# Oral Nitrite in Patients with Pulmonary Hypertension and Heart Failure with Preserved Ejection Fraction (PH-HFpEF)

Michael Risbano, MD
Principal Investigator

NCT03015402

University of Pittsburgh
School of Medicine
Division of Pulmonary, Allergy and Critical Care

# Table of Contents

## PROTOCOL SYNOPSIS

| | |
|---|---|
| Protocol Title: | Oral Nitrite in Patients with Pulmonary Hypertension and Heart Failure with Preserved Ejection Fraction (PH-HFpEF) |
| Protocol Number: | PRO16110594 |
| NCT Number: | 03015402 |
| Version # and Date: | Version 14.0/February 14, 2022 |
| Clinical Phase: | Phase II clinical investigation |
| Investigational Drug: | Oral Nitrite ($NO_2^-$) |
| Trial Site: | Single-Center Trial |
| Sponsor: | Mark Gladwin, MD<br>Professor of Medicine<br>Chair, Department of Medicine<br>University of Pittsburgh |
| Investigator: | Michael Risbano, MD,<br>Assistant Professor of Medicine<br>University of Pittsburgh<br>UPMC PACCM |
| Sub-Investigators: | Mark T. Gladwin, MD<br>Marc Simon, MD<br>Adil Yunis, MD<br>Vinaya Mulkareddy, MD<br>Michael Bashline, MD<br>Frederico Toledo, MD<br>Carl Koch, MD<br>Jessica Huston, MD |
| Study Monitor: | Mark T. Gladwin, MD<br>Chairman, Department of Medicine<br>University of Pittsburgh School of Medicine<br>3550 Terrace St. - 1218 Scaife Hall<br>Pittsburgh, PA 15261 |
| Research Facilities: | UPMC Presbyterian Hospital<br>200 Lothrop Street<br>Pittsburgh, PA 15213 |
| Clinical Laboratories: | University of Pittsburgh Medical Center<br>3477 Euler Way<br>Pittsburgh, PA 15213 |

| | |
|---|---|
| Manufacturer: | Triangle Compounding Pharmacy, Inc.<br>3700 Regency Parkway, Suite 140<br>Cary, NC 27518<br><br>University of Iowa Pharmaceuticals<br>Iowa City, Iowa 52242 |
| Study Rationale: | A strong foundation of knowledge supports that nitrite, as a source of nitric oxide (NO), can prevent and reverse experimental pulmonary hypertension. |
| Study Hypothesis: | We hypothesize that:<br><br>$\boldsymbol{NO_2^-}$ 40mg given orally three times a day for 10 weeks will improve hemodynamics, with a reduction in peak mean pulmonary arterial pressure (mPAP) during submaximal exercise as compared to placebo (primary endpoint), as well as resting mPAP, pulmonary capillary wedge pressure (PCWP) at rest and with submaximal exercise, right atrial pressure at rest and with submaximal exercise, and an increase in pulmonary artery compliance at rest and with submaximal exercise (all secondary endpoints).<br><br>$\boldsymbol{NO_2^-}$ 40mg given orally three times a day for 10 weeks will improve exercise performance, including exercise time and maximal oxygen consumption during maximal and submaximal exercise as compared to placebo (secondary endpoint).<br><br>$\boldsymbol{NO_2^-}$ 40mg given orally three times a day for 10 weeks will improve insulin sensitivity, fasting blood glucose, and HgbA1c, compared to placebo (exploratory endpoint). |

| | |
|---|---|
| Study Aims: | <u>Specific Aims:</u><br><br>1. To evaluate the clinical efficacy and signaling mechanisms of a new FDA-IND approved oral formulation of nitrite (IND# 119526) on exercise tolerance and hemodynamics in PH-HFpEF.<br><br>2. To monitor *in vivo* formation and metabolism of this new class of signaling electrophile using new FDA IND approved oral nitrite.<br><br>3. To leverage the methodological tools and insights gleaned over this time to explore fundamental signaling mechanisms of the reactive nitrogen species, nitrite ($\boldsymbol{NO_2^-}$), in the context of pulmonary hypertension (PH) in the setting of the metabolic syndrome. |

| | |
|---|---|
| | The study aims to assess the use of oral nitrite vs. placebo on exercise capacity and hemodynamic measurements in subjects with PH-HFpEF.<br><br>Subjects will undergo screening procedures to determine that eligibility requirements are met. Prior to receiving the study drug or placebo, subjects will complete baseline assessments.<br><br>Subjects (n=26) who meet the inclusion criteria and none of the exclusion criteria will be randomized to study drug (oral nitrite or placebo) for 10 weeks. Subjects will then cross-over to the alternative therapy for an additional 10 weeks and complete all the same testing procedures.<br><br>**Drug**: oral formulation of sodium nitrite dose strength in 20 or 40 mg capsules or matching placebo three times daily or TID (once in the morning, in the early afternoon and again in the evening) for the duration of the study.<br><br>Subjects will be evaluated as outpatients with the first dose of study drug. Subjects will return at weeks 4 and 8 for safety visits after the start of each study arm. The safety visit will occur to assess adverse events (AE), methemoglobin level, interval histories, brief physical exams, medication compliance review, and dispensing of study drug.<br><br>Subjects will also be contacted by the study investigator and/or the study coordinator for a follow-up phone assessment approximately 30 days following the completion of the study. |
| Planned Sample Size: | 26 subjects |
| Duration of Treatment: | 22 weeks |
| Inclusion Criteria: | Age 18 years and older<br><br>PH-HFpEF confirmed diagnosis by RHC:<br>• Mean Pulmonary Arterial Pressure (mPAP) ≥ 25 mmHg<br>AND<br>• Pulmonary capillary wedge pressure (PCWP) ≥ 15 mmHg<br>AND<br>• Transpulmonary Gradient (TPG) ≥ 12 mmHg<br><br>Patient has one or more of the following heart failure associated signs or symptoms: shortness of breath with activity, overweight, atrial fibrillation and/or systemic hypertension. |
| Exclusion Criteria: | • Age less than 18 years;<br>• SBP > 170 or < 110 mmHg<br>• DBP >95 or < 60 mmHg |

| | |
|---|---|
| | • Hemoglobin A1C > 10<br>• Positive urine pregnancy test or breastfeeding<br>• Ejection Fraction (EF) < 40%<br>• Dementia<br>• End-stage malignancy<br>• Major cardiovascular event or procedure within 6 weeks prior to enrollment<br>• Severe valvular disease<br>• Known chronic psychiatric or medical conditions that may increase the risk associated with study participation in the judgment of the investigator, would make the subject inappropriate for entry into this study<br>• Smoker<br>• Hemoglobin <9 g/dL<br>• Serum creatinine > 3.0 mg/dL<br>• Recent (<3 months) addition or change in dosing of hormonal contraceptive medications (OCP, IUD, DepoProvera)<br>• Receipt of an investigational product or device, or participation in a drug research study within a period of 15 days<br>• RHC < 2 weeks from study screening RHC unless clinically indicated |
| Study Endpoints: | <u>Primary endpoint:</u><br>The primary endpoint of the trial will be mean pulmonary artery pressure (mPAP) during submaximal exercise as compared between placebo and nitrite at 10 weeks (i.e. the week 10 RHC of placebo vs week 10 RHC of nitrite).<br><br><u>Secondary endpoints:</u><br>1.The differences in 6MWT with Borg dyspnea scale assessments and pulse oxygen saturation measurements<br>2. RHC hemodynamics (right atrial pressure, pulmonary capillary wedge pressure, pulmonary artery pressures and resistance, pulmonary artery compliance, cardiac output, diastolic pressure gradients, and transpulmonary gradient)<br>3. Doppler-echocardiography<br>4. NT-proBNP plasma levels<br>5. NYHA functional classification changes<br>6. $NO_2^-$ metabolites<br>7. Endurance exercise time (ET), changes in CPET measurements taken at isotime (oxygen consumption [VO2, L/min], minute ventilation [VE, L/min], ventilatory equivalent [VE/VO2], dead space to tidal volume ratio [Vd/Vt], Borg dyspnea and leg score, and changes in time constants for increases of Vt, respiratory rate [RR], VE, and heartrate)<br>8. Changes in fasting glucose and insulin sensitivity |

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

## 1.1 OBJECTIVE

The main objective of this study is to determine the clinical efficacy of oral inorganic nitrite verses placebo and the therapeutic response with regards to exercise tolerance in patients with Group II PH-HFpEF.

## 1.2 SPECIFIC AIMS

Hypothesis:

1. Acute open-label dosing of 40 mg of oral nitrite in the cardiac catheterization laboratory will safely vasodilate the pulmonary circulation and reduce left ventricular filling pressures of patients with Group II PH-HFpEF.
2. Blinded chronic oral nitrite supplementation (40 mg PO TID for 10 weeks) will improve exercise capacity, hemodynamics, and insulin resistance compared with placebo.

Specific Aims:

Aim 1: To evaluate the clinical efficacy and signaling mechanisms of a new FDA-IND approved oral formulation of nitrite (IND# 119526) on exercise tolerance and hemodynamics in PH-HFpEF.

Aim 2: To monitor *in vivo* formation and metabolism of this new class of signaling electrophile using new FDA IND approved oral nitrite.

Aim 3: To leverage the methodological tools and insights gleaned over this time to explore fundamental signaling mechanisms of the reactive nitrogen species, nitrite ($NO_2^-$), in the context of pulmonary hypertension (PH) in the setting of the metabolic syndrome.

## 1.3 BACKGROUND

### 1.3.1 Harnessing the nitrate-nitrite-NO pathway for PH therapeutics

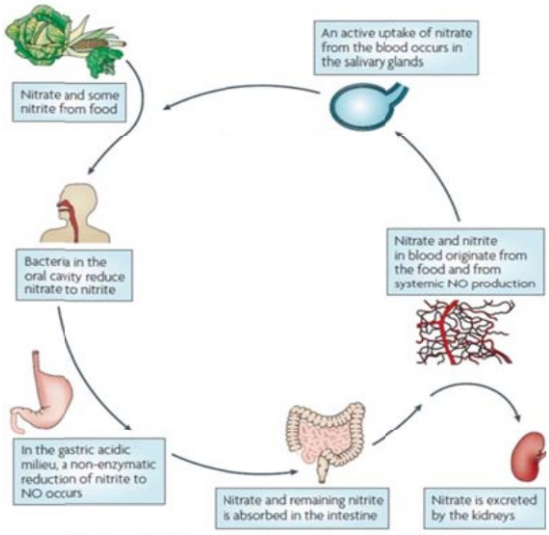


**Fig 1. Nitrate and Nitrite and the Enterosalivary Circulation Pathway**
Lundberg JO, Weitzberg E, Gladwin M. *Nat Rev DrugDiscov* 2008[11]

Reduced bioavailability and impaired production of nitric oxide (NO) are thought to contribute to the development of both metabolic syndrome and PH.[1-6] An increasing number of therapeutic approaches, which enhance NO generation and bioactivity, have been proposed over the past decade and include administration of nitrite and nitrate.[7] Nitrite, the active metabolite of dietary nitrate, has been recognized as a natural reservoir of NO.[7],[8] This is now referred to as the nitrate-nitrite-NO pathway and involves a series of oxygen-independent and NO synthase-independent single electron transfer reactions.[7] Nitrate accumulates in the plasma from oral intake of foods rich in nitrate, such as green leafy vegetables and root plants, or from the intravascular oxidation of NO to nitrate by oxyhemoglobin (**Figure 1**). Nitrate is then concentrated in the saliva and reacts with oral commensal bacteria, which contain nitrate

reductase enzymes.[7, 9] Humans do not possess nitrate reductase enzymes so require these bacteria for conversion of nitrate to nitrite. Nitrite is then swallowed and systemically absorbed where it can be further reduced via one-electron transfer reactions with hemoglobin, myoglobin, neuroglobin, and molybopterrin-containing enzymes (such as xanthine oxidase, aldehyde oxidase and mARC).[10-13] Nitrite has now been shown to regulate blood pressure, hypoxic vasodilation, mitochondrial efficiency, and exercise performance. From a therapeutic standpoint, nitrite has been shown to exhibit therapeutic efficacy in mouse, rat, and sheep pre-clinical models of PH.[14-16] Additionally, oral supplementation of nitrate and nitrite prevents the development of hypoxia- and monocrotaline-induced PH in mice.[17] Furthermore, a recent study suggests that dietary supplementation of nitrate reverses the features of metabolic syndrome in eNOS-deficient mice, yet the mechanism behind this observation and the role of nitrite on metabolic syndrome remain uncertain.[18]

**Nitrate-nitrite-NO signaling improves exercise capacity and efficiency**

In addition to effects on improving pulmonary hypertension, nitrate and nitrate-containing foods such as beet root juice, and purified nitrate solutions, improve exercise efficiency (3-6% increase VO2/watts), exercise time to exhaustion (≈16%), increase tolerance to high intensity exercise (≈25%), and improve mitochondrial efficiency (P/O ratios).[19-21] Studies in highly trained athletes show that higher plasma baseline levels of nitrite are associated with superior exercise capacity and nitrate therapy increases time trial performance (the time required to achieve a pre-specified work level) for several sports.[22-33] A recent meta-analysis of 17 studies investigating the effect of inorganic nitrate supplementation on exercise performance for several types of exercise, including cycling, walking, running, and knee extension exercise, showed a significant benefit on performance for time to exhaustion tests, performance for time trials, and graded exercise tests.[26] Studies in patients with cardiopulmonary disease are now being published including a recently completed trial of a high nitrate containing beet root juice in 15 subjects with COPD (placebo-controlled cross over trial) that increased plasma nitrite levels by 379% and increased median submaximal constant work rate exercise time by 28.8 seconds.[34] A recently completed study evaluated a single dose of 12.9 mmoles of nitrate in beetroot juice compared with nitrate depleted juice in a double-blinded placebo-controlled cross over trial of HFpEF patients without PH and found significant increases in exercise duration, VO2 max, and total work performed.[35, 36] These studies provide relevant background to test oral nitrite in this longer term trial and we expect additional benefit in the targeted enrollment population of patients with HFpEF that also have an increased pulmonary vascular resistance and metabolic syndrome.

