicine, Division of Renal-Electrolyte. Her research focuses on exercise benefits for CKD patients, including translational focus on gene expression changes in skeletal muscle that are induced by exercise routines. Dr. Forman is acting in a mentoring role to Dr. Jhamb as she learns skeletal muscle biopsy and analytic techniques. She will also assist in this protocol with patient visits and call coverage for 24 hour physician investigator availability to subjects.

Gavin Hickey, MD is an Assistant Professor of Medicine in the Department of Medicine-Cardiology. He will assist with oversight of nCPET testing, medical review of patients, patient visits and call coverage for 24 hour physician investigator availability to subjects.

Jessica Bon Field, MD, MS is an Assistant Professor of Medicine in Division of Pulmonary Allergy and Critical Care Medicine in the Department of Medicine at the University of Pittsburgh. Dr. Field's work emphasizes the impact of COPD on function. She will assist with oversight of nCPET testing, medical review of patients, performance of muscle biopsies, patient visits and call coverage for 24 hour physician investigator availability to subjects.

Andrea Levine, MD is a fellow in Pulmonary Medicine. Her research focuses on changes in protein expression in muscle in cardiovascular disease. She will be doing analysis of samples and also can provide assistance for coverage of functional testing and other study visits.

Consultants

Greg Lewis, MD is Assistant Professor of Medicine, Harvard Medical School, Director, Massachusetts General Hospital Cardiopulmonary Exercise Laboratory, and Director,

Massachusetts General Hospital Cardiology Intensive Care Unit. Dr. Lewis is a world expert in iCPET with particular insight in the application of iCPET to distinguish between central (cardiac; pulmonary) vs. peripheral (arterial oxygen utilization) mechanisms that determine exercise performance. His insights and skills for data analysis will complement those of Dr. Forman in nCPET.

Rebecca Vanderpool, PhD is formerly a Postdoctoral Associate at the University of Pittsburgh, now faculty at the University of Arizona Department of Medicine, Division of Pulmonary, Allergy and Critical Care Medicine and Pulmonary Hypertension Program. Dr. Vanderpool brings extensive expertise in the analysis of invasive cardiopulmonary stress tests. She will work in conjunction with Drs. Simon and Lewis in the analysis of the iCPET data, particularly in regard to assessments of RV-PA coupling.

8.2 SOURCES OF SUPPORT

National Institutes of Aging Grant 1R56AG051637-01A1

9. 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. 9:473-482.
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. Larsen FJ, Schiffer TA, Borniquel S, et al. Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab*. 2011;13:149-59.
8. Lai YC, Tabima DM, Dube JJ, Hughan KS, Vanderpool RR, Goncharov DA, St Croix CM, Garcia-Ocaña A, Goncharova EA, Tofovic SP, Mora AL, Gladwin MT. SIRT3-AMP-activated protein kinase activation by nitrite and metformin improves hyperglycemia and normalizes Pulmonary Hypertension Associated with heart failure with preserved ejection fraction. *Circulation*. 2016 Feb 23;133(8):717-31.
9. 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.
10. Forman DE, Ahmed A, Fleg J. Heart failure in very old adults. *Curr Heart Fail Rept*. 2013;10:387-400.
11. Hummel SL, Kitzman. Update on heart failure with preserved ejection fraction. *Curr Cardiovasc Risk Rep*. 2013;7:495-502.
12. Oktay AA, Rich JD, Shah SJ. The emerging epidemic of heart failure with reserved ejection fraction. *Curr Heart Fail Rep*. 2013;10:401-10.

