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CLINICAL TRIAL PROTOCOL

Targeting Pulsatile Load to Increase Exercise Capacity and Quality of Life After Aortic Valve Replacement for Severe Aortic Stenosis (PULSE AS)

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Table of Contents

Table of Contents 2

1. SPECIFIC AIMS 4

2. TRIAL SUMMARY TABLE (OBJECTIVES AND ENDPOINTS) 6

3. BACKGROUND AND SIGNIFICANCE 7

4. STUDY DESIGN AND POPULATION 8

4.1. Overview of study design 8

4.2 Study Sites 8

4.3. Study Population 8

4.3.1. Inclusion Criteria 8

4.3.2. Exclusion Criteria 9

4.3.3. Criteria that will prompt exclusion from the trial at the phase 1 visit 9

4.4. Randomized intervention 10

5. STUDY VISITS AND PROCEDURES 10

5.1. Initial Visit 11

5.2. Randomization procedure 13

5.3. Intervention Phase 1 13

5.4. Washout period 14

5.5. Intervention Phase 2 14

5.6. Adverse Event Reporting 16

5.7. Subject withdrawal / Early termination 16

5.8. Concomitant Medication 16

6. DRUG DISPENSING, ACCOUNTABILITY AND DESTRUCTION 16

6.1. Rules for un-blinding 17

7. ADVERSE EVENTS 17

7.1. Key definitions 17

7.2. Classification of AE/ADEs regarding causal relationship to study interventions and severity 18

7.3. Recording and Reporting of Adverse Events 19

7.4. Follow-up 20

7.5. Management of Suspected Unexpected Serious Adverse Reaction 20

7.6. Pregnancy 20

8. STUDY MEASUREMENTS AND DATA COLLECTION 21

8.1. Assessment of exercise capacity 21

8.2. Quality of life 21

8.3. Doppler Echocardiography 21

8.4. Measurement of late systolic load and arterial wave reflections: 22

8.5. Blood nitrate/nitrite level measurements 22

8.6. Flow-mediated brachial artery dilation 23

8.7. Other assessments 23

9. STUDY CENTER MANAGEMENT PLAN 23

9.1. Principal Investigator **23**
9.2. Investigators Committee **24**
9.3. Operational Affairs Committee **24**
9.4. Data Management **24**
9.5. Quantification Core Laboratories **25**
10. STATISTICAL CONSIDERATIONS **26**
10.1. Power calculations **26**
10.2. Data Analysis Plan **26**
11. PROTECTION OF HUMAN SUBJECTS **28**
11.1. Potential benefits of the proposed research to the study subjects and importance of the knowledge to be gained, and risk/benefit ratio **28**
11.2. Risks to study subjects **28**
11.2.1. Potential Risks of study intervention (potassium nitrate) 29
11.2.2. Potential Risks of study procedures 30
11.3. Adequacy of Protection Against Risks **31**
11.3.1. Recruitment and Informed Consent 31
11.3.2. Protection against risks associated with cardiopulmonary exercise tests 32
11.3.3. Protection against risks of potassium nitrate administration: 32
11.3.4. Measures to minimize the risk of breach in confidentiality: 33
11.3.6 Other measures to minimize risk: 33
12. REGULATORY STANDARDS **33**
12.1. Informed consent **33**
12.2. Institutional Review Board (IRB) **33**
12.3. Source document handling and archiving **34**
13. DATA AND SAFETY MONITORING PLAN: **34**
14. SUBJECT STIPEND **34**
15. REFERENCES **35**
APPENDIX 1. SCHEDULE OF EVENTS **43**

1. SPECIFIC AIMS

Moderate or severe aortic stenosis (AS) affects 2.8% of people aged ≥ 75 years and is one of the most common valvular disorders worldwide[1]. Without intervention, severe AS carries an extremely poor prognosis with a 1 and 5 year survival of ~60% and ~30%, respectively[2,3]. Surgical and transcatheter aortic valve replacement (AVR) are to-date the only interventions that have been shown to improve morbidity and mortality in this population[4,5]. However, there is heterogeneity in clinical course after AVR; even after a successful procedure, some patients show minimal symptomatic or hemodynamic improvement[6].

Symptoms in aortic stenosis result from an inability of the left ventricle (LV) to overcome the systolic load caused by a fixed outflow obstruction. After correction of the stenotic valve, residual LV afterload results primarily from arterial load and is associated with poor outcomes. Indeed, higher pulsatile arterial load after AVR is significantly associated with mortality[7]. An important component of pulsatile load is the magnitude of wave reflections in the arterial tree[8,9]. Wave reflections arise in peripheral arterial sites and travel back to the heart, arriving while the LV is still ejecting blood, increasing the late systolic workload of the LV. Wave reflections are highly sensitive to nitric oxide (NO).

The increased LV afterload during systole results in myocyte hypertrophy and increased wall thickness that is accompanied by a diffuse interstitial fibrosis in the myocardium[10]. In fact, the amount of myocardial fibrosis, as determined by either histology or cardiac MRI, is an independent predictor of LV function improvement and late all-cause mortality after aortic valve replacement [6]. However, myocardial fibrosis does not appear to regress significantly after aortic valve replacement[11,12]. This suggests that arterial factors play a role in the pathogenesis and maintenance of fibrosis even after AVR. This additionally indicates that persistent myocardial fibrosis may contribute to poor quality of life after AVR. Thus, reducing fibrosis, by reducing wave reflections and arterial load, may be an effective strategy towards improving quality of life and exercise capacity in patients after aortic valve replacement for severe aortic stenosis.