**Sirtuins and AMPK as regulators of metabolism, activated by nitrite therapy**

AMPK is a cellular energy sensor that participates in many metabolic processes, and controls liver and skeletal muscle insulin receptor-independent glucose uptake.[37] Dysregulation of AMPK and its signaling network have been associated both with metabolic syndrome and idiopathic PAH.[38-41] Several studies have shown that NO donors increase glucose uptake and GLUT4 expression via AMPK activation,[42, 43] and our group has shown that nitrite increases AMPK activation in cardiomyocytes, identifying AMPK as a candidate signaling pathway for the effects of nitrate and nitrite on glucose homeostasis.[44] As shown in **Figure 2**, activation of AMPK can be mediated by upstream kinases, including liver kinase B1 (LKB1), calcium-sensitive calmodulin-dependent protein kinase II (CaMKII), calcium/calmodulin-dependent protein kinase-beta (CaMKKβ), and TGFβ-activated kinase-1 (TAK1).[45-47] Recently, sirtuin-3 (SIRT3), a major mitochondrial deacetylase that is

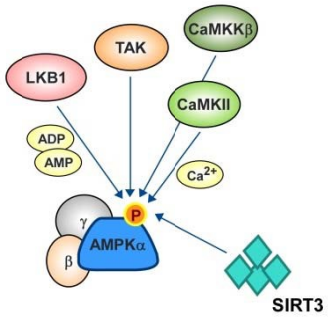


**Figure 2.** Regulation of AMPK.

upregulated in skeletal muscle with exercise, has been shown to activate AMPK in skeletal muscle and neurons.[48-50] On the basis of such observations, we hypothesize that nitrite, similar to metformin, might improve metabolic syndrome via the activation of AMPK signaling pathways. In the skeletal muscle, we propose that nitrite and metformin activate mitochondrial SIRT3, which in turn promotes the phosphorylation and activation of AMPK and membrane translocation of the glucose transporter 4 (GLUT4). Nitrite also directly activates pulmonary vascular smooth muscle AMPK via alternative SIRT3-independent pathways that will be carefully explored. Systemic improvements in metabolic syndrome and localized effects on the pulmonary vasculature suggest that nitrite may be an ideal therapy for PH-HFpEF in the setting of metabolic syndrome.

**Development of sodium nitrite administration for human use**

A phase 1a and 1b dose escalation study designed to define the maximum tolerated and safe doses of inhaled sodium nitrite delivered to normal volunteers following electronic nebulization has shown an increase in heart rate (+24 ± 5 bpm) without clinically significant changes in systolic (-3 ± 2 mmHg) or diastolic (-3 ± 2 mmHg) blood pressure at the maximal tolerated dose (MTD of 125 mg).[51] At the highest delivered dose (176 mg), there was a significant decrease in systemic blood pressure and increase in heart rate. Reduction of nitrite to NO was demonstrated by an increase in exhaled NO. Methemoglobin levels increased at the highest doses administered, but remained less than 3.5% in all subjects. Pharmacokinetic modeling demonstrated dose-proportional increases in peak and AUC plasma nitrite. There is an ongoing phase 2 study (clinicaltrials.gov NCT01431313) of inhaled nitrite therapy in patients with group II PH (PH-HFpEF), Group I PAH, and Group III PH (PH due to lung disease), with data suggesting likely efficacy in patients with PH-HFpEF at doses of both 45 and 90 mg.[52-54] We have also now completed phase 1 safety and PK analyses of a newly developed FDA-IND approved oral nitrite preparation in normal volunteers, with and without 15-nitrogen labeling (both with matching placebos).[55, 56] The oral bioavailability of nitrite is excellent (95-98%) and similar to intravenous delivery.[57] An ongoing safety and efficacy trial of 3 months of treatment with oral nitrite (20 mg, twice daily) in patients with metabolic syndrome (clinical trials.gov NCT01681810) continues to accrue.

## 1.4 SIGNIFICANCE

Group II pulmonary hypertension (PH) or post-capillary pulmonary venous hypertension is known to occur secondary to left ventricular (LV) systolic or diastolic dysfunction. The latter is more commonly referred to as heart failure with preserved ejection fraction (HFpEF) or non-systolic heart failure, and is the most common cause of Group II PH. With chronic elevations in the diastolic filling pressure of the left heart, the pulmonary vasculature can vasoconstrict and undergo pathological remodeling and secondary right ventricular (RV) hypertrophy and dilation, leading to an elevated pulmonary vascular resistance (PVR) and high transpulmonary pressure gradients.[58-61] It is now appreciated that features of the metabolic syndrome, including systemic hypertension, obesity, diabetes, and hyperlipidemia, represent risk factors for the development of pulmonary vascular disease. In fact, patients with pulmonary arterial hypertension (Group I PAH) exhibit an increased prevalence of glucose intolerance (increased hemoglobin A1c levels).[60-64] Patients with Group II PH-HFpEF commonly present with two or more features of the metabolic syndrome.[63] Mouse models of metabolic syndrome, such as the apo E knock-out mice fed a high fat diet, have been shown to develop pulmonary hypertension.[65] There are no currently approved therapies for PH-HFpEF and most medications approved for Group I PAH have been shown to be ineffective in patients with left heart disease. New therapies that target both the metabolic syndrome and the pulmonary vasculature are being tested in pre-clinical models and in patients, including peroxisome proliferator-activated receptor-γ activators, and, in our studies, metformin and

nitrate/nitrite.[18, 65-68] To date, there are no established animal models of PH-HFpEF associated with metabolic syndrome and no specific therapy for patients with PH-HFpEF.

# 2. RESEARCH DESIGN AND METHODS

## 2.1 CLASSIFICATION AND METHODOLOGICAL DESIGNS

This is a single-center, 22-week, double-blind, randomized, placebo-controlled cross-over trial of oral nitrite on exercise capacity and hemodynamic measurements in subjects with PH-HFpEF.

## 2.2 DETAILED DESCRIPTION OF STUDY DESIGN

Potential subjects will undergo screening visit(s) to determine that eligibility requirements are met. An echocardiogram, bloodwork, and screening right heart catheterization hemodynamic measurements will determine subject's eligibility. Subjects who meet all the inclusion criteria, and none of the exclusion criteria, will be included in the study. Once eligibility is confirmed, subjects will complete a series of baseline procedures prior to receiving study drug. The study population (n=26) will receive either oral sodium nitrite or matching placebo as follows:

**Drug**: 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. Oral formulation of sodium nitrite, total dose strength of 40 mg or placebo three times daily or TID (once in the morning and again in the early afternoon and again in the evening).

During the treatment period, subjects will undergo periodic clinical evaluations and weekly follow-up phone calls in between clinic visits. Throughout the study period, subjects will be asked to remain diligent in recording in a diary the date and time of study drug dosing and any associated symptoms.

### 2.2.1. Study Drug Preparation and Distribution

Sodium nitrite are prepared at Triangle Compounding Pharmacy, Inc. (Cary, NC 27518) and matching placebos were prepared at NIH pharmacy. The labeling and packaging will be conducted according to Good Clinical Practice and regulatory requirements. The University of Pittsburgh Medical Center Investigational Drug Pharmacy Service (UPMC-IDS) will be utilized for dispensing of the study drugs (nitrite and placebo). The study drug is administered only to subjects enrolled in the study and in accordance with the protocol.

<u>Sodium nitrite</u>
Standard sodium nitrite will be supplied as capsules for three times daily oral administration with a capsule dose strength of 20 mg or 40 mg. Total dose strength will be 40mg TID.

<u>Matching placebo</u>
Placebo will be supplied as matching capsules for three times daily oral administration.

### 2.2.2. Treatment Period

Subjects will undergo study treatment duration of 10 weeks of sodium nitrite three times daily or matching placebo. This is considered appropriate to demonstrate the potential effects of nitrite in adults with PH-HFpEF. Upon completion of the 1st 10 weeks plus a 2-week wash-out period, the subjects will cross-over to receive the alternative therapy (nitrite or matching placebo) and undergo the same study procedures as noted for the 1st 10 weeks. Subjects will be given a diary to monitor study drug administration and will be asked to return the diary at each clinic visit to monitor compliance. Upon completion of the cross-over portion of the trial, subjects will be given the option to receive open-label drug for 6 months.

### 2.2.3. Safety Monitoring:

Weekly phone follow-up (+/- 7 days) assessments will occur while on active study treatment (when subjects are not followed up in clinic) that will include symptom review, assessment of adverse events, review of concomitant medications, and interval histories. Subjects may return to clinic for outpatient assessments at weeks 4 and 8 (both Arm 1 and Arm 2) if the PI deems it necessary to bring the patient in for an evaluation. No weekly phone follow up may be necessary if subjects are evaluated in clinic. Clinical visits are completed in the outpatient clinics at UPMC Presbyterian outpatient cardiology and/or Falk Clinic, Comprehensive Lung Center, or Translational Research Center (TRC). The in-person clinical visits include a brief physical exam, NYHA functional class assessment, vital signs including oxygen and methemoglobin levels via co-oximetry, NTproBNP lab, 6MWT and Borg scale, interval histories, medication compliance review, AE assessment/symptom review, and dispensing of study drug.

In addition to these formal evaluations, subjects will be encouraged to immediately contact the study investigator and/or the study coordinator with questions, concerns, or to report new symptoms that occur during their study participation. If there are particular concerns that need to be addressed sooner than the in-person visits, the subject will be asked to return to the clinic as soon as possible for evaluation. If appropriate, based upon the evaluation, medical treatments will be provided to subjects, including appropriate referral to physicians or other services at the UPMC.

### 2.2.4. Medication Compliance

Randomization to study drug verses placebo will occur after eligibility has been confirmed by screening RHC and baseline procedures are completed. Randomization of sequence of nitrite-placebo versus placebo-nitrite followed by cross-over to the alternative treatment in a 1:1 ratio according to a computer generated pseudo-random code.

Subjects will self-administer their first dose of study medication or placebo under supervision of the physician investigator or the primary study coordinator after eligibility has been confirmed.

Subjects will also be dispensed their study medication for home use and a diary to track drug administration. Subjects will return the previously dispensed study medication and diary at every study visit thereafter, and medication compliance will be assessed

The study investigator and/or the study coordinator will review the daily diary. In the event that the compliance rate is < 80%, subjects will be re-educated on medication compliance. If medication compliance falls outside of the acceptable range more than 2 times, the study investigators will discuss subject eligibility for continued participation in the study.

### 2.2.5. Medication Accountability

The study investigators or the study coordinator will document the amount of study drug dispensed from UPMC-IDS. The study drug accountability records will be maintained at UPMC-IDS throughout the course of the clinical trial.

## 2.3 STUDY ASSESSMENT

The complete study assessment and procedures are outlined in Table 1.
Subjects will undergo study procedures and screening RHC to confirm that eligibility criteria have been met. The RHC procedure may be performed on a separate day. If a RHC has been completed within 12 months from enrollment, test results may be considered for baseline measurements.

### 2.3.1. Screening Procedures

The outpatient visits will take place in various outpatient clinics and/or procedural areas throughout the University of Pittsburgh Medical Center (UPMC) Presbyterian and UPMC Montefiore Hospitals. The screening procedures will take place over 2 days if screening RHC is needed. Subjects will be asked to fast a minimum of 8 hours prior to each visit. These screening procedures will determine eligibility for the study.

- Obtain written informed consent
- Complete history and physical examination to include body weight, height
- Vital signs that include: blood pressure (BP), pulse, respirations, and pulse oximetry
- Obtain waist measurement
- Review of concomitant medications
- NYHA functional class assessment
- 6 minute walk test (6MWT) and Borg scale
- MetHb level via co-oximetry (pre single dose and 30 minutes post single dose) . Due to unknown reasons the co-oximeter device may not be able to read metHb levels. In that event, a blood sample will be collected to test metHb levels at approximately 30-45 minutes after the 40mg dose.
- Blood samples: fasting glucose, comprehensive metabolic panel, hemoglobin A1C, fasting lipid profile, PT/PTT, complete blood count, platelet and differential, NT-proBNP. The total volume of blood drawn is approximately 1 ½ tablespoons (20 mL). If any of the above tests were done within 30 days before the screening visit, they may or may not be repeated for research purposes.
- Urine pregnancy test for women of childbearing potential
- Echocardiogram (if subject has had an echocardiogram within the past year we may not obtain during screening)
- Right heart catheterization (with optional micromanometry), research blood samples for plasma nitrite concentrations, red cell iron-nitrosyl-hemoglobin and platelet studies, and open label 40mg oral nitrite dosing with methemoglobin level. **Research blood samples will be collected prior to taking the 40mg oral nitrite and approximately 30-45 minutes post-dose.**
- A RHC may be performed to determine the PH-HFpEF eligibility criteria. The RHC will be conducted at UPMC Presbyterian Hospital, 3rd FL Catheterization Laboratory. The RHC hemodynamic measurement parameters for PH-HFpEF eligibility are as follows: mPAP ≥ 25 mm Hg; PCWP ≥15 and TPG ≥ 12. If a RHC has been completed within 12 months from enrollment, test results may be considered for baseline measurements. The total volume of blood drawn is approximately 1 ¾ tablespoons

(26mL). Subjects who report using PDE-5 inhibitor medications will be asked to hold the medication 5 days before the screening RHC and throughout the study.