13. Kaila K, Haykowsky MJ, Thompson RB, Paterson DI. Heart failure with preserved ejection fraction in the elderly: scope of the problem. *Heart Fail Rev.* 2012;17:555-62.
14. Massie BM, Carson PE, McMurray JJ, et al. Irbesartan in patients with heart failure and preserved ejection fraction. *N Engl J Med.* 2008;359:2456-67.
15. Pitt B, Pfeffer MA, Assmann SF, Boineau R, et al. Spironolactone for heart failure with preserved ejection fraction. *N Engl J Med.* 2014;370:1383-92.
16. Paulus WJ, Tschöpe C. A novel paradigm for heart failure with preserved ejection fraction: comorbidities drive myocardial dysfunction and remodeling through coronary microvascular endothelial inflammation. *J Am Coll Cardiol.* 2013;62:263-71.
17. Kitzman DW, Upadhyia B, Vasu S. What the dead can teach the living: the systemic nature of heart failure with preserved ejection fraction. *Circulation.* 2014.
18. Pandey A, Parashar A, Kumbhani D, Agarwal S, Garg J, Kitzman D, Levine BD, Drazner M, Berry JD. Exercise training in patients with heart failure and preserved ejection fraction: a meta-analysis of randomized control trials. *Circ Heart Fail.* 2014.
19. Edelmann F, Gelbrich G, Düngen HD, Fröhling S, Wachter R, Stahrenberg R, Binder L, Töpper A, Lashki DJ, Schwarz S, Herrmann-Lingen C, Löffler M, Hasenfuss G, Halle M, Pieske B. Exercise training improves exercise capacity and diastolic function in patients with heart failure with preserved ejection fraction: results of the Ex-DHF (Exercise training in Diastolic Heart Failure) pilot study. *J Am Coll Cardiol.* 2011;58:1780-91.
20. Haykowsky MJ, Brubaker PH, Stewart KP, Morgan TM, Eggebeen J, Kitzman DW. Effect of endurance training on the determinants of peak exercise oxygen consumption in elderly patients with stable compensated heart failure and preserved ejection fraction. *J Am Coll Cardiol.* 2012;60:120-8.
21. Lavie CJ, Arena R, Swift DL, Johannsen NM, Sui X, Lee DC, Earnest CP, Church TS, O'Keefe JH, Milani RV, Blair SN. Exercise and the cardiovascular system: clinical science and cardiovascular outcomes. *Circ Res.* 2015;117:207-19.
22. Fleg JL, Cooper LS, Borlaug BA, Haykowsky MJ, Kraus WE, Levine BD, Pfeffer MA, Piña IL, Poole DC, Reeves GR, Whellan DJ, Kitzman DW. National Heart, Lung, and Blood Institute Working Group. Exercise training as therapy for heart failure: current status and future directions. *Circ Heart Fail.* 2015;8:209-20.
23. Haykowsky MJ, Kitzman DW. Exercise physiology in heart failure and preserved ejection fraction. *Heart Failure Clin.* 2014;10:445-52.
24. Gielen S, Sandri M, Kozarez I, Kratzsch J, Teupser D, Thiery J, Erbs S, Mangner N, Lenk K, Hambrecht R, Schuler G, Adams V. Exercise training attenuates MuRF-1 expression in the skeletal muscle of patients with chronic heart failure independent of

age: the randomized leipzig exercise intervention in Chronic Heart Failure and aging catabolism study. *Circulation*. 2012;125:2716-27.

25. 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.
26. 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.
27. Forman DE, Sanderson BK, Josephson RA, Raikhelkar J, Bittner V. Heart failure as a newly approved diagnosis for cardiac rehabilitation: challenges and opportunities. *J Am Coll Cardiol*. 2015 Jun 23;65(24):2652-9.
28. Reeves GR, Houston-Miller N, Ellis SJ, Whellan D, O'Connor C. Predictors of adherence to exercise training in heart failure: a controlled trial investing outcomes in exercise training study. Paper presented at: American Heart Association Scientific Session; November, 2002; Chicago, IL.
29. Vanderpool R, Gladwin MT. Harnessing the nitrate-nitrite-nitric oxide pathway for therapy of heart failure with preserved ejection. *Circulation*. 2015;131:334-6.
30. Lundberg JO, Gladwin MT, Weitzberg E. Strategies to increase nitric oxide signaling in cardiovascular disease. *Nat Rev Drug Discov*. 2015;14:623-41.
31. Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol*. 2007;191:59-66.
32. Simonsick EM, Schrack JA, Glynn NW, Ferrucci L. Assessing fatigability in mobility-intact older adults. *J Am Geriatr Soc*. 2014;62:347-51.
33. Schnelle JF, Buchowski MS, Ikizler TA, Durkin DW, Beuscher L, Simmons SF. Evaluation of two fatigability severity measures in elderly adults. *J Am Geriatr Soc*. 2012;60:1527-33.
34. Eldadah BA. Fatigue and fatigability in older adults. *PM R*. 2010;2:406-13.
35. Avlund K, Damsgaard MT, Schroll M. Tiredness as determinant of subsequent use of health and social services among nondisabled elderly people. *J Aging Health*. 2001;13:276-86.
36. Avlund K, Pedersen AN, Schroll M. Functional decline from age 80 to 85: Influence of preceding changes in tiredness in daily activities. *Psychosom Med*. 2003;65:771-7.