Prior data demonstrates that a single oral dose of inorganic nitrate reduces late systolic load in other populations. Dietary nitrate increases plasma nitrite, an endothelium-independent source of the potent vasodilator nitric oxide (NO). Thus, augmentation of the nitrate-nitrite-NO pathway has the potential for chronic “disease-modifying” benefits in aortic stenosis. Therefore, nitrate supplementation represents a logical intervention to improve exercise capacity and quality of life in aortic stenosis.

The current trial is designed to assess the safety and efficacy of sustained oral administration of inorganic nitrate in patients with severe aortic stenosis and to assess the mechanisms by which inorganic nitrate enhances oxygen uptake and exercise capacity in this population. This is a randomized double-blind crossover clinical trial, in which 22 subjects who underwent surgical or transcatheter AVR for severe AS three or more months prior to enrollment will receive the following 2 interventions, in randomized order: (1) Potassium nitrate (KNO_3), at a dose of 12-18 mmol/d by mouth for 4 ± 1 weeks, or; (2) Potassium chloride (KCl), at a dose of 12-18 mmol/d by mouth for 4 ± 1 weeks. A 1-week ± 3 day washout period will be introduced between the 2 interventions. The purpose of the trial is to test the safety of KNO_3 and its efficacy on a number of clinical and physiologic endpoints in subjects who underwent AVR for severe AS.

Our specific aims are:

Specific Aim 1: To assess whether KNO_3 therapy improves endpoints with direct clinical relevance: aerobic capacity and quality of life (QOL)

> Hypothesis 1a: KNO_3 will improve exercise capacity, quantified as: (1) Total work performed during a maximal-effort exercise test; (2) Peak oxygen consumption (VO_2) during a symptom-limited maximal effort exercise test. These will be the co-primary endpoints of the trial.
>
> Hypothesis 1b: KNO_3 will improve QOL, assessed using the Kansas City Cardiomyopathy Questionnaire.

Specific Aim 2: To dissect the effects of KNO_3 on specific physiologic adaptations to exercise

> Hypothesis 2a: KNO_3 improves the systemic vasodilator response to exercise, measured as the change in systemic vascular resistance during a symptom-limited maximal effort exercise test.
>
> Hypotheses 2b**:** Since nitrates may exert venodilating effects and/or myocardial effects, we will test the hypotheses that KNO_3 impacts LV diastolic filling parameters (measured with echocardiography at rest and after peak exercise) and improves myocardial strain (assessed with speckle-tracking echocardiography).

Specific Aim 3: To assess whether KNO_3 reduces late systolic LV load from wave reflections (assessed via comprehensive aortic pressure-flow relations, using arterial tonometry and Doppler echocardiography).

This trial has the potential to establish a novel, inexpensive, and readily implementable therapeutic paradigm for post-AVR patients and to characterize specific mechanisms involved, enhancing our understanding of this disease.

2. TRIAL SUMMARY TABLE (OBJECTIVES AND ENDPOINTS)

Trial Title / Acronym	Targeting **Pu**lsatile **L**oad to Increa**s**e **E**xercise Capacity and Quality of Life After Aortic Valve Repair for Severe **A**ortic **S**tenosis (PULSE AS)
Phase	Phase IIb Randomized Clinical Trial (RCT)
Number of Subjects	22
Patient Population	Ages 50-90; 3 or more months after AVR for severe AS
Study Site	University of Pennsylvania
Study Design	Randomized, double-blinded crossover study
Randomized Intervention	Potassium Nitrate (KNO_3) capsules, at a dose of 6 mEq (1 capsule) three times daily for 4 ± 1 weeks, versus, potassium chloride (KCl) capsules administered at a dose of 6 mEq (1 capsule) three times daily for 4 ± 1 weeks
Primary Aim	To assess whether KNO_3 therapy improves exercise capacity, quantified as: (1) Total work performed during a maximal-effort exercise test; and (2) Peak oxygen consumption (VO_2) during a symptom-limited maximal effort exercise test. These will be the co-primary endpoints of the trial.
Secondary Aims	To assess whether KNO_3: • improves quality of life • improves the systemic vasodilator response to exercise • improves LV diastolic function and myocardial systolic strain • reduces late systolic LV load from wave reflections
Exploratory Aims	To assess whether KNO_3 improves: • Ventilatory threshold, VO_2 kinetics • Natriuretic peptide levels • Physical activity To assess the relationship between nitrate and nitrite levels achieved and the therapeutic response achieved in each subject (e.g., improvement in endpoints in response to KNO_3 vs control). To assess the effect on RNA expression.
Primary Endpoints	Peak oxygen uptake (VO_2) during a symptom-limited maximal effort exercise test after 4 ± 1 weeks of KNO_3 vs KCl Quality of life score, assessed using the Kansas City Cardiomyopathy Questionnaire
Secondary Endpoints	Measures of LV diastolic function (E/e', left atrial volume index),and peak global systolic myocardial longitudinal and circumferential strain Augmentation index, late systolic wall stress and aortic input impedance

3. BACKGROUND AND SIGNIFICANCE

Calcific aortic stenosis (AS) occurs in 3-5% of people in the United States aged ≥ 75 years[13]. Without intervention, severe AS carries an extremely poor prognosis with a 1 and 5 year survival of ~60% and ~30%, respectively[2,3]. Surgical and transcatheter aortic valve replacement (AVR) are currently the only interventions that have been shown to improve morbidity and mortality in this population[4,5]. Over the last decade, TAVR has been shown to result in a dramatic improvement of survival and quality of life for many patients who were deemed to be inoperable candidates for SAVR[4,5]. However, there is heterogeneity in clinical course after AVR; even after a successful procedure, some patients show minimal symptomatic or hemodynamic improvement[6]. Among the extreme risk cohort of patients in the CoreValve US Pivotal trial, 39% of patients had a poor outcome 6 months after TAVR with 22% dying, 6% having poor quality of life by KCCQ and 1.4% with with a decline in their quality of life after the procedure[14]. This was redemonstrated in reports from the PARTNER trial, in which 35% of patients had a poor outcome at 6 months post-procedure, with 19% subsequently dying and 16% with poor quality of life[15]. This suggests an unmet need to identify and intervene upon physiologic abnormalities in patients who do not benefit from TAVR.