### 2.3.2. Baseline Procedures

After eligibility has been confirmed, the following procedures will take place in various outpatient clinics and/or procedural areas throughout the University of Pittsburgh Medical Center (UPMC) Presbyterian Hospital, UPMC Montefiore Hospital, Kaufmann Building, and/or Falk Clinic. Subjects will need to fast a minimum of 2 hours prior to these visits. These procedures will be completed over 2-3 days.

- Cardiopulmonary exercise test (CPET): Incremental exercise testing (IET), a practice constant work rate ($CWR_P$) test, and baseline constant workrate ($CWR_B$) test, Spirometry- Forced vital capacity (FVC) maneuver only
- Accelerometer – activity monitor to be worn for about 7-10 days
- Completion of The Kansas City Cardiomyopathy Questionnaire (KCCQ), 36-Item Short Form Health Survey (SF-36), and Pulmonary Arterial Hypertension – Symptoms and Impact Questionnaire (PAH-SYMPACT).

A physical exam with NYHA functional class assessment may be performed and this will be completed on a case by case basis at the discretion of the study physician.

Subjects who report using PDE-5 inhibitor medications and do not need a screening RHC will be asked to hold the medication 5 days before the initial study drug or placebo dose and throughout the study. Patients who are fatigued or short of breath after stopping PDE5i, if specifically done for the study, will be evaluated and if it is felt to be related to stopping PDE5i will be restarted and they will continue in the trial with monitoring as per the trial.

## Randomization

Randomization to study drug verses placebo will occur upon completion of all screening and baseline procedures and confirmation by a study physician that subjects have met all eligibility criteria. Randomization of sequence of nitrite-placebo versus placebo-nitrite followed by cross-over to the alternative treatment in a 1:1 ratio according to a computer generated pseudo-random code.

Subjects will self-administer their first dose of study medication or placebo under supervision of the physician investigator or the primary study coordinator after eligibility has been confirmed. Each patient will have their vital signs and methemoglobin measured before taking the study medication and approximately 30-45 minutes after taking their first dose.

Subjects will also be dispensed their study medication for home use including a medication administration diary. Subjects will return the previously dispensed study medication and diary at every study visit thereafter, and medication compliance will be assessed.

### 2.3.3. Treatment Period and Monitoring

The follow up visits will take place in various outpatient clinics and/or procedural areas throughout the University of Pittsburgh Medical Center (UPMC) Presbyterian Hospital, UPMC Montefiore Hospital, Kaufmann Building, and/or Falk Clinic. The intravenous glucose tolerance test (IVGTT) will be performed at the Montefiore Clinical and Translational Research Center (CTRC).

Phone Visits: Weeks 4 and 8 (+/- 7 Days) following the start of each treatment arm

• Interval history (ER visits/hospitalizations)
• Review of concomitant medications
• Review for compliance and symptoms
• AE/SAE assessment

***If the PI deems it necessary to bring a participant to clinic, the following will be performed in addition to the items listed under the Phone visits:**

These visits may take up to 2 hours to complete. Subjects will be instructed to hold the morning or afternoon dose of study drug depending on the appointment time.

- Self-administration of drug by subject under direct supervision of study team
- Interval history
- Physical examination including vital signs, pulse co-oximetry, and body weight
- Methemoglobin percentage level via co-oximetry (approximately 30-45 minutes post study medication)
- Review of concomitant medications
- Collection of used study drug and diary; review for compliancy and symptoms
- Functional Class assessment
- NT-proBNP blood sample. The total volume of blood drawn for this sample is approximately 1 teaspoon.
- 6MWT and Borg scale
- Dispense study drug and new diary cards (if needed)
- AE/SAE assessment

Clinic Visit Week 10 (+/- 14 and End of Treatment (+/- 14) to begin testing visits)

These visits may take up to 4 days to complete all the assessments. Subjects e instructed to arrive in a fasted state and hold study medication the morning of these scheduled visits. Participants will take their morning or afternoon dose under direct supervision of the study team and will continue to take the study medication TID until all testing is completed.

- Self-administration of drug by subject under direct supervision of study team at the start of each visit
- Interval history
- Physical examination including vital signs, pulse co-oximetry, and body weight
- Waist measurement
- Methemoglobin percentage level via co-oximetry (approximately 30-45 minutes post study medication)
- Review of concomitant medications
- Collection of used study drug and diary; review for compliancy and symptoms
- Functional Class assessment
- 6MWT and Borg scale
- Accelerometer
- Urine pregnancy if applicable
- Echocardiogram

- Blood samples: fasting glucose, comprehensive metabolic panel, hemoglobin A1C, fasting lipid profile, PT/PTT, complete blood count, platelet and differential, and NT-proBNP. The total volume of blood drawn for these samples is approximately 1 ½ tablespoons (20 mL).
- Cardiopulmonary exercise test (CPET): Constant Work Rate Test
- RHC: Hemodynamic measurements will be completed at rest. Once the resting measurements are completed, the patient will take a single dose of their randomly assigned study medication. Another set of hemodynamic measurements will be taken approximately 30-45 minutes after the study medication is taken. Following these measurements, the patient will complete a constant workrate supine exercise test lasting about 4-12 minutes. Patients will pedal until exhaustion. The information collected from the baseline constant workrate exercise test during the baseline procedures will determine the watts used for the constant workrate supine exercise tests in the catheterization lab during weeks 10 and EOT. Hemodynamic measurements will be taken about every 2 minutes during the exercise test.
- Micromanometry (optional) during RHC
- Research blood samples (plasma nitrite concentrations, red cell iron-nitrosyl-hemoglobin, and platelet studies) during RHC: pre-dose and approximately 30-45 minutes post dosing of study drug. The total volume of blood drawn is approximately 1 ¾ tablespoons (26 mL).
- AE/SAE assessment
- IVGTT (optional)
- Completion of the KCCQ, SF-36 and PAH-SYMPACT Questionnaires
- Once all of the testing is completed, subjects will be given a diary card to keep track of any symptoms during the 2-week washout period and again after the EOT testing for the 30 day follow up phone call.

<u>2-Week Washout Period (+ 7 days)</u>
Upon completion of the first 10 weeks of study treatment, a 2-week washout period will occur. Subjects will not receive the study drug or placebo for two (2) weeks. Subjects will be given a diary card to keep track of any symptoms, doctor appointments, etc. during this time period. Subjects will return to clinic after the washout to cross over to alternative therapy and undergo the same study procedures as the first 10 weeks.

<u>Cross-Over Visit</u>

This visit may be completed over 2 days dependent on patient and staff availability. Subjects will be instructed to fast for a minimum of 8 hours on the day of the bloodwork. Patients will only need to fast for 2 hours prior to the constant work rate exercise test.

- Self-administration of drug by subject under direct supervision of study team
- Interval history
- Collection of diary; review for compliancy and symptoms
- Physical examination including vital signs, pulse co-oximetry, and body weight
- Urine pregnancy if applicable
- Methemoglobin percentage level via co-oximetry (approximately 30-45 minutes post study medication)
- Review of concomitant medications
- Functional Class assessment
- 6MWT and Borg scale
- Constant work rate exercise test
- Accelerometer

- Blood samples: fasting glucose, comprehensive metabolic panel, hemoglobin A1C, fasting lipid profile, PT/PTT, complete blood count, platelet and differential, NT-proBNP, research blood samples: plasma nitrite concentration, red cell iron-nitrosyl-hemoglobin, and platelet studies. The total volume of blood drawn for these samples is approximately 2 ½ tablespoons (35 mL).
- Dispense study drug and diary cards
- AE/SAE assessment
- Completion of the KCCQ, SF-36 and PAH-SYMPACT Questionnaires

Weekly phone follow-up (+/- 7 days) assessments will occur while on active study treatment (when subjects are not followed up in clinic) that will include symptom review, assessment of adverse events, review of concomitmant medications, and interval histories. Subjects will return to clinic at week 10 and undergo study procedures as noted in the first 10 weeks.

### 2.3.4. End of Trial and Follow-Up Period

Post Study Drug Telephone Assessment (approximately 30 days after the final day of testing)

- Telephone assessment for interval histories and AEs.

### 2.3.5. Open Label Period

Upon completion of the end of treatment visit, subjects will be given the option to receive open-label drug for 6 months. Subjects will receive open-label of Oral Nitrite ($NO_2^-$). Subjects may be asked to return monthly for blood pressure, methemoglobin checks (optional). Oral nitrite will be dispensed at each of these visits. Patients will be asked to return in-person for month 3 and month 6 of the open label period, and will be asked to complete 3 questionnaires (KCCQ, SF-36, and PAH-SYMPACT), perform a six-minute walk test, and have a blood collection to measure NT-proBNP level. The total amount of blood drawn is 4ml.

Currently HFpEF is a disease with no approved treatments. The main purpose of the open label period is to provide specific treatment to those patients that have a response during the crossover trial. This can also be a benefit to the patients if they are getting relief from their symptoms from the oral nitrite. It will also provide longer term safety data. Patient reported outcomes such as QOL are collected.

### 2.3.6. Schedule of Assessments

The table below summarizes the protocol procedures that will be performed throughout the study trial

**TABLE 1**

| Study Phase | Screening | Baseline | Weekly Phone Call +/- 7 days | Weeks 4 and 8 (per PI only) +/- 7 days | Week 10 +/-14 days | Cross-over Visit | Weekly Phone Call +/- 7 days | Weeks 4 and 8 (per PI only) +/- 7 days | End of Treatment (EOT) | Follow-up Period Phone Call |
|---|---|---|---|---|---|---|---|---|---|---|
| Informed Consent | X | | | | | | | | | |
| I/E Criteria | X | | | | | | | | | |
| Medical History & Demographics | X | | | | | | | | | |
| Physical Exam | X | | | X | X | X | | X | X | |
| Vital Signs & Body Weight | X | X | | X | X | X | | X | X | |
| Oxygen Saturation | X | | | X | X | X | | X | X | |
| Blood Samples: fasting glucose,CMP,hemoglobin A1C, fasting lipid profile, PT/PTT, complete blood count, platelet and differential, research labs | X | | | | X | X | | | X | |
| α NTproBNP blood test | X | | | X | X | X | | X | X | |
| Urine Pregnancy test (if applicable) | X | | | | X | X | | | X | |
| NYHA functional class assessment | X | | | X | X | X | | X | X | |
| α 6 minute walk test (6MWT) | X | | | X | X | X | | X | X | |
| Echocardiogram | X∞ | | | | X | | | | X | |
| ▪ Spirometry lung function test | | X | | | | | | | | |
| Incremental exercise testing (IET) | | X | | | | | | | | |
| Practice Constant Work Rate ($CWR_P$) | | X | | | | | | | | |
| Constant Work Rate (CWR) test | | X | | | X | X | | | X | |
| (RHC): RA, RV, PA, PCWP, CO/CI Resting hemodynamics and (sub-maximal exercise at Week 10 and EOT only) | X† | | | | X | | | | X | |
| ɜ Micromanometry (optional) | X | | | | X | | | | X | |
| Accelerometer | | X | | | X | X | | | X | |
| ɲ IVGTT | | | | | X | | | | X | |
| MetHgb levels via co-oximetry | X | X | | X | X | X | | X | X | |
| Randomization | | X | | | | | | | | |
| Dispense and study drug administration | | X | | X | X | X | | X | If patient wants to continue open label | |
| Study drug compliancy assessment | | | | X | X | | | X | X | |
| Dispense subject diary | | X | | X | X | X | | X | X | |
| α KCCQ, SF-36 and PAH-SYMPACT questionnaires | | X | | | X | X | | | X | |
| AE Assessment | | | X | X | X | X | X | X | X | X |
| Review of concomitant medications | X | | X | X | X | X | X | X | X | |
| Interval History Assessment | | | X | X | X | X | X | X | X | X |

∞ If subject has had an echocardiogram within the past year, we may not obtain during screening.
▪ **Spirometry lung function test may not be needed at baseline visit if results are available within 6 months from enrollment.**
† **A screening RHC may not be performed if a RHC was completed within 12 months from enrollment per PI discretion.**
ג **Micromanometry is optional and completed during RHC.**
ת **Insulin-dependent diabetics will not receive the IVGTT.**
α **At month 3 and month 6 of open label period, subjects will complete three questionnaires, six-minute walk test and NT-proBNP blood draw.**

## 2.4 DESCRIPTION OF STUDY PROCEDURES

### 2.4.1 Echocardiogram

Transthoracic echocardiograms will be completed to assess RV function by measures such as TAPSE as well as to assess the Doppler waveform of the pulmonary artery. This is a noninvasive test.

### 2.4.2 Six Minute Walk Test (6MWT and Borg Scale)

The six-minute walk test (6MWT) will be conducted in accordance with local standard operating procedures. The 6MWT measures the distance an individual is able to walk over a total of six minutes on a hard, flat surface. The subject is allowed to self-pace and rest as needed as they traverse back and forth along a marked walkway. The test is used as a performance-based measure of functional exercise capacity. It evaluates the global and integrated responses of all the systems involved during exercise, including the pulmonary and cardiovascular systems, systemic circulation, peripheral circulation, blood, neuromuscular units, and muscle metabolism. The self-paced 6MWT assesses the submaximal level of functional capacity. Most subjects do not achieve maximal exercise capacity during the 6MWT; instead, they choose their own intensity of exercise and are allowed to stop and rest during the test. However, because most activities of daily living are performed at submaximal levels of exertion, the 6MWT may best reflect the functional exercise level for daily physical activities.