37. Richardson CA, Glynn NW, Ferrucci LG, Mackey DC. Walking energetics, fatigability, and fatigue in older adults: The Study of Energy and Aging Pilot. *J Gerontol A Biol Sci Med Sci*. 2015;70:487-94.
38. Glynn NW, Santanasto AJ, Simonsick EM, Boudreau RM, Beach SR, Schulz R, Newman AB. The Pittsburgh Fatigability Scale for older adults: development and validation. *J Am Geriatr Soc*. 2015;63:130-5.
39. Kitzman DW, Nicklas B, Kraus WE, Lyle MF, Eggebeen J, Morgan TM, Haykowsky. 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.
40. Leeuwenburgh C. Role of apoptosis in sarcopenia. *J Gerontol A Biol Sci Med Sci*. 2003;58:999-1001.
41. 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 iSIRENTE study. *Age Ageing*. 2013;42:203-9.
42. Katz A, Sahlin K. Regulation of lactic acid production during exercise. *Appl Physiol*. (1985). 1988;65:509-18.
43. 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.
44. Conley KE, Jubrias SA, Esselman PC. Oxidative capacity and ageing in human muscle. *The Journal of Physiology*. 2000;526:203-210.
45. Waters DL, Mullins PG, Qualls CR, Raj DSC, Gasparovic C, Baumgartner RN. Mitochondrial function in physically active elders with sarcopenia. *Mechanisms of Ageing and Development*. 2009;130:315-319.
46. Waters DL, Brooks WM, Qualls CR, Baumgartner RN. Skeletal muscle mitochondrial function and lean body mass in healthy exercising elderly. *Mechanisms of Ageing and Development*. 2003;124(3):301-309.
47. Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S, Nair KS. Decline in skeletal muscle mitochondrial function with aging in humans. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:5618-23.
48. Rooyackers OE, Adey DB, Ades PA, Nair KS. Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle. *Proceedings of the National Academy of Sciences*. December 24, 1996 1996;93:15364-9.