There are a number of factors that determine a patient's functional health status after AVR. Given the relative advanced age of patients who undergo TAVR, it should come as no surprise that presence of co-morbidities and overall frailty, in addition to age and gender, are critical. An analysis of patients from the PARTNER trial identified a number of significant pre-procedure risk factors that were predictive of poor outcomes after TAVR. Poor functional capacity, measured by Six-Minute Walk Test (6MWT) and lower mean aortic valve radients were the most predictive; other predictors were renal dysfunction, oxygen-dependent lung disease, and poor baseline cognitive function[16]. There are also clear cardiac and systemic hemodynamic characteristics that are associated with poor outcomes after AVR. Low-gradient AS and tricuspid valve regurgitation were shown to be associated with all-cause 6 month post-procedure mortality; severe mitral regurgitation and moderate/severe postprocedural aortic regurgitation appear to be independent predictors of poor response to treatment[17]. Higher pulsatile arterial load after aortic valve repair is significantly associated with mortality[7].

Symptoms in aortic stenosis result from an inability of the left ventricle to overcome the systolic load caused by a fixed outflow obstruction. Left ventricle (LV) afterload in patients with severe AS can be conceptualized as a series circuit with additive contributions by both the aortic valve and systemic arterial tree[8,9,18]. After correction of the stenotic valve, residual LV afterload results primarily from arterial load and is associated with poor outcomes. Indeed, higher pulsatile arterial load after AVR is significantly associated with mortality[7]. An important component of pulsatile load is the magnitude of wave reflections in the arterial tree[8,9]. Wave reflections arise in peripheral arterial sites and travel back to the heart, arriving while the LV is still ejecting blood, increasing the late systolic workload of the LV. Available evidence causally link late systolic load with LV remodeling, diastolic dysfunction, and HF risk. Wave reflections are highly sensitive to NO.

The increased LV afterload during systole results in myocyte hypertrophy and increased wall

thickness, which allows the ejection fraction to be maintained by decreasing LV wall stress[19]. This myocyte growth is accompanied by a diffuse interstitial fibrosis in the myocardium, as fibroblasts increase collagen synthesis in response to the elevated intraventricular pressures[10]. In fact, the amount of myocardial fibrosis, as determined by either histology or cardiac MRI, is an independent predictor of LV function improvement and late all-cause mortality after aortic valve replacement[6]. However, myocardial fibrosis does not appear to regress significantly after aortic valve replacement[11,12]. This suggests that nonvalvular factors may play a role in the pathogenesis and maintenance of fibrosis even after AVR.

Our trial will test a novel intervention (inorganic nitrate) to modify key physiologic abnormalities (arterial vasodilator reserve and late systolic LV load from arterial wave reflections), which have the potential for both immediate-term improvements in exercise tolerance and long-term "disease-modifying" effects. The trial is designed to address the safety and potential clinical benefit of this approach in severe aortic stenosis using endpoints with direct clinical relevance, and also to characterize the specific physiologic mechanisms involved. This will potentially identify a new therapeutic paradigm and a readily implementable therapeutic intervention.

4. STUDY DESIGN AND POPULATION

4.1. Overview of study design

In this phase IIb, double-blind, cross-over trial, we will assign 22 subjects who are post-AVR for severe AS to: (A) Potassium nitrate administered by mouth at a dose of 6 mEq three times daily for 4 ± 1 weeks, or (B) Potassium chloride (KCl) at identical doses. The order of the interventions (AB-BA design) will be randomized, with a 1-week ± 3 day washout period separating each intervention. A crossover design will enable each subject to receive both treatments, reducing inter-individual variability and maximizing our power to detect effects of potassium nitrate vs. control (KCl). The active drug (KNO_3-) and control (KCl) will be prepared at the Investigational Drug Pharmacy at the University of Pennsylvania and dispensed by an investigational drug pharmacist, blinded to both the subjects and the investigators.

4.2 Study Sites

The site for this trial will be the Hospital of the University of Pennsylvania.

4.3. Study Population

Subjects will be identified from clinic visits at the University of Pennsylvania for post-TAVR follow up. We will enroll 22 subjects meeting the following criteria:

4.3.1. Inclusion Criteria

1. Adults aged 50-90 years of age
2. Diagnosis of severe aortic stenosis prior to aortic valve repair
3. Successful trancatheter aortic valve repair via transfemoral procedural approach or successful surgical aortic valve repair at least three months prior to enrollment

4. Stable medical therapy: no addition/removal/changes in antihypertensive medications, or beta-blockers in the preceding 30 days.