The Borg Scale is a method of rating perceived exertion during exercise and will be used while the subject performs a 6MWT.

### 2.4.3 Accelerometer

Physical activity: The SenseWear Pro Armband Accelerometer (BodyMedia, Pittsburgh, PA and/or Actigraph, Pensacola, FL) multi-sensor device will be used to provide an objective measure of physical activity for about 7-10 days.

### 2.4.4 Spirometry

Spirometry testing at baseline will consist of the Forced Vital Capacity (FVC) maneuver. A trained pulmonary function technician will administer the pulmonary function testing. The FVC is the maximal volume of air exhaled with a maximally forced effort from a maximal inspiration. The FVC maneuver will be performed using the Easy on-PC (NDD) with the subject in a seated position. Within the FVC maneuver, the maximal volume of air exhaled in the first second of a forced expiration (FEV1) will be obtained as well. The best of three efforts will be defined as the highest FEV1 and the highest FVC, each obtained on any of three efforts that meet the American Thoracic Society (ATS) guidelines for acceptability and repeatability criteria (with a maximum of eight attempts). The highest FEV1 and FVC will be selected regardless of whether they come from different spirometric maneuvers or from the same maneuver.

If the ratio of FEV1/FVC is less than 0.70, then salbutamol (albuterol) will be administered. After a gentle and incomplete expiration, a dose of 100 $\mu$ g of salbutamol (albuterol) is inhaled

in one breath to total lung capacity. The breath is inhaled for 10 seconds before the subject exhales. Four separate doses (total dose 400 $\mu$ g) are delivered at approximately one minute intervals. Three additional, acceptable post-bronchodilator FVC manoeuver tests will be recorded 15 to 30 minutes after the last dose of salbutamol (albuterol) is inhaled.

### 2.4.5 Cardiopulmonary Exercise Testing (CPET) and Gas Exchange Measurements:

CPET will be performed using an electromagnetically braked cycle ergometer (Ergoline 800, SensorMedics) and the gas exchange measurements will be analyzed using the Viasys Vmax Encore metabolic cart system.

CPET at baseline will consist of an incremental exercise test (IET) and a constant work rate test for practice ($CWR_P$). The $CWR_P$ test will determine the constant workrate (CWR) that will be used at baseline, Week 10, crossover, and EOT.

Determining Workload (WL) and CWR at Baseline

- IET:The work rate will increase in a stepwise manner, using an individualized incremental WL (based on age, height, weight, and gender) to achieve a targeted duration of 4 to 12 minutes to volitional exhaustion. The incremental WL will be determined to the nearest multiple of 5 watts [69].

- $CWR_P$: A resting period of at least 2 hours will be scheduled between exercise tests. The CWR is a set work rate that remains unchanged throughout the duration of the test. The $CWR_P$ will be performed around 70-75% of the maximal WL from the IET [70, 71]. Further adjustment in CWR will be based on the exercise endurance time (ET) of $CWR_P$ to optimize CWR at baseline, Week 10, crossover, and EOT to achieve a target duration of 4 to 12 minutes. ET will be measured from the start of loaded pedaling to volitional exhaustion and the WL has returned to unloaded pedaling. If a patient is unable to reach 4 minutes, the patient may be asked to return on a separate day to repeat the $CWR_P$ to adjust to a workload the patient can endure for at least 4 minutes. The second test will be optional.

CPET for baseline, Week 10, crossover, and EOT

The CWR determined from $CWR_P$ will be used for baseline, Week 10, crossover, and EOT. ET will be measured from the start of loaded pedaling to volitional exhaustion and the WL has returned to unloaded pedaling. The endurance exercise challenge will be used to compare oral nitrite to placebo.

CPET will measure the integrated response of the pulmonary mechanics, gas exchange, cardio/pulmonary vascular, peripheral muscle systems, and the subject's perceived exertion. For each subject, isotime measurements of cardiopulmonary metabolic responses will be compared over the minimum ET of Week 10 and EOT. Data will be reported on an exercise test form.

A detailed description of the CPET procedures is provided by the CPET lab.

Emergency equipment including a defibrillator will be readily available.

### 2.4.6 Laboratory Testing

The following peripheral blood samples will be evaluated at the screening/baseline, week 10, cross-over, and end of treatment visits for follow-up comparison purposes: fasting glucose, comprehensive metabolic panel, hemoglobin A1C, fasting lipid profile, PT/PTT, complete blood count, platelet and differential, NT-proBNP, and research blood samples for plasma

nitrite concentrations, red cell iron-nitrosyl-hemoglobin, and platelet studies. Urine pregnancy test will be performed on females of child-bearing potential at baseline, week 10, cross-over visit, and at the end of study treatment. The samples will be collected and analyzed at the UPMC Presbyterian laboratories. The NT-proBNP blood sample will be sent to the Translational Research Core Laboratory (TRCL) for storage and analysis. Research samples will be sent to Shiva/Gladwin labs in Vascular Medicine Institute (VMI) for storage and analysis.

#### 2.4.6.1 Research Laboratory Profiles

Blood samples for platelet mitochondrial analysis, plasma nitrite concentrations and red cell iron-nitrosyl-hemoglobin may be collected at screening/baseline, Week 10, crossover, and EOT visits. These de-identified blood samples will be sent to the Shiva/Gladwin labs in the Vascular Medicine Institute for storage and analysis under the supervision of Drs. Gladwin, Risbano,and Shiva.

### 2.4.7 Methemoglobin Levels

The Masimo Rainbow SET® CO-Oximeter device that will be used in this clinical trial has been validated to provide accurate determinations of methemoglobin levels under conditions of hypoxia and methemoglobinemia. Measurements will be obtained before initial dose and approximately 30-45 minutes post initial dose and approximately 30-45 minutes after the dose is given at the clinical visits. Due to unknown reasons the co-oximeter device may not be able to read metHb levels. In that event, a blood sample will be collected to test metHb levels.

### 2.4.8 Right Heart Catheterization, Simultaneous Pressure and Doppler Flow Velocity Measurement, and Exercise RHC

Right heart catheterization (RHC) is performed routinely as a clinical standard of care procedure for diagnostic purposes in this disease population. The standard right heart catheterization is performed with a balloon tipped, flow-directed pulmonary artery catheter (Swan-Ganz catheter), inserted through a sheath in the internal jugular vein. As per standard clinical protocol, hemodynamic recordings of right atrial, right ventricular, pulmonary artery pressures, pulmonary capillary wedge pressure, in addition to cardiac output, are made at baseline.

RHC may be completed at screening for eligibility purposes, week 10, and end of treatment visits. Resting and supine constant workrate exercise RHC measurements will be performed at week 10 and end of treatment. Hemodynamic measurements may be obtained every 2 minutes during the exercise test.

Subjects may also undergo additional hemodynamic measurements using a micromanometer pressure catheter that may be inserted via the Swann-Ganz catheter distal port. Measurements with micromanometer and/or Swann-Ganz catheter may be made with simultaneous flow velocity from transthoracic echocardiographic doppler ultrasound at resting and after the exercise portion of RHC. This portion of the study that involves the micromanometer instrumentation is expected to add an additional 5-10 minutes onto the procedure at each time point.

Cardiac output (CO) is the amount of blood pumped through the left ventricle of the heart in a time interval of one minute. There are a number of ways to calculate CO. Thermodilution and/or Fick cardiac index are two means that will be used for measuring CO for this protocol. The CO will be the average of the recorded CO collected.

Cardiac index (CI) is a measurement that relates the CO from the left ventricle to body surface area (BSA), therefore determining cardiac function to size of the individual. CI is calculated by CO/BSA.

Pulmonary capillary wedge pressure (PCWP) provides an indirect estimate of left atrial pressure (LAP). PCWP is measured by the balloon-tipped, multi-lumen catheter (Swan-Ganz catheter) while it is in a branch of the pulmonary artery. Just behind the tip of the catheter is a small balloon that can be inflated with air (~1 cc). The catheter has one opening (port) at the tip (distal to the balloon) and a second port several centimeters proximal to the balloon. These ports are connected to pressure transducers. When properly positioned in a branch of the pulmonary artery, the distal port measures pulmonary artery pressure and the proximal port measures right atrial pressure. The balloon is then inflated, which occludes the branch of the pulmonary artery. When this occurs, the pressure in the distal port rapidly falls, and after several seconds, reaches a stable lower value that is very similar to left atrial pressure (normally about 8-10 mmHg). The balloon is then deflated and pulmonary artery pressure can again be recorded. The same catheter can be used to measure cardiac output. Anticoagulants would need to be held prior to the right heart catheterization as this is standard clinically for that procedure. Antiplatelets and beta blockers are continued, also clinical standard of care. We will be holding only the morning dose of oral hypoglycemics for all non-insulin dependent diabetics until they complete the study later that morning - this is also standard clinically for this procedure. Holding diuretics along with potassium may also occur as this is often done prior to RHC but will be addressed on a case by case basis by study investigators.

### 2.4.9 Intravenous Glucose Tolerance Test (IVGTT)

This visit will take place in the Montefiore CTRC. This test will occur at least 48 hours after the last exercise bout to account for the effects of acute exercise on insulin resistance. Subjects will be asked not to drink or eat anything other than plain water for 8 hours prior to their arrival for this visit.

Both glucose and insulin will be injected intravenously into the arm. Blood samples will be taken over the course of 3.5 hours, totaling about 15 1/2 tablespoons.

The IVGTT is considered a gold-standard test to measure insulin secretion and sensitivity. It has been standardized and used worldwide for more than 20 years. In our study, the expected duration is 180 mins after the test is started with an IV glucose bolus of 0.3g/kg. After the glucose bolus, a small dose of human regular insulin is given IV to moderate the plasma glucose rise. The insulin dose is 25 mUnits/Kg body weight. Patients taking oral hypoglycemics may be asked to hold those medications the morning of the IVGTT but can resume taking them following the completion of the IVGTT the same day.

### 2.4.10 Self Administered Health Questionnaires

The Kansas City Cardiomyopathy Questionnaire (KCCQ) helps quantify physical function, symptoms, social function, self-efficacy and knowledge and quality of life. The 36-Item Short Form Health Survey (SF-36) measures physical, mental and general health. The Pulmonary Arterial Hypertension – Symptoms and Impact Questionnaire aims to assess pulmonary hypertension symptoms and their impact on quality of life.

## 2.5 SPECIMEN COLLECTION AND MANAGEMENT

### 2.5.1 Specimen Handling and Labeling (De-Identification)

Specimens collected will be properly labeled. All research biological specimens and all records associated with the samples will be labeled only with a unique code that contains no personal identifiers. The information linking these code numbers to the corresponding subject's identity will be kept in a secure location in the investigator's office, and will not be available to staff managing samples at the research laboratories.

Immediately upon receipt of the biological specimens, all attempts will be made to process, isolate, collect, and store the specimens. The code number and date on which the specimen is frozen, all other information about the specimen, and subsequent processing will be entered on the specimen processing worksheet.

### 2.5.2 Specimen Management and Storage

Specimens in excess of immediate assay requirements may be stored indefinitely in a locked freezer under the control of the principal investigator. Subject's de-identified biological samples and data will be stored for future research analyses and may be shared with other researchers', their laboratories or federal repositories. Samples will be sent to the Lai lab at Indiana University, Indianapolis for further analysis. Future sharing outside the institution will be done under an approved data or specimen sharing agreement.

The blood samples will be stored after appropriate coding to remove patient identifiers. The coding information linking patient identifiers to the stored samples will be maintained in a locked, secure area that will be accessible only to the study investigator. Subjects may request to have their samples destroyed at any time. These samples will be destroyed immediately upon receipt of the subjects' written request to do so. Identification of which samples to destroy will be available from the coding information linking patient identifiers to the stored samples as described earlier in this paragraph.

### 2.5.3 Restrictions to Direct Access of Specimens

Specimens will be kept in the responsible study investigators' laboratories indefinitely and will be under the control of the principal investigator. Investigators or other personnel not involved with the management or operations of the study are not permitted direct access to the specimens.

## 2.6 ENDPOINTS

### 2.6.1 Primary Endpoints

The primary endpoint of the trial will be mean pulmonary artery pressure (mPAP) during submaximal exercise as compared between placebo and nitrite at 10 weeks (i.e. the week 10 RHC of placebo vs week 10 RHC of nitrite).

### 2.6.2 Secondary Endpoints

<u>Secondary endpoints:</u>

1.The differences in 6MWT with Borg dyspnea scale assessments and pulse oxygen saturation measurements
2. RHC hemodynamics (right atrial pressure, pulmonary capillary wedge pressure, pulmonary artery pressures and resistance, pulmonary artery compliance, cardiac output, diastolic pressure gradients, and transpulmonary gradient)
3. Doppler-echocardiography
4. NT-proBNP plasma levels

5. NYHA functional classification changes
6. $NO_2^-$ metabolites
7. Endurance exercise time (ET), changes in CPET measurements taken at isotime (oxygen consumption [VO2, L/min], minute ventilation [VE, L/min], ventilatory equivalent [VE/VO2], dead space to tidal volume ratio [Vd/Vt], Borg dyspnea and leg score, and changes in time constants for increases of Vt, respiratory rate [RR], VE, and heartrate).
8. Changes in fasting glucose and insulin sensitivity

## 2.7 SUBJECT WITHDRAWAL

A subject may voluntarily discontinue participation in this study at any time. The investigator may also, at his or her discretion, discontinue a subject from this study at any time. Every effort should be made by the investigator to keep the subject in the study.