49. Santanasto AJ, Glynn NW, Jubrias SA, Conley KE, Boudreau RM, Amati F, Mackey DC, Simonsick EM, Strotmeyer ES, Coen PM, Goodpaster BH, Newman AB. Skeletal muscle mitochondrial function and fatigability in older adults. *J Gerontol A Biol Sci Med Sci*. 2015;70:1379-85.
50. Coen PM, Jubrias SA, Distefano G, Amati F, Mackey DC, Glynn NW, Manini TM, Wohlgemuth SE, Leeuwenburgh C, Cummings SR, Newman AB, Ferrucci L, Toledo FG, Shankland E, Conley KE, Goodpaster BH. Skeletal muscle mitochondrial energetics are associated with maximal aerobic capacity and walking speed in older adults. *J Gerontol A Biol Sci Med Sci*. 2013;68:447-55.
51. Borlaug BA, Olson TP, Lam CSP, Flood KS, Lerman A, Johnson BD, Redfield MM. Global cardiovascular reserve dysfunction in heart failure with preserved ejection fraction. *J Am Coll Cardiol*. 2010;56:845-54.
52. Borlaug BA, Melenovsky V, Russell SD, Kessler K, Pacak K, Becker LC, Kass DA. Impaired chronotropic and vasodilator reserves limit exercise capacity in patients with heart failure and a preserved ejection fraction. *Circulation*. 2006 Nov 14;114:2138-47.
53. Mohammed SF, Hussain S, Mirzoyev SA, Edwards WD, Maleszewski JJ, Redfield MM. Coronary microvascular rarefaction and myocardial fibrosis in heart failure with preserved ejection fraction. *Circulation*. 2015 Feb 10;131(6):550-9.
54. Kitzman DW, Herrington DM, Brubaker PH, Moore JB, Eggebeen J, Haykowsky MJ. Carotid arterial stiffness and its relationship to exercise intolerance in older patients with heart failure and preserved ejection fraction. *Hypertension*. 2013;61:112-9.
55. Piepoli MF, Kaczmarek A, Francis DP, Davies LC, Rauchhaus M, Jankowska EA, Anker SD, Capucci A, Banasiak W, Ponikowski P. Reduced peripheral skeletal muscle mass and abnormal reflex physiology in chronic heart failure. *Circulation*. 2006;114:126-34.
56. Van Tassel BW, Raleigh JM, Abbate A. Targeting interleukin-1 in heart failure and inflammatory heart disease. *Curr Heart Fail Rep*. 2015;12:33-41.
57. Mentz RJ, Kelly JP, von Lueder TG, Voors AA, Lam CS, Cowie MR, Kjeldsen S, Jankowska EA, Atar D, Butler J, Fiuzat M, Zannad F, Pitt B, O'Connor CM. Noncardiac comorbidities in heart failure with reduced versus preserved ejection fraction. *J Am Coll Cardiol*. 2014;64:2281-93.
58. Jones AM. Dietary nitrate supplementation and exercise performance. *Sports Med*. 2014;44:S35-S45.
59. Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, Cabrales P, Fago A, Feelisch M, Ford PC, Freeman BA, Frenneaux M, Friedman J, Kelm M, Kevil CG, Kim-Shapiro DB, Kozlov AV, Lancaster JR Jr, Lefer DJ, McColl K, McCurry K, Patel RP, Petersson J, Rassaf T, Reutov VP, Richter-Addo GB, Schechter A, Shiva S,

- Tsuchiya K, van Faassen EE, Webb AJ, Zuckerbraun BS, Zweier JL, Weitzberg E. Nitrate and nitrite in biology, nutrition and therapeutics. *Nat Chem Biol*. 2009;5:865-9.
60. Jones AM. Influence of dietary nitrate on physiological determinants of exercise performance: a critical review. *Appl Physiol Nutr Metab*. 2014;39:1019-28.
61. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov*. 2008;7:156-67.
62. Gladwin MT, Raat NJ, Shiva S, Dezfulian C, Hogg N, Kim-Shapiro DB, Patel RP. Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. *Am J Physiol Heart Circ Physiol*. 2006;291:H2026-35.
63. Lai YC, Kviklyte S, Vertommen D, Lantier L, Foretz M, Viollet B, Hallén S, Rider MH. A small-molecule benzimidazole derivative that potently activates AMPK to increase glucose transport in skeletal muscle: comparison with effects of contraction and other AMPK activators. *Biochem J*. 2014;460:363-75.
64. Zamani P, Rawat D, Shiva-Kumar P, Geraci S, Bhuvra R, Konda P, Doulias PT, Ischiropoulos H, Townsend RR, Margulies KB, Cappola TP, Poole DC, Chirinos JA. Effect of inorganic nitrate on exercise capacity in heart failure with preserved ejection fraction. *Circulation*. 2015;131:371-80.
65. Hughan KS*, Delmastro-Greenwood M*, Helbling N, Corey C, Bellavia L, Potti G, Grimes G, Wendell SG, Kim-Shapiro D, Freeman BA, Shiva S, Gladwin MT. Conjugated linoleic acid modulates the bioactivity of oral nitrite and nitrate in humans. (*equal contribution) (Submitted, in review: *Hypertension*).
66. ATS Statement: Guidelines for the Six-Minute Walk Test. *Am J Respir Crit Care Med* 2002;166:111–117.
67. Wert DM, Brach JS, Perera S, VanSwearingen JM. The association between energy cost of walking and physical function in older adults. *Arch Gerontol Geriatr*. 2013;57:198-203.
68. Shiroma EJ, Freedson PS, Trost SG, Lee IM. Patterns of accelerometer assessed sedentary behavior in older women. *JAMA*. 2013;310:2562-3.
69. Jefferis BJ, Sartini C, Shiroma E, Whincup PH, Wannamethee SG, Lee IM. Duration and breaks in sedentary behaviour: accelerometer data from 1566 community-dwelling older men (British Regional Heart Study). *Br J Sports Med*. 2014.
70. Rowlands AV, Olds TS, Hillsdon M, Pulsford R, Hurst TL, Eston RG, Gomersall SR, Johnston K, Langford J. Assessing sedentary behavior with the GENEActiv: introducing the sedentary sphere. *Med Sci Sports Exerc*. 2014;46(6):1235-47.