4.3.2. Exclusion Criteria

1. Supine systolic blood pressure (SBP) < 100 mmHg OR supine diastolic blood pressure (DBP) <60 mmHg
2. Poorly controlled hypertension, as defined as SBP > 160 mmHg OR DBP > 100 mmHg
3. Pregnancy. Women of childbearing potential will undergo a pregnancy test during the screening visit
4. Atrial fibrillation within the prior 8 weeks before enrollment
5. Inability/unwillingness to exercise
6. Moderate or greater mitral regurgitation or aortic/perivalvular regurgitation, any degree of mitral stenosis, severe right-sided valvular disease, or presence of a mitral prosthetic valve.
7. Moderate or severe patient prosthesis mismatch, as defined by Effective Orifice Area Index < 0.85 cm^2/m^2
8. Hypertrophic, infiltrative, or inflammatory cardiomyopathy
9. Pericardial disease
10. Current angina
11. Acute coronary syndrome or coronary intervention within the past 2 months
12. Primary pulmonary arteriopathy
13. Clinically significant lung disease as defined by: Chronic Obstructive pulmonary disease meeting Stage III or greater GOLD criteria, treatment with oral steroids within the past 6 months for an exacerbation of obstructive lung disease, or the use of daytime supplemental oxygen
14. Ischemia on stress testing without subsequent revascularization (during the screening visit)
15. Treatment with phosphodiesterase inhibitors that cannot be withheld
16. Treatment with organic nitrates
17. Significant liver disease impacting synthetic function or volume control (ALT/AST > 3x ULN, Albumin <3.0 g/dL)
18. eGFR < 30 mL/min/1.73 m^2
19. G6PD deficiency. For males of African, Asian or Mediterranean decent, this will be evaluated prior to drug administration. A qualitative test positive for deficiency or a quantitative test with clinically significant G6PD deficiency (<60% of normal activity) will prompt exclusion from the trial (prior to drug administration).
20. History of methemoglobinemia or methemoglobin level >5% at baseline visit
21. Serum K>5.0 mEq/L
22. Severe right ventricular dysfunction.
23. Any medical condition that, in the opinion of the investigator, will interfere with the safe completion of the study.

4.3.3. Criteria that will prompt exclusion from the trial at the phase 1 visit

1. Acute coronary syndrome or coronary intervention for unstable coronary disease after enrollment
2. New onset ischemia on stress testing since the time of enrollment
3. New treatment with organic nitrates or phosphodiesterase inhibitors that cannot be withheld.

4. Serum K>5.5 mEq/L.
5. Any medical condition that, in the opinion of the investigator, will interfere with the safe completion of the study, or the validity of endpoint assessments. These ad-hoc discontinuations will be discussed with the PI of the trial and the medical director of the IND.

In the following situations, subjects who meet the criteria below will not be immediately discontinued, but will be scheduled for an ad hoc visit for reassessment of values prior to drug initiation in phase 2:

1. Supine systolic blood pressure < 90 mm Hg
2. Acute kidney injury with eGFR < 30 mL/min/1.73m2
3. Methemoglobinemia – baseline methemoglobin level > 5%

If these criteria persist during the ad-hoc visit, the subject will be discontinued from the study.

4.4. Randomized intervention

The randomized interventions will be the following (in random order, separated by a 1-week ± 3 day washout period)

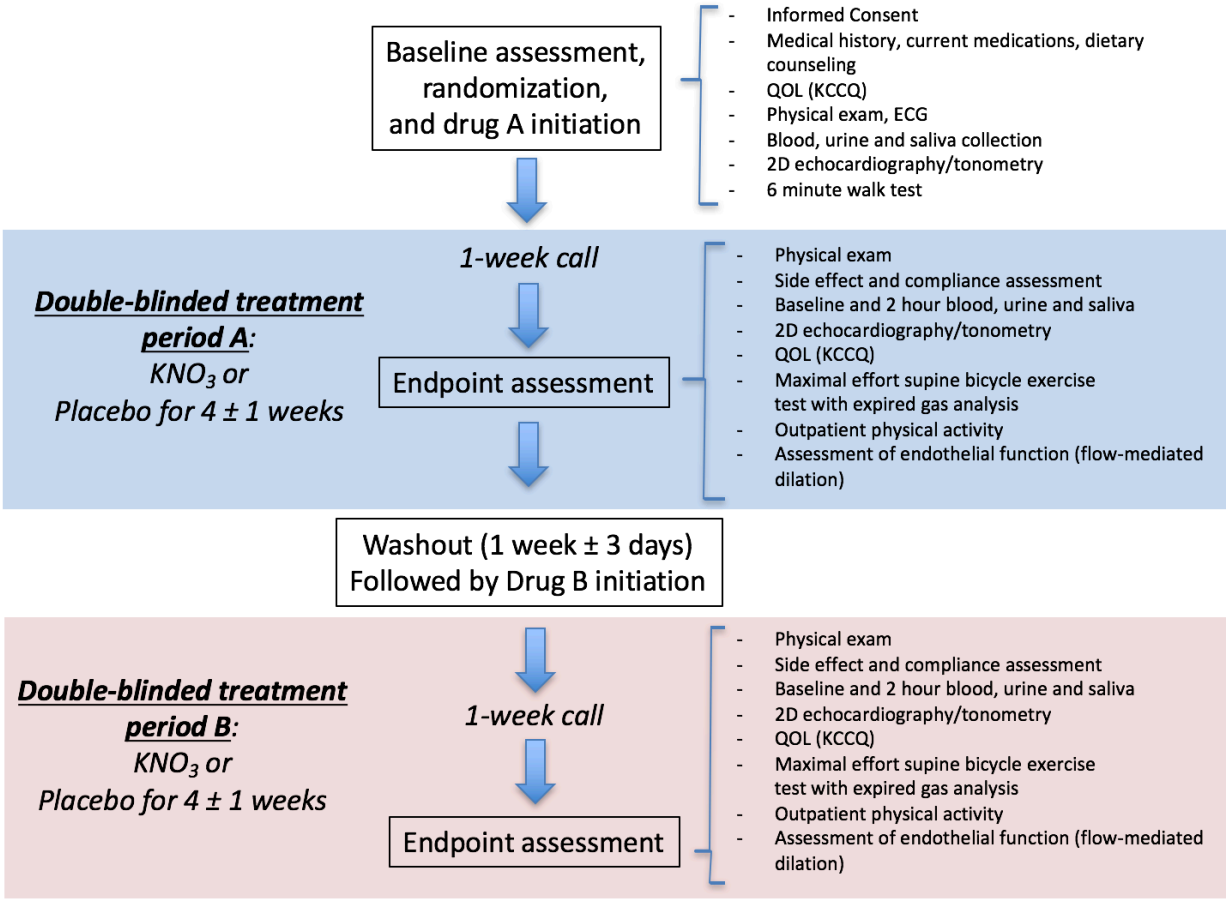
1. Potassium nitrate: Interventional drug capsules will consist of potassium nitrate (KNO_3-) crystals [610 mg, corresponding to 6.03 mmoles of NO3-] with 190mg of lactose monohydrate, spray dried, NF. The dose for this trial will be 18 mmoles of NO_3-per day, given as one capsule (6 mmoles) three times a day.