Subjects may be withdrawn from the study prior to completion if any of the following criteria are observed:

- Intercurrent illness or an unexpected fatal or life-threatening adverse event, which requires discontinuation of study treatment
- Pregnancy
- Subject reached protocol-defined stopping criteria
- Request by the subject to withdraw from the study
- Protocol violations
- Persistent non-compliance
- Lost to follow-up
- Investigator discretion
- Study closed/terminated

### 2.7.1. Dropouts and withdrawals

To be considered complete, a subject must complete all study visits as specified in the protocol without violations of the protocol so significant as to obscure the response to study treatment.

Subjects who fail to complete all study required visits will not be considered complete and may not enroll at a later date and will not be replaced. A record will be kept of all subjects who fail to complete all study visits and their primary reasons for discontinuation.

In the event of subject withdrawal, subjects will be encouraged to continue all follow-up visits for safety monitoring or to continue follow up as directed by their personal primary physicians, unless the subject withdraws consent at any time (without having to justify the decision). All available data from subjects who discontinued during the study, for whatever reason, will be included in the safety analysis.

## 2.8 STATISTICAL ANALYSIS

### 2.8.1 Sample Size and Power

**Sample size:** Prior study has indicated that oral nitrate reduces baseline mean PAP -7 (SD = 4) mm Hg vs. -3 (4) mmHg in placebo group. A sample size of 12 in each group provides 88%

power to detect this difference. This sample size will provide 80% power to detect -10 (6) vs. -5 (6) decrease in post-exercise mPAP in treatment and placebo group, respectively.

**Statistical analysis:** Pre-randomization characteristics of two groups will be presented using the median (interquartile range) or frequency tables at each study sequence. The difference of mPAP during submaximal exercise as compared between placebo and nitrite at 10 weeks (i.e. the week 10 RHC of placebo vs week 10 RHC of nitrite) using the parametric PK-cross analysis (i.e. an ANOVA which determines the sequence, period, carryover, and treatment effects). Post-exercise values of before and after crossover will be compared between two groups using similar approach. For secondary outcomes, we will compare the difference of baseline and follow-up value and compare these differences with a parametric test. Alternatively a nonparametric test will be used for these comparisons. For both primary and secondary outcomes, treatment effect in each sequence as well as mean (SD) total treatment effect and corresponding P values will be reported.

We will also test the effect of treatment on pre-exercise mPAP and secondary outcomes using mixed effect models. In all analysis, missing data will be imputed and sensitivity analysis with imputed data will be performed. All analyses will be performed by ITT approach.

# 3. HUMAN SUBJECTS

## 3.1 SUBJECT POPULATION

The racial, gender and ethnic characteristics of the proposed subject population in this research protocol shall reflect the demographics of the population of Pittsburgh and the surrounding area. We shall attempt to recruit subjects in proportion to these demographics. No exclusion criteria shall be based on race, ethnicity, or gender.

Every effort will be made to keep subjects in the study until they complete all study procedures.

### 3.1.1 Inclusion of Women and Minorities

Both men and women of all races and ethnic groups are eligible for this trial. Women who meet the inclusion criteria, and have none of the exclusion criteria, will be enrolled without restriction as dictated by the study protocol. Because of the use of a study medication, woman of child bearing potential must meet specialized inclusion/exclusion criteria to minimize this risk.

### 3.1.2 Inclusion of Children

Children under the age of 18 will not be recruited for this protocol because of the need to explore potential adverse effects of the study treatment more fully in adults.

## 3.2 INCLUSION CRITERIA

Potential study subjects must satisfy the following criteria to be enrolled in the study:

1. Age 18 years and older
2. PH-HFpEF

Confirmed diagnosis by RHC:

- Mean Pulmonary Arterial Pressure (mPAP) ≥ 25 mm Hg
  AND
- Pulmonary capillary wedge pressure (PCWP) ≥ 15 mm Hg

AND

- Transpulmonary Gradient (TPG) ≥ 12 mm Hg

3. Patient has one or more of the following heart failure associated signs or symptoms: shortness of breath with activity, overweight, atrial fibrillation and/or systemic hypertension.

### 3.3 EXCLUSION CRITERIA

Subjects meeting any of the exclusion criteria at baseline will be excluded from participating in study.

- Age less than 18 years
- SBP > 170 or < 110 mmHg
- DBP > 95 or < 60 mmHg
- Hemoglobin A1C > 10
- Positive urine pregnancy test or breastfeeding
- Ejection Fraction < 40%
- Dementia
- End-stage malignancy
- Major cardiovascular event or procedure within 6 weeks prior to enrollment
- Severe valvular disease
- Known chronic psychiatric or medical conditions that may increase the risk associated with study participation in the judgement of the investigator would make the subject inappropriate for entry into this study
- Smoker
- Hemoglobin < 9 g/dL
- Serum creatinine > 3.0 mg/dL
- Recent (<3 months) addition or change in dosing of hormonal contraceptive medications (OCP, IUD, DepoProvera)
- Receipt of an investigational product or device, or participation in a drug research study within a period of 15 days;
- RHC < 2 weeks from study screening RHC unless clinically indicated

## 4. RECRUITMENT AND INFORMED CONSENT PROCEDURES

### 4.1 RECRUITMENT METHODS

- 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, internal medicine clinics, or other UPMC or community facilities. For UPMC Presbyterian/Montefiore-based clinics, we will obtain physician and HRPO approval for screening of subject medical records for identification of eligible candidates. He will engage with additional cardiologists, internal medicine physicians, 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 engage the attending cardiologist as to appropriateness for the study. As noted, permission to speak with subject will be obtained from the Cardiology attending.

b. Research Registry

The HRPO submission will include the request to use the Clinical Translational Science Institute's Pitt+Me Research Registry, which includes a database of over 90,000 individuals who have indicated their interest in participating in research studies. The Pitt+Me initiative includes the use of social media to engage subjects. We will also request to use the University of Pittsburgh's Pepper Center Research Registry which includes a database of older individuals interested in research, fostering inclusion of older adults and covers individuals in the community, assisted living and skilled nursing facilities.

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.

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

- 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 study physician.

b) Setting dates for patient visits will also occur prior to screening visit to maintain timeliness of visits per protocol.

c) 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 final eligibility will be confirmed.

*d)* To minimize the possibility that subjects will feel obligated to participate, investigators will reinforce with their subjects that participation is voluntary, that they do not have to participate, and that the decision not to participate will not affect their care, now or in the future. The investigator will also allow subjects to make further inquiries if they are interested.

## 4.2 INFORMED CONSENT PROCEDURES

Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continues throughout the individual's study participation. Extensive discussion of risks and possible benefits of participation in this study will be provided to the subjects and their families. Consent forms describing in detail the study procedures and risks are given to the subject and written documentation of informed consent is required prior to enrolling in the study. Consent forms will be IRB approved and the subject will be asked to read and review the document. Upon reviewing the document, the investigator will explain the research study to the subject and answer any questions that may arise. The subjects will sign the informed consent document prior to being enrolled in the study. The subjects should have the opportunity to discuss the study with their surrogates or think about it prior to agreeing to participate. The subjects may withdraw consent at any time throughout the course of the study. A copy of the informed consent document will be given to the subjects for their records. The rights and welfare of the subjects will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

Prior to performing any of the research study procedures or interventions, subjects must provide informed consent. The investigator will verbally explain the study to the potential subject in a language understandable to subjects, providing all pertinent information (purpose, procedures, risks, benefits, alternatives to participation, etc.), and will allow potential subjects ample opportunity to ask questions to elicit a better understanding of the study. Following this verbal explanation, potential subjects will be provided with a local IRB approved consent form and will be asked to read and review the document. Upon reviewing the document, the investigator will provide adequate opportunity for the subject to consider all options, and answer any additional questions the potential subject may have. Every effort will be made to ensure that subjects have comprehended the study information prior to obtaining subject's voluntary agreement to participate.

Remote Consenting

Remote consenting may be used as opposed to in person consenting where possible to reduce unnecessary in person encounters. In the event remote consenting is utilized, subjects will be provided with a copy of the informed consent prior to the teleconsent meeting either via email, fax, mail or previously provided during an in person visit. After the physician investigator and subject review the consent form via video conferencing (e.g., telephone, conference call, videoconference, telemedicine, etc.), the subject will be offered the opportunity to ask any questions and have those questions answered. The subject will sign and date the informed consent.

The physician investigator will verify the subject physically signed the consent document either by viewing via video conference or obtaining a photo of the complete signed consent signature page or obtaining verbal confirmation from the subject that he/she signed the consent form or agreed to participate electronically. The signed document is then mailed, emailed, photo/scanned to text or faxed to the study team.

If the signed consent is provided as an electronic copy (emailed, photo/scanned, or faxed), the subject will return the original signed document on their first in person visit or by mail. If the signed consent form is mailed to the study team, the physician investigator will sign their copy which they possess after the subject has acknowledged signature on their copy. Once the subject's original copy is received the study team's copy will be attached to make a single document.

The study team will document the remote consent process, including the date and time the conversation occurred, how it occurred (e.g., telephone, video conference), the names of the participants, confirmation that the subject received the informed consent, and confirmation of verbal informed consent to participate.

In addition, older potential participants whose competency to consent is in question will be tested for sufficient comprehension and recall of the information presented. Prospective subjects who do not remember the important facts about participation in the research study after repeated testing will not be included in the study.

The subjects may withdraw consent at any time throughout the course of the study. A copy of the informed consent document will be given to the subjects for their records. The rights and welfare of the subjects will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

## 5. POTENTIAL RISKS AND BENEFITS

### 5.1. Risk of Experimental Drug Intervention

Numerous studies have evaluated acute, subacute, and chronic drinking water exposures of nitrite in laboratory animals, and drinking water and dietary exposures in humans. Recent studies are available using a high dose of nitrite by oral route in the form of beet root juice. Recent studies have evaluated acute exposures of oral preparations of nitrite on PK and blood pressure and are characterized below. More extensive human data is available on parenteral sodium nitrite as it is currently available and approved by the FDA for use in the emergency treatment of cyanide poisoning. [72, 73] It is also notable that neutrapharmaceutical preparations are currently being sold with levels of nitrite (12.7 mg per tablet).

<u>Nitrite:</u>
Sodium nitrite has been used commercially as a food preservative, an anti-corrosive agent, a coloring agent, and an anti-anginal agent, with additional uses in laxatives, burn ointments, and liniments. Amyl nitrite has been inhaled or ingested as an euphoric stimulant. Nitrite has also been found as a contaminant in well water. Literature searches generated case reports of nausea, vomiting, abdominal pain, dizziness, headache, flushing, cyanosis, tachypnea, dyspnea, hypotension, and death attributed to excess nitrite (high-dose) exposure from these sources as a consequence of methemoglobinemia due to oxidation of heme-iron in oxyhemoglobin. Normal background methemoglobin production is 1-3%. If levels of methemoglobin rise above approximately 30% of total hemoglobin, a subject may appear cyanotic and experience dyspnea, due to the reduced oxygen carrying capacity of hemoglobin

(methemoglobin cannot bind oxygen). Levels above 50% can cause seizures, hypotension, coma, and death. Sodium nitrite administration for cyanide poisoning at the labeled dosage of 300 mg causes methemoglobinemia, a desirable effect, as methemoglobin binds to cyanide, thus protecting cellular mitochondria. A standard dose of nitrite used for cyanide poisoning is 300 mg up to 600 mg. Note that methemoglobin levels have never risen higher than 3% at the currently used therapeutic doses (< 75 mg) in 80 volunteers in phase I studies at the NIH.

The Sponsor of this study proposal, Dr. Gladwin, has previously held an IND for sodium nitrite (IND # 70,411) for cardiovascular applications and currently has an approved IND for the use of sodium nitrite for lung transplant recipients (IND # 111,643). The cardiovascular IND involved the administration of sodium nitrite to 69 normal volunteers in 4 phase I-II clinical trials without observed adverse effects. He has also treated 11 subjects with sickle cell disease on this IND without observed adverse effects. The lower doses of nitrite used in these investigational treatment regimens – 60-120 mg daily or 20-40% of the dose (300mg) 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[33-35]. In another study by Gladwin et al., 18 healthy adults received an infusion of sodium nitrite totaling 75 mg (15 minutes each x 2 infusions). This was associated with a 7 mmHg decrease in mean arterial pressure, a peak methemoglobin of less 3%, and no other significant effects[34]. Note this single dose is 1.9 times the single dose per time of day we plan to use in this trial.

In an open-label three-way crossover study, 9 healthy adult subjects received two single high dose oral sodium nitrite aqueous solutions (0.12 and 0.06 mmol $NaNo_2$/mmol Hb, equivalent to 290-380 mg and 140-190 mg sodium nitrite, respectively, depending on the total body hemoglobin level of the person) and one intravenous sodium nitrite dose (0.12 mmol $NaNo_2$/mmol Hb)[18]. Note that this is 1.2-3.2 times the daily dose we plan to use in this trial. There was a washout period of at least 7 days between each of the treatments. Mild headache occurred in 44-55% of subjects and was the most frequent complaint during each treatment session, which the authors ascribed to the sodium nitrite, not methemoglobinemia, as the percentage of methemoglobinemia stayed below clinically toxic levels (<15%). By report, up to 22% experienced nausea, which subsided within half an hour[18]. The pharmacokinetic analysis of this study indicated similar bioavailability of oral and IV delivery of nitrite, as well as similar side effect and safety profiles.