71. Lyden K, Keadle SK, Staudenmayer J, Freedson PS. A method to estimate free-living active and sedentary behavior from an accelerometer. *Med Sci Sports Exerc.* 2014;46(2):386-97.
72. Buysse DJ, Reynolds DF, Monk TH, Berma SR, Kupfer DJ. The Pittsburgh Sleep Quality Index (PSQI): A new instrument for psychiatric research and practice. *Psychiatry Research.* 1989;28(2):193-213.
73. Gilbody S, Richards D, Brealey S, Hewitt C.J. Screening for depression in medical settings with the Patient Health Questionnaire (PHQ): a diagnostic meta-analysis. *Gen Intern Med.* 2007;22:1596-602.
74. Sullivan MD, Andrea Z, Russo J, Katon WJ. Self-efficacy and self-reported functional status in coronary heart disease: a six-month prospective study. *Psychosomatic Med.* 1998;60: 473-8.
75. Melzack R. The McGill pain questionnaire: from description to measurement. *Anesthesiology.* 2005;103:199-202.
76. Charlson ME, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis.* 1987;40(5):373-83.
77. Rockwood K, Song X, MacKnight C, Bergman H, Hogan DB, McDowell I, Mitnitski A. A global clinical measure of fitness and frailty in elderly people. *CMAJ.* 2005;173:489-95.
78. Nasreddine ZS, Phillips NA, Bedirian V, Charbonneau S, Whitehead V, Collin I, Cummings JL, Chertkow H. The Montreal Cognitive Assessment, MoCA: a brief screening tool for mild cognitive impairment. *J Am Geriatr Soc.* 2005; 53: 695–699.
79. 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;35:1245–1255.
80. Stewart, A. L., K. M. Mills, P. G. Sepsis, et al. Evaluation of CHAMPS, a physical activity promotion program for older adults. *Ann. Behav. Med.* 19: 353–361, 1997.
81. Glynn NW, Santanasto AJ, Simonsick EM, Boudreau RM, Beach SR, Schulz R, Newman AB. The Pittsburgh Fatigability scale for older adults: development and validation. *J Am Geriatr Soc.* 2015 Jan;63(1):130-5. doi: 10.1111/jgs.13191.
82. Jubrias SA, Crowther GJ, Shankland EG, Gronka RK, Conley KE. Acidosis inhibits oxidative phosphorylation in contracting human skeletal muscle in vivo. *The Journal of Physiology.* 2003;553(2):589-99.

83. Stefan D, Di Cesare F, Andrasescu A, Popa E, Lazariev A, Vescovo E, et al. Quantitation of magnetic resonance spectroscopy signals: the jMRUI software package. *Measurement Science and Technology*. 2009;20:104035.
84. Prasad A, Hastings JL, Shibata S, Popovic ZB, Arbab-Zadeh A, Bhella PS, Okazaki K, Fu Q, Berk M, Palmer D, Greenberg NL, Garcia MJ, Thomas JD, Levine BD. Characterization of static and dynamic left ventricular diastolic function in patients with heart failure with a preserved ejection fraction. *Circ Heart Fail*. 2010;3:617-26.
85. National Toxicology Program National Institutes of Health, Department of Health and Human Services. Toxicology and carcinogenesis studies of sodium nitrite (CAS NO. 7632-00-0). F344/N rats and B6C3F1 mice (drinking water studies). *Technical Report Series* 2001; 495:7–273.
86. 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; 19:10-17.
87. 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 Nov; 64A(11): 1190-1198. PMID: PMC2981453.
88. Resnick JL, Gupta N, Wagner J, Costa G, Martin L, Koritsky DA, Perera S, Eid K, Roberts M, Greenspan SL, Abu-Elmagd K. Skeletal integrity and visceral transplantation. *American Journal of Transplantation*. 2010 Oct; 10(10): 2331-2340.
89. Baron RM, Kenny DA. The moderator-mediator distinction in social psychological research: conceptual, strategic and statistical considerations. *Journal of Personality and Social Psychology*. 1986; 51 1173-1182.
90. Preacher KJ, Hayes AF. SPSS and SAS procedures for estimating indirect effects in simple mediation models. *Behavior Research Methods, Instruments, and Computers*. 2004; 36:717-731.
91. Sobel ME. Asymptotic confidence intervals for indirect effects in structural equation models. In: *Sociological Methodology*, Leinhardt S, ed. San Francisco. Jossey-Bass. 1982;290-312.
92. Preacher KJ, Hayes AF. Asymptotic and resampling strategies for assessing and comparing indirect effects in multiple mediator models. *Behavior Research Methods*. 2008;40:879-891.
93. Rubin DB. Multiple Imputation for Nonresponse in Surveys. John Wiley and Sons; 1987.
94. Rubin DB. Multiple imputation after 18+ years. *Statistics in Medicine* 1991;14:1913-1925.