2. Control: Capsules will consist of potassium chloride (KCl), granular, USP (450mg) plus lactose monohydrate, spray dried, NF (300mg). We chose potassium chloride as the control in order to isolate the effects of nitrate from any potential effect of potassium supplementation. The dose for this trial will be 18 mmoles of KCl per day, given as one capsule (6 mmoles) three times a day.

5. STUDY VISITS AND PROCEDURES

An overview of the study design and flow of study visits is presented in Figure 3.

Figure 3. Overview of the study design and procedures.



The following investigational devices will be used during the study:

Portalite: continuous wave near infrared spectroscopy (NIRS) system to measure oxygenation of muscle tissue

Portamon: continuous wave near infrared spectroscopy (NIRS) system to measure oxygenation of muscle tissue

5.1. Initial Visit

During the baseline study visit, inclusion and exclusion criteria will be reviewed to ensure subject suitability. After eligibility has been confirmed, written informed consent will be obtained from the subject using Institutional Review Board (IRB)-approved documents. Informed consent will be

obtained before any study procedures. Subjects will be given the opportunity to have all questions regarding their participation answered in detail in a private setting before entering the study.

Following informed consent, a physical examination, with measurement of orthostatic blood pressures, will be performed. A urine pregnancy test will be performed in women with child-bearing potential. Blood will be collected for measurement of (a) comprehensive metabolic panel; (b) complete blood count; (d) methemoglobin. A pregnancy test (for women of childbearing potential) and Glucose-6-phosphate dehydrogenase (G6PD) deficiency screening (for males of males of African, Asian or Mediterranean decent) will be performed as needed. Blood, urine, and saliva samples will be collected and frozen for nitrate/nitrite and biomarker level determination. The Kansas City Cardiomyopathy Questionnaire (KCCQ) will be administered. Subjects will also be given an informational brochure regarding foods that are high in nitrate/nitrite and thus should be avoided during the study.

Echocardiography will then be performed using a standardized protocol. Images will be obtained from the parasternal long axis, short axis, apical 5-, 4-, 3-, and 2-chamber, subcostal, and suprasternal views for offline analysis of myocardial strain. Dedicated ventricular chamber images will be obtained in the 4- and 2-chamber apical positions for determination of left ventricular volumes. Mitral inflow velocities, including color M-mode interrogation, will be assessed in the 4-chamber view. Tissue Doppler imaging will be performed at the mitral septal position, approximately 1-cm apical to the mitral valve plane. Additional images will be obtained in the parasternal short axis at the level of the papillary muscles, 2-chamber, and 4 chamber apical views for assessment of myocardial strain. Pulse-wave Doppler interrogation of the left ventricular outflow tract (LVOT) will be performed in the apical 5-chamber view.

Concurrent arterial tonometry will be performed using a high-fidelity tonometer at the carotid, femoral, and radial arteries, using a Sphygmocor device. Waveforms will be calibrated using the brachial artery blood pressures, obtained using a validated oscillometric device. Waveforms will be digitally-stored for off-line analysis. Where available, we will also measure blood pressure using a BP+ device (Uscom, Sydney, Australia) , which is an FDA-approved device that measures brachial and central blood pressure using a standard brachial blood pressure cuff. Body surface measurements will be made to determine distance between the suprasternal notch to the carotid, radial, and femoral arteries. A 6-Minute Walk Test will also be performed during this visit. Patients who demonstrate significant desaturation during exercise ≤94% or a fall in Spo2 ≥5%) will be discontinued from the study at this timepoint.

Initial visit procedures

- Informed consent*
- Eligibility assessment
- Medical history, review of concomitant medications
- Dietary counseling
- KCCQ
- Physical Exam (including orthostatic and anthropometric measurements)
- ECG, laboratory tests (CBC, comprehensive metabolic panel, NTproBNP, methemoglobin; pregnancy test and G6PD deficiency screening as needed
- Blood, urine, saliva collection
- Echocardiogram, arterial tonometry

- 6 minute walk test with oxygen saturation

* Informed consent will be obtained before any study procedures

5.2. Randomization procedure

A blocked randomization will be performed. Each block will contain an equal number of allocations to KNO_3 followed by KCl versus KCl followed by KNO_3. The order of treatment sequences within each block will be randomized.