A recent study determined the safety and feasibility of prolonged intravenous nitrite infusion. Twelve adult volunteers received increasing starting doses of sodium nitrite, 4.2 to 533.8 µg/kg/hr for 48 hours. Dose limiting toxicity occurred at 445.7 µg/kg/hr (10.6 mg/kg/day) and was limited to asymptomatic transient decreases of arterial blood pressure of up to 20 mmHg and asymptomatic increases of methemoglobin levels above 5%. No tolerance or clinically significant rebound was observed[36]. Note, this is 8.2 times the daily dose we plan to use in this trial (based on an adult body weight of 95 kg).

For nitrite, two retrospective case-control studies have shown that high maternal dietary nitrite intake from cured meat or drinking water during pregnancy might be associated with risk of childhood brain tumors and possibly gastric and esophageal cancer. This evidence is only based on retrospective case-control studies; cohort studies have found no significantly increased risks[24].

In the 2001 National Toxicology Program (NTP) Report summarizing 2-year rodent drinking water studies, there was no evidence of carcinogenic activity of sodium nitrite in male or

female F344/N rats exposed to up to 130 mg/kg/day in males and 150 mg/kg/day in females, or in male B6C3F1 mice exposed to up to 220 mg/kg/day. There was equivocal evidence of carcinogenic activity of sodium nitrite in the highest dose of 165 mg/kg/day in female B6C3F1 mice based on the positive trend in the incidences of squamous cell papilloma or carcinoma (combined) of the forestomach. Exposure to sodium nitrite in drinking water resulted in increased incidences of epithelial hyperplasia in the forestomach. However, no chromosomal damage (genetic toxicity) was observed in three studies conducted in rats and mice *in vivo*[37]. Taken together, these findings suggest minimal carcinogenic nitrite-mediated risk.

In the current study, we will use lower doses of nitrite than used safely in any of these studies. Our nitrite dose of 40 mg three times daily is lower than the dose used on the cardiovascular IND and < 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: none
- Rare: flushing, tachycardia, hypotension

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

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

### 5.2 Risk of Study Procedures

Risks of Blood Drawing

The amount of blood to be drawn over the course of this research study could be a maximum of 40 tablespoons. To minimize the risks of blood tests, all blood draws are performed by trained RN, MD, technician or phlebotomist.

Common risks include temporary discomfort, bruising which may last for several days, redness, swelling, lower hemoglobin level. Infrequent risks include a subject may feel

lightheaded or faint when blood is drawn. This is usually due to nervousness and is not usually serious. Rare risks include infection and bleeding.

Risks of Echocardiography
This is a noninvasive procedure. There is no known risk associated with this procedure. Gel will be applied during the test which may cause coldness and/or irritation.

Risks of Accelerometer Use
Common risks associated with wearing the accelerometer are redness, irritation, and chaffing.

Risks of Right heart Catheterization
Common risks: pain at the needle entry site and slight risk of bleeding around the site, bruising at site, lightheadedness or dizziness during the needle stick.

Infrequent risks: puncturing the lung which would require a chest tube insertion, irregular heartbeats which usually stop when the long tube is removed from the heart.

Very rare complications include cardiac arrhythmias, cardiac tamponade, low blood pressure, infection, or embolism caused by blood clots at the tip of the catheter.

Risks of the Use of Micromanometer Catheter during Catheterization
The micromanometer pressure and flow catheters are FDA approved, and have been used for clinical/investigative indications for over 25 years. Moreover, these catheters do not pose any inherent risk to patients. The catheter will be placed through the clinical Swann-Ganz catheter that is within the preexisting jugular venous sheath. This obviates the need for an additional venipuncture. This catheter has successfully been used without complications by the study Principal Investigator for the past 5+ years. Risks associated with this procedure are estimated to be rare, which include abnormal heart rhythm, bleeding, or perforation of the heart or pulmonary artery.

Risks of Medications used during Catheterization
The medications used for sedation are relatively brief in duration and should wear off within several hours. The side effects are listed below for each drug.

- 1% Lidocaine: will be used to numb the area prior to the insertion of the cardiac catheter into either the arm, neck or groin vein. A common side effect is slight burning at the site which dissipates quickly.
- Fentanyl: Common side effects include temporary light-headedness, dizziness, and nausea, vomiting or sweating.
- Midazolam: Common side effect includes drowsiness. Infrequent side effects are nausea, vomiting. Breathing problems are rare. Allergic reactions (e.g., hives, itching, etc.) to lidocaine, fentanyl, or midazolam are rare.

Risks of Fluoroscopy during the Catheterization
An x-ray may be done, if indicated, on participants whose neck vein is used for the catheterization. An X-ray performed for the purpose of this research study involves exposure to radiation. Each x-ray will result in a radiation dose of approximately 3 rems to the neck with minimal exposure of other body areas. A total of 9 rems if all three RHC's are performed. For comparison, radiation workers are permitted, by Federal regulation, to receive a maximum annual radiation dose of 20 rems to the most sensitive organs of their body. There is no minimum amount of radiation exposure that is recognized as being totally free of the risk of causing genetic mutations (abnormal cells) or cancer. However, the risk associated with the

amount of radiation exposure received from participation in this study is considered to be low and comparable to everyday risks.

Risks of Cardiopulmonary Exercise Test (CPET)

CPET is a well-established clinical tool. The risk of serious or fatal complications is rare. The risks include but are not limited to the following:

- Common risks include shortness of breath, mild angina, musculoskeletal discomfort.
- Infrequent risk includes hypotension, chest pain.
- Rarely, exercise may cause moderate to extreme pain which could be due to muscle sprains, muscle strains, broken bones, or chest pain, changes include shortness of breath, abnormal blood pressure, or fainting, myocardial infarction, prolonged cardiac arrhythmia, stroke, or sudden death are rare (occur in <1% of people).

Risk of Spirometry

Spirometry infrequently can make the chest tight or lead to shortness of breath. Albuterol will be available if this occurs. Rarely, a subject may have coughing or lightheadedness with spirometry. If Albuterol is given, the subject will get up to 4 puffs of albuterol with these tests. Albuterol may make a subject feel slightly jittery or nervous, but the feelings are temporary.

Risks of Intravenous Glucose Tolerance Test (IVGTT)

- IV Catheter Insertion Common Risks: bruising, soreness, phlebitis. Infrequent Risks: infiltration (a leakage of anything that has been given through the vein (such as the saline, dextrose, deuterated glucose, insulin) into the arm tissue that surrounds the vein and holds the IV). Other Risks: infection, dizziness/fainting, bleeding.

- IVGTT Common Risks: (occurring in less than 25% of people) include: phlebitis (inflammation of a vein), transient hyperglycemia (high blood sugar not requiring treatment). Rare side effects (occurring in less than 1% of people) include: Hypoglycemia (low blood sugar) which may cause symptoms such as shakiness, nervousness, sweatiness, hunger, dizziness, or fast heart rate. If severe, hypoglycemia can cause coma, seizure, or even death.

Reproductive Risks

It is not known if the study drug can affect an unborn baby. Therefore, subjects should not become pregnant or father a baby while on this study. If subjects are physically able to father a baby, subjects must use an effective method of birth control while on this study. If subjects become aware that they or their sexual partner is pregnant during the course of their participation in this research study, subjects must contact, as soon as possible, the study investigator.

Risk of Collection and Storage of Private Health Information and Internet Usage

Although we are taking many steps to protect the participants' information, there is always a chance that their information or identity could be disclosed. We will continue to review and improve the ways we keep their information private. To protect their information, paper-based records will be kept in a secure location and only be accessible to personnel involved in the study. Paper charts will contain subject identifiers but will be in locked cabinets within a locked office on a unit that has restricted access.

## 5.3 ALTERNATIVE TREATMENTS

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

## 5.4 POTENTIAL BENEFITS

Participation in the proposed research may or may not provide a direct benefit to participants in this research. This research involves interventions and/or procedures that present greater than minimal risk to the involved adults, but which holds out the potential for direct individual benefit. The risk is justified by the extent of potential benefit to the involved adults, which includes possibly revealing previously-undiagnosed morbidities commonly seen in obesity such as hyperlipidemia, impaired fasting glucose, or type 2 diabetes mellitus. Information obtained from the proposed research will provide information about the relationship between metabolic syndrome and PH-HFpEF and patient outcome.

Based on the preceding assessment of risks and potential benefits, the risks to subjects are reasonable in relation to anticipated benefits. The research presents a balance of risks and expected direct benefits similar to that available in the clinical setting.

## 5.5 DATA SAFETY MONITORING PLAN

### 5.5.1 Data Safety Monitoring Board

A Data and Safety Monitoring Board (iDSMB) independent of the study investigators will monitor this clinical trial for additional measure of subject protection. The iDSMB consists of clinicians completely independent of the investigators who have no financial, scientific, or other conflict of interest with the trial.

The iDSMB will conduct interim monitoring of 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.

### 5.5.2 Data Safety Monitoring Plan

Assuring patient safety is an essential component of this protocol. The study Principal Investigator has primary responsibility for the oversight of the data and safety monitoring. The study investigators will evaluate all adverse events. All subjects who have AEs, whether considered associated with the use of the study medication or not, must be monitored to determine the outcome. The clinical course of the AE will be followed up according to accepted standards of medical practice, even after the end of the period of observation, until a satisfactory explanation is found or the Principal Investigator considers it medically justifiable to terminate follow-up.

All untoward medical occurrences observed in subjects receiving the study drug will be recorded on the participants' adverse event worksheets by the study coordinator under the supervision of the principal investigator. The worksheets will then be reviewed for completeness and internal consistency. In addition to internal safeguards built into a computerized system, external safeguards will be put in place to ensure that access to the computerized system and to the data is restricted to authorized personnel. Training conducted by qualified individuals on a continuing basis will be provided to individuals in the specific

operations with regard to computerized systems that they are to utilitze during the course of the study.

The Sponsor and Investigator will prepare a detailed written summary of serious, unexpected, and treatment-related adverse events, and will compare and contrast each event with prior events. The written summary will be provided to the DSMB and the IRB.

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

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

Stopping Rule

For safety reasons, we propose to temporarily suspend enrollment and review with our DSMB if any two subjects experience the following despite dose reduction:

1. A single occurrence of venous methemoglobin level >5%
   If subjects report change in skin tone and dizziness or other signs of methemoglobinemia for ≥24 hours, an interim visit will be scheduled (if not already being seen for a research visit) and the following steps will occur. The visit can be conducted in UPMC Presbyterian or the TRC.

   The physician investigator will factor relevant subject symptoms and hemodynamics and:

   - *Determine if unblinding is needed (if methemoglobin % >5) and,*
   - *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*
   - *Subject should be withdrawn*

   Interim visit: If methemoglobin is 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.

2. Blood pressure (BP) and related symptoms: If subjects experience lightheadedness or dizziness, we request that they call the study coordinator who will assess their symptoms, compare to baseline levels, and discuss need for interim visit with study physician. If a visit is indicated, BP, RR, heart rate, pulse co-oximetry and methemoglobin level via co-oximetry will be measured.
    - If systolic BP ≤ 90mm Hg, the study drug will be reduced from 40 TID to 20 TID. Subjects will be reassessed via telephone to ensure that the patient is stable on the new dose. If stable and asymptomatic, subjects will continue with the half dose.
    - If subjects are symptomatic with dizziness, lightheadedness for ≥ 24 hours, and require fluid replacement or other therapy for hypotension, subjects will be requested to return to clinic for an interim visit for assessment of vitals, methemoglobin percent, and evaluation by an investigator-physician to reevaluate the subject's suitability for continued participation based on symptoms.
    - If SBP remains ≤ 90mm Hg and symptomatic and at the lowest dosage of 20mg TID, subject will be withdrawn.

    If an unexpected fatal event or any of the above life-threatening events occur despite dose reduction, the study will be halted, until data review by investigators and the Data Safety Monitoring Board has rendered a final recommendation about study continuation.

Other potential reasons for stopping participation in study activities

1. Subject participation will be discontinued if a participant has a positive pregnancy test at any point during study participation.
2. In the event that the medication compliance rate is < 80%, subjects will be re-educated on medication compliance. If medication compliance falls outside of the acceptable range more than 2 times, the study investigators will discuss subject eligibility for continued participation in the study.

### 5.5.3 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 (eg. pertinent scientific literature reports or therapeutic development, results of related studies) that may have an impact on the safety and study participants or the ethics of the research study.

- A review of study procedures designed to protect the privacy of the research subjects and the confidentiality of their research data.

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

- Grade 1 (Mild): asymptomatic or mild symptoms; clinical or diagnostic observation only; intervention not indicated.

- Grade 2 (Moderate): minimal, local, or noninvasive intervention indicated; limiting age-appropriate ADL (Activities of Daily Living).

- Grade 3 (Severe): medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care ADL.

- Grade 4 (Life-threatening): consequences; urgent intervention indicated.

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

### 5.5.4 Frequency of Monitoring

The Investigator will review subject safety data as it is generated. The Investigator and the research staff will meet at approximately monthly intervals to re-evaluate study goals, subject recruitment, data coding and retention, documentation and identification of adverse events, complaints, and confidentiality of subjects. There will be an evaluation of the progress of the research study, including assessments of data quality, time lines, participant recruitment, accrual, and retention. The Investigator will also review the outcome and adverse event data to determine whether there is any change to the anticipated benefit-to-risk ratio of study participation and whether the study should continue as originally designed or should be re-evaluated and changed.