95. Little RJ, D'Agostino R, Cohen ML, Dickersin K, Emerson SS, Farrar JT, Frangakis C, Hogan JW, Molenberghs G, Murphy SA, Neaton JD, Rotnitzky A, Scharfstein D, Shih WJ, Siegel JP, Stern H. The prevention and treatment of missing data in clinical trials. *N Engl J Med*. 2012;367(14):1355-60.
96. 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.
97. 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.
98. Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, Partovi K, Pelletier MM, Oldfield EH, Cannon RO 3rd, Schechter AN, Gladwin MT. Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation. *Circulation*. 2007;116:1821-31.
99. Mack AK, McGowan li VR, Tremonti CK, Ackah D, Barnett C, Machado RF, Gladwin MT, Kato GJ. Sodium nitrite promotes regional blood flow in patients with sickle cell disease: a phase I/II study. *Br J Haematol*. 2008;142(6): 971–978.
100. Hunault CC, van Velzen AG, Sips AJ, Schothorst RC, Meulenbelt J. Bioavailability of sodium nitrite from an aqueous solution in healthy adults. *Toxicology Letters*. 2009;190:48-53.
101. Pluta RM¹, Oldfield EH, Bakhtian KD, Fathi AR, Smith RK, Devroom HL, Nahavandi M, Woo S, Figg WD, Lonser RR. Safety and feasibility of long-term intravenous sodium nitrite infusion in healthy volunteers. *PLoS One*. 2011 Jan 10;6(1):e14504.
102. Huber JC Jr, Brender JD, Zheng Q, Sharkey JR, Vuong AM, Shinde MU, Griesenbeck JS, Suarez L, Langlois PH, Canfield MA, Romitti PA, Weyer PJ. The National Birth Defects Prevention Study. Maternal dietary intake of nitrates, nitrites and nitrosamines and selected birth defects in offspring: a case-control study. *Nutr J*. 2013; 12: 34.
103. Evans DC, Doraiswamy VA, Prosciak MP, Silveira M, Seamon MJ, Rodriguez Funes V, Cipolla J, Wang CF, Kavuturu S, Torigian DA, Cook CH, Lindsey DE, Steinberg SM, Stawicki SP Complications associated with pulmonary artery catheters: a comprehensive clinical review. *Scand J Surg*. 2009;98:199.
104. Borlaug BA, Koepp KE, Melenovsky V. Sodium Nitrite Improves Exercise Hemodynamics and Ventricular Performance in Heart Failure With Preserved Ejection Fraction. *J Am Coll Cardiol*. 2015;66:1672-82.
105. Greenspan SL, Nace D, Perera S, Ferchak M, Fiorito G, Medich D, Zukowski K, Adams D, Lee C, Knoblock S, Saul M, Resnick NM. Lessons learned from an osteoporosis clinical trial in frail long term care residents. *Clinical Trials*. 2012; 9: 247-256.

106. Murphy, AL, Kratz, AL, Schepens Niemiec, SL. Assessing fatigability in the lab and in daily life in older adults with osteoarthritis using perceived, performance and ecological measures. *J. Gerontology*. 2017; 72 (1): 115-120.