5.3. Intervention Phase 1

Subjects will be randomized to receive either active drug (KNO_3) or control (KCl) as the "first" drug for phase A. The initial dose will be one 6 mmol capsule administered twice daily, to be taken with meals. We will provide subjects with a 14-day supply of study medication to account for potential delays in receiving the 2nd batch of medication (i.e., after the 1-week call).

1-week call: Subjects will be called by telephone ~1 week later to assess if they are experiencing any side-effects. Subjects will receive reinforcement of the dietary restrictions imposed during the study. Subjects will be interrogated about headaches and dizziness. If these symptoms are not present, or are not worse compared to the subject's baseline, the drug dose will be up titrated to 18 mmol daily (One 6-mmol capsule taken 3 times per day). The presence of orthostatic symptoms (i.e. sustained lightheadedness upon standing) will prompt a visit to assess for orthostatic vital signs. The presence of symptomatic orthostatic hypotension (20 mmHg reduction in systolic blood pressure with associated symptoms such as dizziness) measured at this subsequent assessment visit will prompt exclusion of patients from the trial.

For subjects who have a serum K at the initial visit between 4.7 and 5.0 mEq/L in the presence of either: (a) potassium-sparing diuretic use, or (b) Estimated glomerular filtration rate of 30-39 mL/min/1.73m2, we will check a serum potassium 1 week after the implementation of the 18 mmol/d dose as a safety measure. A serum potassium > 5.5 mEq/L will prompt exclusion at this point.

At 3 weeks into each interventional phase, the Actigraph devices will be mailed to the subjects. The goal will be for the subjects to wear this device on their wrist during the final week of each interventional phase to look for differences with each therapy. The devices will be brought back by the subject at the endpoint visit.

Phase 1 endpoint assessment: Following 4 ± 1 weeks of therapy with KNO_3/KCl, subjects will return for endpoint assessments. Physical examination with measurement of orthostatic blood pressure, KCCQ, dietary counseling, and side effect assessments will be performed. A urine pregnancy test will be performed in women with child-bearing potential. An intravenous catheter will be placed in the antecubital vein and securely fixed in place. Blood, urine and saliva samples will be collected prior to, and approximately 2 hours after the morning dose administration, given with a standardized low-nitrate breakfast. Blood RNA samples will be collected prior to the

morning dose administration using Paxgene tubes (BD Biosciences, Franklin Lakes, NJ). Subjects will then undergo repeat echocardiography, arterial tonometry and blood pressure measurements (including BP+ central blood pressure measurements). Subjects will then perform a maximal-effort peak oxygen consumption (VO_2) test using a supine bicycle exercise test with expired gas analysis. Cardiac output at rest will be measured immediately prior to exercise using pulsed wave Doppler echocardiography. Flow-mediated dilation of the brachial artery to assess endothelial function will be performed (Unex Co. Ltd., Nagoya, Japan). An additional blood sample will be obtained at peak exercise. The Kansas City Cardiomyopathy Questionnaire (KCCQ) will be administered. Download of actigraph device data will also be done.

Phase 1 visit

- Interim medical history, review of concomitant medications
- Physical Exam (including orthostatic vital signs)
- Blood, urine, saliva collection
- Blinded drug administration
- ECG, laboratory tests (CBC, comprehensive metabolic panel, methemoglobin; pregnancy test as needed, BD PAXgene RNA samples
- KCCQ
- Blood, urine and saliva collection approximately 2 hours post-administration
- Echocardiogram, arterial tonometry
- Cardiopulmonary test with resting cardiac output measurements, blood draw at peak exercise
- Download of Actigraph data and continuation of outpatient physical activity
- Endothelial function assessment with flow-mediated brachial artery dilation

5.4. Washout period

Following the endpoint assessment, subjects will enter a 1-week ± 3 day washout period during which they will not receive any study medications.

At 3 weeks into each interventional phase, the Actigraph device will be mailed to the subjects. The goal will be for the subjects to wear this devices on their wrist during the final week of each interventional phase to look for differences with each therapy. The device will be returned by the subject at the endpoint assessment.

5.5. Intervention Phase 2

Following the washout period, subjects will receive either active drug (KNO_3) or control (KCl) during phase 2 of the trial. Subjects will receive the intervention (KNO_3 or KCl) that was not administered to them in phase 1, such that each subject will receive both study interventions in this cross-over design. The ~1-week call and the ~4-week study visit will be repeated as above. We will provide subjects with a 14-day supply of study medication to account for potential delays in receipt of the 2nd batch of medication (i.e., after the 1-week call).

1-week call: Subjects will be called by telephone ~1 week later to assess if they are experiencingside-effects. Subjects will receive reinforcement of the dietary restrictions imposed during the study. Subjects will be asked about headaches and dizziness. If these symptoms are not present, or are not different than the subject's baseline, the drug dose will be up-titrated to 18 mmol daily (One 6-mmol capsule taken 3 times per day). The presence of orthostatic symptoms will prompt a visit to assess for orthostatic vital signs. The presence of symptomatic orthostatic hypotension (20 mmHg reduction in systolic blood pressure with associated symptoms such as dizziness) at the follow up visit will prompt exclusion of patients from the trial.