The DSMB will also be expected to meet as needed, but not less than every six months to provide an overall summary status report to the regulatory agencies.

### 5.5.5 Reportable Adverse Events

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

1. Fatal or immediately life threatening
2. Permanently disabling, or severely incapacitating
3. Requires or prolongs inpatient hospitalization
4. Important medical events that may not result in death, be life threatening, or require hospitalization may be considered serious adverse events when, based upon appropriate medical judgment, they may jeopardize the patient, or subject, and may require medical or surgical intervention to prevent one of the serious outcomes listed above.

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

### 5.5.6 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 DSMB and the IRB within 24 hours of discovery of the incident with subsequent follow-up submission of a detailed written report.

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

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

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

## 5.6 RISKS MANAGEMENT PROCEDURES

### 5.6.1 Protection Against Risks

General Risks of Study Protocol and Procedures

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

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

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

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

The Investigator will retain the data for the entire period of this study. The Investigator may continue to use and disclose subjects' de-identified information for the purpose of this study for a minimum of seven years after final reporting or publication of the study. If the subject

decides to withdraw or be withdrawn from study participation, they may request that the study data and samples be destroyed.

### 5.6.2 Protection Against Potential Risks of Experimental Intervention

The study will include involvement by trained staff / investigators with experience in the administration of the study drug. CPET will be conducted by experienced exercise physiologists along with a physician to continuously observe a 12 lead ECG monitor (GE Cardiosoft software) that will utilize electrodes and detection electronics which minimize motion artifact during the IET. A 3 lead ECG will be used during the CWR tests. If ECG changes develop or the participant becomes distressed the test will be stopped. Subjects' vital signs are monitored closely throughout the procedure.

Continuous monitoring by the Data and Safety Monitoring Board.

Required Education in the Protection of Human Research Participants

- The Investigator and all sub-investigators listed on University of Pittsburgh Institutional Review Board approved protocol are required to complete the CITI modules. This web-based tutorial is a requirement of the IRB for protocol submission.

## 6. STUDY ADMINSTRATION

### 6.1 QUALITY CONTROL AND QUALITY ASSURANCE

Independent monitoring of the clinical study for protocol and GCP compliance will be conducted periodically (i.e., at a minimum of annually) by qualified staff of the Education and Compliance Office – Human Subject Research, Research Conduct and Compliance Office, University of Pittsburgh.

The Investigator and Sponsor and the University of Pittsburgh and University of Pittsburgh Medical Center will permit direct access of the study monitors and appropriate regulatory authorities to the study data and to the corresponding source data and documents to verify the accuracy of this data.

### 6.2 DATA HANDLING AND RECORD-KEEPING

#### 6.2.1 Data recording

Study worksheets will be completed for each subject enrolled into the clinical study. The Investigator will review, sign and date completed worksheets; the Investigator's signature serving as attestation of the Investigator's responsibility for ensuring that all clinical and laboratory data are complete, accurate, and authentic.

Appropriate coded identifications (i.e. Subject ID number) will be used. Every effort will be made to collect complete data for each study visit. Causes of *missing data* will be fully documented. With respect to safety evaluation, it is not planned to impute missing data.

#### 6.2.2 Record maintenance and retention

The Sponsor and Investigator will maintain records in accordance with Good Clinical Practice guidelines, to include:

- FDA correspondence related to the IND and clinical protocol, including copies of submitted Safety Reports and Annual Reports
- IRB correspondence (including approval notifications) related to the clinical protocol; including copies of adverse event reports and annual or interim reports
- Current and past versions of the IRB-approved clinical protocol and corresponding IRB-approved consent form(s) and, if applicable, subject recruitment advertisements
- Signed FDA Form 1572 Statements of Investigator (i.e., for the Sponsor and all identified sub-investigators)
- Financial disclosure information (Investigator-sponsor and clinical protocol sub-investigators)
- Curriculum vitae (Sponsor and clinical protocol sub-investigators)
- Certificates of required training (e.g., human subject protections, Good Clinical Practice, etc.) for Sponsor and listed sub-investigators
- Listing of printed names/signatures of Investigator-sponsor and listed sub-investigators
- Normal value(s)/range(s) for medical/laboratory/technical procedures or tests included in the clinical protocol
- Laboratory certification information
- Instructions for on-site preparation and handling of the investigational drug(s), study treatment(s), and other study-related materials (i.e., if not addressed in the clinical protocol)
- Decoding procedures for blinded trials
- Master randomization list
- Signed informed consent forms
- Completed worksheets; signed and dated by Investigator
- Source Documents or certified copies of Source Documents
- Monitoring visit reports
- Copies of Sponsor communications to the Investigator and copies of Investigator communications to sub-investigators
- Subject screening and enrollment logs
- Subject identification code list
- Investigational drug accountability records, including documentation of drug disposal
- Retained biological specimen log
- Interim data analysis report(s)
- Final clinical study report

Subject-specific data and will be coded and the subject identification code list will be stored so as to protect the subjects' confidentiality. Subject names or other directly identifiable information will not appear on any reports, publications, or other disclosures of clinical study outcomes.

The Investigator and Sponsor and will retain the specified records and reports for up to 2 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 2 years after investigations under the IND have been discontinued and the FDA so notified.

## 6.3 ETHICS

Institutional Review Board (IRB) Approval

The Investigator will obtain, from the University of Pittsburgh Human Research Protection Office (HRPO), prospective approval of the clinical protocol and corresponding informed consent form(s), modifications to the clinical protocol and corresponding informed consent forms, and brochures (i.e., directed at potential research subjects and clinical faculty/staff) for study recruitment.

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

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

In the event that the University of Pittsburgh IRB requires, as a condition of approval, substantial changes to a clinical protocol submitted under an FDA-accepted IND application, or in the event of the Investigator's decision to modify the previously accepted clinical protocol the Investigator will submit (i.e., in advance of implementing the change) a Protocol Amendment to the IND describing any change to this Phase 2 clinical protocol that significantly affects the safety of the subjects. For changes that do not affect critical safety assessments, the revisions to the clinical protocol will be addressed in the Annual Report to the IND.

Ethical and scientific conduct of the clinical study
The clinical study will be conducted in accordance with the current IRB-approved clinical protocol; ICH Guidelines on GCP; and relevant policies, requirements, and regulations of the University of Pittsburgh HRPO, University of Pittsburgh and UPMC, Commonwealth of Pennsylvania, and applicable federal agencies.

The Investigator will make certain that appropriate processes and procedures are in place to ensure that ongoing questions and concerns of enrolled subjects are adequately addressed and that the subjects are informed of any new information that may affect their decision to continue participation in the clinical study. In the event of substantial changes to the clinical study or the risk-to-benefit ratio of study participation, the Investigator will obtain the informed consent of enrolled subjects for continued participation in the clinical study.

# 7. COSTS AND PAYMENTS

## 7.1 COSTS

Study drug and all costs associated with this research protocol will be supported by ongoing research grants.

## 7.2 PAYMENTS

Subjects may be paid a total of $1,400 on a 'WePay' debit card if all study procedures are completed. This is a breakdown of payments:

- Screening – patients will be compensated $200 if the screen fail following the screening RHC

- Baseline visits with screening RHC - $200
- Baseline visits with no screening RHC - $100

- Completion of Week 10 procedures - $400
  - Day 1 - $100
  - Day 2 - $200 - RHC
  - Day 3 - $100 – IVGTT

- Cross-over visit - $50

- Completion of end of treatment procedures - $750
  - Day 1 - $200
  - Day 2 - $450 - RHC
  - Day 3 - $100 - IVGTT

Subjects will receive the first payment following the completion of week 10 and will receive the final payment at the final visit for EOT. Subjects will also be provided a ticket for outpatient parking costs at all visits. In the event that a subject must be withdrawn due to a study related adverse event, they will be reimbursed for the individual study visits that were completed to date per the above reimbursement schedule. Travel and hotel may be provided in certain circumstances. Subjects may also receive meals or meal tickets for visits greater than 4 hours in length.

Subjects who require an interim visit will be compensated $25 and provided parking.

## 8. QUALIFICATIONS AND SOURCE OF SUPPORT

### 8.1 QUALIFICATIONS OF THE INVESTIGATORS

Sponsor:

**Mark Gladwin, M.D.,** is Professor of Medicine, University of Pittsburgh. Dr Gladwin is the Chair of the Department of Medicine and Director of the Heart, Lung, Blood, and Vascular Medicine Institute at the University of Pittsburgh. He is an internationally recognized authority in the field of sodium nitrite including both the basic science and a broad range of clinical applications in cardiovascular disease. He is a current IND holder for the investigation of sodium nitrite in lung transplant.

Investigator:

**Michael Risbano, MD, MA** is an Assistant Professor in the Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine. Dr. Risbano's clinical interests are primarily focused on the diagnosis, management, and treatment of pulmonary hypertension. He is an attending physician in the Comprehensive Pulmonary Hypertension Program. Dr. Risbano's past research has focused on the discovery and implementation of biomarkers for the early diagnosis of pulmonary hypertension in patients with scleroderma (SSc-PAH). He is interested in the hemodynamic evaluation of subjects with pulmonary hypertension and correlation of hemodynamic values with biomarker levels. Dr. Risbano also has a research interest in the

hemodynamic responses to vasoactive medications. He will be responsible for the overall accrual of patients who are seen at the UPMC Comprehensive Pulmonary Hypertension Program on a regular basis as well as for performance of the cardiac catheterizations, analysis and reporting of all data.

Sub-investigators:

**Marc A. Simon, M.D**., is an Adjunct Professor in the Heart Failure and Cardiac Transplantation Section at the University of Pittsburgh. Dr. Simon has been involved in many research projects. He will assist with confirming eligibility and interpreting cardiac catheterizations, and assist with analysis of thedata.

**Adil Yunis, MD, Vinaya Mulkareddy, MD,** and **Michael Bashline, MD** are cardiology fellows in the Heart and Vascular Institute workingin pulmonary hypertension research.

**Frederico Toledo, MD:** Dr. Toledo is an Associate Professor of Medicine. Dr. Toledo is board-certified in endocrinology and has been a clinical researcher for one and a half decades. He has performed more than 500 euglycemic clamps/IVGTTs as part of his studies and as a collaborator in others.

**Carl Koch, MD**, is a Clinical Instructor of Medicine in the Division of Pulmonary, Allergy and Critical Care division of the University of Pittsburgh. Since beginning his postdoctoral fellowship at the University of Pittsburgh, Dr. Koch has continued to foster his research into the role of nitric oxide and its metabolites in pulmonary and vascular physiology. He has further developed interest in the role of the microbiome in nitric oxide metabolism as it pertains to the development of pulmonary hypertension and cardiovascular disease. Dr. Koch will assist with with subject enrollment and implementation of the study.

**Jessica Huston, MD**, is an Assistant Professor, Advanced Heart Failure, Pulmonary Hypertension, and Cardiac Transplant In the Division of Cariology at the University of Pittsburgh. Dr. Huston will assist with subject enrollment and implementation of the study.

## 8.2 SOURCE OF SUPPORT

National Institute of Health- tPPG Grant- Mark Gladwin, MD

## REFERENCES

1. Porter, T.R., et al., *Endothelium-Dependent Pulmonary-Artery Responses in Chronic Heart-Failure - Influence of Pulmonary-Hypertension.* Journal of the American College of Cardiology, 1993. **22**(5): p. 1418-1424.
2. Cooper, C.J., et al., *The influence of basal nitric oxide activity on pulmonary vascular resistance in patients with congestive heart failure.* American Journal of Cardiology, 1998. **82**(5): p. 609-614.
3. Fernandez, M.L., et al., *Association of NOS3 gene with metabolic syndrome in hypertensive patients.* Thrombosis and Haemostasis, 2004. **92**(2): p. 413-418.