Phase 2 endpoint assessment: Following 4 ± 1-weeks of therapy with KNO_3 or KCl, subjects will return for a repeat endpoint assessment. Physical examination with measurement of orthostatic blood pressure, KCCQ, dietary counseling, and side effect assessments will be performed. A urine pregnancy test will be performed in women with childbearing potential. An intravenous catheter will be placed in the antecubital vein and securely fixed in place. Blood, urine and saliva samples will be collected prior to, and approximately 2 hours after, morning dose administration, given with a standardized low-nitrate breakfast. Blood RNA samples will be collected prior to the morning dose administration using Paxgene tubes (BD Biosciences, Franklin Lakes, NJ). Subjects will then undergo repeat echocardiography, arterial tonometry and blood pressure measurements (including BP+ central blood pressure measurements). Subjects will then perform a maximal-effort peak oxygen consumption (VO_2) test using a supine bicycle exercise test with expired gas analysis. Cardiac output at rest will be measured immediately prior to exercise using pulsed wave Doppler echocardiography. An additional blood sample will be obtained at peak exercise. Flow-mediated dilation of the brachial artery to assess endothelial function will be performed (Unex Co. Ltd., Nagoya, Japan). The Kansas City Cardiomyopathy Questionnaire (KCCQ) will be administered. Download of actigraph device data will also be done.

Following the final endpoint assessment, subjects' participation in the study will be over.

Phase 2 visit (final visit)

- Interim medical history, review of concomitant medications
- Physical Exam (including orthostatic vital signs)
- Blood, urine, saliva collection
- Blinded drug administration
- ECG, laboratory tests (CBC, comprehensive metabolic panel, methemoglobin; pregnancy test as needed, BD PAXgene RNA samples
- KCCQ
- Blood, urine and saliva collection approximately 2 hours post-administration
- Echocardiogram, arterial tonometry
- Cardiopulmonary test with resting cardiac output measurements, blood draw at peak exercise
- Download of Actigraph data and continuation of outpatient physical activity
- Endothelial function assessment with flow-mediated brachial artery dilation

5.6. Adverse Event Reporting

All adverse events and adverse device effects will be reported following FDA guidelines. The research team will keep a log of all adverse events that occur in the trial, and any reportable events will be reported as described in Section 7 of this protocol. The study team in charge of the conduct of the trial is up to date on all trainings pertaining to safety guidelines and adverse event reporting. Adverse Events will be reported to the site IRB, the Sponsor and the NIH Program Office in a timely fashion, as specified in section 7.

5.7. Subject withdrawal / Early termination

Subjects may voluntarily withdraw from the study at any time and for any reason, or this may be at the investigator's discretion. The investigator may withdraw a patient from the study due to protocol non-compliance, incorrect enrollment or randomization, or for any other reasons related to subject safety. The reason for study discontinuation will be recorded on the source documents and all such subjects will be asked to complete an early termination visit.

During this visit, we will document: (1) vital signs; (2) compliance with the medications (3) Adverse effects. (4) Specific reason for withdrawal.

Subject Early Termination Visit Procedures
• Vital Signs and Physical Exam
• Medication adherence log
• Safety Labs
• Adverse event assessment
• Documentation of reason for withdrawal

5.8. Concomitant Medication

Subjects should be treated with standard of care medications for AS or associated comorbidities. As per inclusion criteria, subjects should be on a stable medical regimen prior to entry. Further adjustment of blood pressure medications during the study period is discouraged and should only be performed according to new and clinically compelling worsening of clinical status. Therapy with organic nitrates or phosphodiesterase-5 inhibitors is contraindicated during the study period.

6. DRUG DISPENSING, ACCOUNTABILITY AND DESTRUCTION

KNO_3 and KCl capsules will be prepared at the University of Pennsylvania Investigational Drug Service [3600 Spruce Street, Ground floor Maloney Building, Philadelphia, PA 19104]. Drug bottling, labeling and dispensing will also be managed by the Penn Investigational Drug Pharmacy. Prior to initiation of each phase, participants will receive a sufficient supply of KNO3 or control capsules to last until the end of each treatment phase, allowing for 100% compliance with the regimen for the entire treatment phase, including a window of ±7 days for the 1-week up-

titration (to ensure an adequate supply of medication before additional medications are received by the subject) and ±1 week for the endpoint assessment visit.

Subjects will be instructed to take the medication as required by the protocol, and compliance will be assessed via pill count performed by IDS.

Subjects will be instructed to return unused capsules at the end of each treatment phase. A pill count will be performed by IDS prior to destruction of the drug. Returned trial capsules will be stored separately from the non-allocated trial capsules until returned trial capsules are disposed of. The IDS personnel will keep track of all received, used, partly used and unused trial products.

Used and unused study drug will be destroyed at the instruction of the sponsor. A copy of the drug destruction SOP should be maintained in the pharmacy section of the Regulatory Binder. Study drug should not be destroyed until drug accountability has been completed.

6.1. Rules for un-blinding

Randomization data will be kept strictly confidential, accessible only to authorized IDS personnel, until the time of un-blinding. The investigators will be given access to the treatment code for their patients for emergency un-blinding by calling the IDS. This is considered to be a very unlikely occurrence. Any suspected study drug-related events will be treated as though the patient received active (KNO_3). Nevertheless, in the rare event of necessary un-blinding, the site PI and the sponsor must be notified.

7. ADVERSE EVENTS

7.1. Key definitions

Adverse Event: An adverse event (AE) is any untoward medical occurrence associated with the use of a drug in a subject whether or not considered drug or biologic related. An AE can therefore be any unfavorable and unintended sign, symptom or disease temporally associated with the use of the pharmaceutical product.