4. Giaid, A. and D. Saleh, *Reduced Expression of Endothelial Nitric-Oxide Synthase in the Lungs of Patients with Pulmonary-Hypertension.* New England Journal of Medicine, 1995. **333**(4): p. 214-221.
5. Duplain, H., et al., *Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase.* Circulation, 2001. **104**(3): p. 342-345.
6. Fagan, K.A., et al., *The pulmonary circulation of homozygous of heterozygous eNOS-null mice is hyperresponsive to mild hypoxia.* Journal of Clinical Investigation, 1999. **103**(2): p. 291-299.
7. Lundberg, J.O., E. Weitzberg, and M.T. Gladwin, *The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics.* Nature Reviews Drug Discovery, 2008. **7**(2): p. 156-167.
8. Sparacino-Watkins, C.E., Y.C. Lai, and M.T. Gladwin, *Nitrate-Nitrite-Nitric Oxide Pathway in Pulmonary Arterial Hypertension Therapeutics.* Circulation, 2012. **125**(23): p. 2824-2826.
9. Lundberg, J.O.N., et al., *Intragastric Nitric-Oxide Production in Humans - Measurements in Expelled Air.* Gut, 1994. **35**(11): p. 1543-1546.
10. Tiso, M., et al., *Human Neuroglobin Functions as a Redox-regulated Nitrite Reductase.* Journal of Biological Chemistry, 2011. **286**(20): p. 18277-18289.
11. Shiva, S., et al., *Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration.* Circulation Research, 2007. **100**(5): p. 654-661.
12. Sparacino-Watkins, C.E., et al., *Nitrite Reductase and Nitric-oxide Synthase Activity of the Mitochondrial Molybdopterin Enzymes mARC1 and mARC2.* Journal of Biological Chemistry, 2014. **289**(15): p. 10345-10358.
13. Cosby, K., et al., *Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation.* Nature Medicine, 2003. **9**(12): p. 1498-1505.
14. Hunter, C.J., et al., *Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator.* Nature Medicine, 2004. **10**(10): p. 1122-1127.
15. Zuckerbraun, B.S., et al., *Nitrite Potently Inhibits Hypoxic and Inflammatory Pulmonary Arterial Hypertension and Smooth Muscle Proliferation via Xanthine Oxidoreductase-Dependent Nitric Oxide Generation.* Circulation, 2010. **121**(1): p. 98-109.
16. Casey, D.B., et al., *Pulmonary vasodilator responses to sodium nitrite are mediated by an allopurinol-sensitive mechanism in the rat.* American Journal of Physiology-Heart and Circulatory Physiology, 2009. **296**(2): p. H524-H533.
17. Baliga, R.S., et al., *Dietary Nitrate Ameliorates Pulmonary Hypertension Cytoprotective Role for Endothelial Nitric Oxide Synthase and Xanthine Oxidoreductase.* Circulation, 2012. **125**(23): p. 2922-2932.
18. Carlstrom, M., et al., *Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice.* Proc. **107 SRC - GoogleScholar**: p. 17716-20.
19. Larsen, F.J., et al., *Effects of dietary nitrate on oxygen cost during exercise.* Acta Physiologica, 2007. **191**(1): p. 59-66.
20. Larsen, F.J., et al., *Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise.* Free radical biology medicine, 2010. **48 SRC - GoogleScholar**: p. 342-7.
21. Larsen, F.J., et al., *Dietary Inorganic Nitrate Improves Mitochondrial Efficiency in Humans.* Cell Metabolism, 2011. **13**(2): p. 149-159.
22. Totzeck, M., U.B. Hendgen-Cotta, and C. Rammos, *Higher endogenous nitrite levels are associated with superior exercise capacity in highly trained athletes.* Nitric oxide biology and chemistry official journal of the Nitric Oxide Society, 2012. **27 SRC - GoogleScholar**: p. 75-81.
23. Berry, M.J., N.W. Justus, and J.I. Hauser, *Dietary nitrate supplementation improves exercise performance and decreases blood pressure in COPD patients.* Nitric oxide biology and chemistry official journal of the Nitric Oxide Society, 2014.

24. Bond, H., L. Morton, and A.J. Braakhuis, *Dietary nitrate supplementation improves rowing performance in welltrained rowers.* International journal of sport nutrition and exercise metabolism, 2012. **22 SRC - GoogleScholar**: p. 251-6.
25. Camm, A.J. and M.B. Maltz, *A controlled single-dose study of the efficacy, dose response and duration of action of nicorandil in angina pectoris.* The American journal of cardiology, 1989. **63**: p. 61J-5J.
26. Hoon, M.W., et al., *The effect of nitrate supplementation on exercise performance in healthy individuals: a systematic review and meta-analysis.* International journal of sport nutrition and exercise metabolism, 2013. **23 SRC - GoogleScholar**: p. 522-32.
27. Lane, S.C., J.A. Hawley, and B. Desbrow, *Single and combined effects of beetroot juice and caffeine supplementation on cycling time trial performance.* Applied physiology nutrition and metabolism Physiologie appliquee nutrition et metabolisme, 2014. **39 SRC - GoogleScholar**: p. 1050-7.
28. Lansley, K.E., P.G. Winyard, and S.J. Bailey, *Acute dietary nitrate supplementation improves cycling time trial performance.* Medicine and science in sports and exercise, 2011. **43 SRC - GoogleScholar**: p. 1125-31.
29. Muggeridge, D.J., et al., *The effects of a single dose of concentrated beetroot juice on performance in trained flatwater kayakers.* International journal of sport nutrition and exercise metabolism, 2013. **23 SRC - GoogleScholar**: p. 498-506.
30. Muggeridge, D.J., et al., *A single dose of beetroot juice enhances cycling performance in simulated altitude.* Medicine and science in sports and exercise, 2014. **46**: p. 143-50.
31. Murphy, M., et al., *Whole beetroot consumption acutely improves running performance.* Journal of the Academy of Nutrition and Dietetics, 2012. **112 SRC - GoogleScholar**: p. 548-52.
32. Peeling, P., et al., *Beetroot Juice Improves on-Water 500 m Time-Trial Performance, and Laboratory-Based Paddling Economy in National and International-Level Kayak Athletes.* International journal of sport nutrition and exercise metabolism, 2014.
33. Vanhatalo, A., et al., *Dietary nitrate reduces muscle metabolic perturbation and improves exercise tolerance in hypoxia.* The Journal of physiology 589551728, 2011. **589 SRC - GoogleScholar**: p. 5517-28.
34. Kerley, C.P., K. Cahill, and K. Bolger, *Dietary nitrate supplementation in COPD: an acute, double-blind, randomized, placebo-controlled, crossover trial.* Nitric oxide biology and chemistry official journal of the Nitric Oxide Society, 2015. **44 SRC - GoogleScholar**: p. 105-11.
35. Zamani, P., D. Rawat, and P. Shiva-Kumar, *Effect of inorganic nitrate on exercise capacity in heart failure with preserved ejection fraction.* Circulation discussion 80, 2015. **131 SRC - GoogleScholar**: p. 371-80.
36. Vanderpool, R. and M.T. Gladwin, *Harnessing the nitrate-nitrite-nitric oxide pathway for therapy of heart failure with preserved ejection fraction.* Circulation, 2015. **131 SRC - GoogleScholar**: p. 334-6.
37. Hardie, D.G., F.A. Ross, and S.A. Hawley, *AMPK: a nutrient and energy sensor that maintains energy homeostasis.* Nat Rev Mol Cell Biol. **13**: p. 251-62.
38. Bandyopadhyay, G.K., et al., *Increased malonyl-CoA levels in muscle from obese and type 2 diabetic subjects lead to decreased fatty acid oxidation and increased lipogenesis; thiazolidinedione treatment reverses these defects.* Diabetes, 2006. **55 SRC - GoogleScholar**: p. 2277-85.
39. Sriwijitkamol, A., D.K. Coletta, and E. Wajcberg, *Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes: a time-course and dose-response study.* Diabetes, 2007. **56 SRC - GoogleScholar**: p. 836-48.

40. Yu, X., S. McCorkle, and M. Wang, *Leptinomimetic effects of the AMP kinase activator AICAR in leptinresistant rats: prevention of diabetes and ectopic lipid deposition.* Diabetologia, 2004. **47**: p. 2012-21.
41. Viollet, B., S. Horman, and J. Leclerc, *AMPK inhibition in health and disease.* Crit Rev Biochem Mol Biol. **45**: p. 276-95.
42. Higaki, Y., et al., *Nitric oxide increases glucose uptake through a mechanism that is distinct from the insulin and contraction pathways in rat skeletal muscle.* Diabetes, 2001. **50**: p. 241-7.
43. Lira, V.A., et al., *Nitric oxide increases GLUT4 expression and regulates AMPK signaling in skeletal muscle.* American journal of physiology Endocrinology and metabolism, 2007. **293 SRC - GoogleScholar**: p. E1062-8.
44. Mo, L., Y. Wang, and L. Geary, *Nitrite activates AMP kinase to stimulate mitochondrial biogenesis independent of soluble guanylate cyclase.* Free radical biology medicine PMC3477807, 2012. **53 SRC - GoogleScholar**: p. 1440-50.
45. Hawley, S.A., et al., *Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase.* Chem, 1996. **271 SRC - GoogleScholar**: p. 27879-87.
46. Hawley, S.A., D.A. Pan, and K.J. Mustard, *Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase.* Cell metabolism, 2005. **2**: p. 9-19.
47. Momcilovic, M., et al., *Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro.* Chem, 2006. **281 SRC - GoogleScholar**: p. 25336-43.
48. Palacios, O.M., J.J. Carmona, and S. Michan, *Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1alpha in skeletal muscle.* Aging, 2009. **1**: p. 771-83.
49. Jing, E., et al., *Sirtuin-3 (Sirt3) regulates skeletal muscle metabolism and insulin signaling via altered mitochondrial oxidation and reactive oxygen species production.* Proc Natl Acad, 2011. **108 SRC - GoogleScholar**: p. 14608-13.
50. Fu, J., et al., *trans-(-)-epsilon-Viniferin increases mitochondrial sirtuin 3 (SIRT3), activates AMP-activated protein kinase (AMPK), and protects cells in models of Huntington Disease.* Chem, 2012. **287 SRC - GoogleScholar**: p. 24460-72.
51. Rix, P.J., A. Vick, and N.J. Attkins, *Pharmacokinetics, pharmacodynamics, safety, and tolerability of nebulized sodium nitrite (AIR001) following repeat-dose inhalation in healthy subjects.* Clinical pharmacokinetics PMC4335132, 2015. **54 SRC - GoogleScholar**: p. 261-72.
52. Simon, M.A., et al., *Efficacy and safety of inhaled sodium nitrite in pulmonary hypertension associated with heart failure with preserved ejection fraction.* American Thoracic Society. **2016 SRC - GoogleScholar**: p. A3956-A3956.
53. Koch, C.D., et al., *Efficacy And Safety Of Nitrite In Patients With Group 3 Pulmonary Hypertension.* American Thoracic Society, 2014.
54. Simon, M.A., et al., *Acute hemodynamic effects of inhaled sodium nitrite in pulmonary hypertension associated with heart failure with preserved ejection fraction.* e89620 doi101172jciinsight89620, 2016. **1**(18 SRC - GoogleScholar).
55. Lai, Y.C., et al., *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. **133**(8): p. 717-731.
56. Delmastro-Greenwood, M., et al., *Nitrite and nitrate-dependent generation of anti-inflammatory fatty acid nitroalkenes.* Free Radical Biology and Medicine, 2015. **89**: p. 333-341.
57. Hunault, C.C., et al., *Bioavailability of sodium nitrite from an aqueous solution in healthy adults.* Toxicology letters, 2009. **190**: p. 48-53.
58. Rich, S. and M. Rabinovitch, *Diagnosis and treatment of secondary (non-category 1) pulmonary hypertension.* Circulation, 2008. **118 SRC - GoogleScholar**: p. 2190-9.

59. Lai, Y.C., et al., *Pulmonary arterial hypertension: the clinical syndrome.* Circ Res PMC4096686, 2014. **115 SRC - GoogleScholar**: p. 115-30.
60. Thenappan, T., S.J. Shah, and M. Gomberg-Maitland, *Clinical characteristics of pulmonary hypertension in patients with heart failure and preserved ejection fraction.* Circulation Heart failure, 2011. **4 SRC - GoogleScholar**: p. 257-65.
61. Guazzi, M., *Pulmonary hypertension in heart failure preserved ejection fraction: prevalence, pathophysiology, and clinical perspectives.* Circulation Heart failure, 2014. **7 SRC - GoogleScholar**: p. 367-77.
62. Pugh, M.E., et al., *Unrecognized glucose intolerance is common in pulmonary arterial hypertension.* Lung Transplant. **30 SRC - GoogleScholar**: p. 904-11.
63. Robbins, I.M., J.H. Newman, and R.F. Johnson, *Association of the metabolic syndrome with pulmonary venous hypertension.* Chest, 2009. **136 SRC - GoogleScholar**: p. 31-6.
64. Shapiro, B.P., M.D. McGoon, and M.M. Redfield, *Unexplained pulmonary hypertension in elderly patients.* Chest, 2007. **131**: p. 94-100.
65. Hansmann, G., R.A. Wagner, and S. Schellong, *Pulmonary arterial hypertension is linked to insulin resistance and reversed by peroxisome proliferator-activated receptor-gamma activation.* Circulation, 2007. **115**: p. 1275-84.
66. Bueno, M., et al., *Nitrite signaling in pulmonary hypertension: mechanisms of bioactivation, signaling, and therapeutics.* Antioxidants redox signaling PMC366, 1920. **18 SRC - GoogleScholar**: p. 1797-809.
67. Nisbet, R.E., J.M. Bland, and D.J. Kleinhenz, *Rosiglitazone attenuates chronic hypoxia-induced pulmonary hypertension in a mouse model.* American journal of respiratory cell and molecular biology. **42 SRC - GoogleScholar**: p. 482-90.
68. Nisbet, R.E., A.S. Graves, and D.J. Kleinhenz, *The role of NADPH oxidase in chronic intermittent hypoxiainduced pulmonary hypertension in mice.* American journal of respiratory cell and molecular biology, 2009. **40**: p. 601-9.
69. Wasserman, K., *Principles of exercise testing and interpretation*. 2nd ed. 1994, Philadelphia: Lea & Febiger. xvii, 479 p.
70. O'Donnell, D.E., M. Lam, and K.A. Webb, *Measurement of symptoms, lung hyperinflation, and endurance during exercise in chronic obstructive pulmonary disease.* American Journal of Respiratory and Critical Care Medicine, 1998. **158**(5): p. 1557-1565.
71. Oga, T., et al., *The effects of oxitropium bromide on exercise performance in patients with stable chronic obstructive pulmonary disease - A comparison of three different exercise tests.* American Journal of Respiratory and Critical Care Medicine, 2000. **161**(6): p. 1897-1901.
72. Meredith, T.H., et al., *C, eds. Antidotes for Poisoning by Cyanide, Vol 2.* In International Program on Chemical SafetyCommission of the European Communities Evaluations of Antidotes Series Geneva Switzerland World Health Organization and Commission of the European Communities 14280 EN, 1993.
73. Yen, D., et al., *The clinical experience of acute cyanide poisoning.* Am Med, 1995. **13 SRC - GoogleScholar**: p. 524-528.