Suspected Adverse Reaction: A suspected adverse reaction (SAR) is any adverse event for which there is a reasonable possibility that the drug caused the event. “Reasonable possibility” suggests there is a causal relationship between the drug and the adverse event. “Suspected adverse reaction” implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a drug

Serious Adverse Events (SAE): An adverse event or suspected adverse reaction is considered serious if the investigator or sponsor believes any of the following outcomes may occur:

- Death
- Life-threatening AE: Places the subject at immediate risk of death at the time of the event as it occurred. It does not include an AE that, had it occurred in a more severe form, might have caused death.
- Persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions.

- Inpatient hospitalization or prolongation of hospitalization.
- Congenital anomaly or birth defect.
- Important medical events that may not result in death, be life threatening, or require hospitalization may be considered a serious adverse event when, based upon appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition above.

This determination is based on the opinion of either the investigator or sponsor (e.g., if either believes it is serious, it must be considered serious).

Unanticipated Adverse Device Effect (ADE): Any serious adverse effect on health or safety, any life-threatening problem or death caused by, or associated with a device, if that effect, problem, or death was not previously identified in nature, severity, or degree of incidence in the application; or any other unanticipated serious problem associated with a device that relates to the rights, safety, or welfare of subjects.

7.2. Classification of AE/ADEs regarding causal relationship to study interventions and severity

A medically-qualified investigator must assess all AEs in terms of causal relationship to intervention, severity, and "expectedness" using the following guidelines:

Classification of Adverse Events for Causal Relationship to Study Interventions	
Not related	There is not a reasonable causal relationship to the investigational product and the adverse event
Unlikely related	No temporal association or the cause of the event has been identified, or the drug or device cannot be implicated.
Possibly related:	There is reasonable evidence to suggest a causal relationship between the drug and adverse event.
Related	There is evidence to suggest a causal relationship, and the influence of other factors is unlikely.

Classification of Adverse Events Regarding Severity Scale	
1	Mild AE. Awareness of sign, symptom, or event, but easily tolerated; no treatment required
2	Moderate AE. Discomfort enough to cause interference with usual activity and may warrant intervention. In the latter scenario, AE responds to treatment
3	Severe AE. Incapacitating, limiting usual/normal activities or significantly affects clinical status requiring hospitalization or prolongation of hospitalization.
4	Life-threatening or disabling
5	Fatal AE

Expectedness: The expectedness of an AE/ADE or SAR shall be determined according to the specified reference document containing safety information (e.g., most current investigator's brochure or product label). Any AE/ADE that is not identified in nature, severity, or specificity in the current study reference document(s) (e.g. protocol or investigator's brochure) is considered unexpected. Events that are mentioned in the investigator's brochure as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but not specifically mentioned as occurring with the particular drug under investigation are considered unexpected.

The following AEs are expected, disease-related events in patients post-transcatheter or surgical AVR (84).

- Unplanned hospitalization, ER visit or clinic visit
- Arrhythmias
- Permanent pacemaker implantation
- Sudden cardiac death
- Acute coronary syndrome
- Cerebrovascular event
- Paravalvular aortic regurgitation
- Wound dehiscence
- Infective endocarditis
- Aortic dissection
- Sternal infection
- Thromboembolism

The following are potential expected side effects of KNO3:

- Stomach discomfort
- Slight headache
- Dizziness
- Lightheadedness
- Low blood pressure
- Stomach Ache, diarrhea, nausea, or vomiting
- Shortness of breath
- Flushing
- Rash
- Mild orthostatic hypotension
- Clinically significant methemoglobinemia or significant symptomatic orthostatic hypotension (20 mmHg or greater reduction in systolic blood pressure with associated symptom such as dizziness) are potential side effects, although we will consider them unexpected given prior data in this patient population with this particular drug and dosing scheme.

7.3. Recording and Reporting of Adverse Events

The site PI will continuously supervise all aspects of the trial and review the records of the study

subjects following each visit and at the end of their participation. The site PI will be responsible for ensuring that all adverse events are noted, followed and reported to the IRB, Sponsor (at psom-ind-ide@psom.upenn.edu) and IND Medical Director as appropriate.

SAEs occurring from the time of signed informed consent to the final study visit will be captured on the SAE eCRF. AEs will be classified according to the guidelines/definitions specified in section 7 of the protocol. Any AE rated ≥ 3 in severity (i.e., SAE) must be reported by the site investigator or qualified designee within 1 working day of first becoming aware of the event, to the sponsor via email and regulatory medical director. The IRB should be notified as per institutional guidelines. The medical director and site IRB, as appropriate, will make an immediate determination about the necessity to modify the protocol, include additional information in the consent form, inform previous participants, temporarily hold enrollment of patients, or terminate the study. The study will proceed only if the medical director and site IRB all agree on this course of action.

The investigator or qualified designee will enter the required information regarding the SAE into the appropriate module of the eCRF, All study procedures and cumulative adverse events are subject to full IRB review at least yearly.

Events significant enough to necessitate modification of study drug dosing will be captured on an appropriate eCRF module ("Study Drug Dosing" page). Should the PI determine that modification to the study drug is appropriate, the PI must notify the sponsor.

7.4. Follow-up

The Investigator will record safety follow-up information according to the same process used for reporting the initial event as described above. The Investigator will follow all reportable events until resolution, stabilization or the event is otherwise explained.

7.5. Management of Suspected Unexpected Serious Adverse Reaction

AEs that meet the criteria of serious, related to study intervention, and unexpected for the study intervention, qualify for expedited reporting by the sponsor to the regulatory authorities. The Site Investigator will assess all SAEs and evaluate for "unexpectedness" and relationship to study drug. The Site Investigator is required to complete a report for any events identified as serious, study drug related and unexpected, using the CIOMS format. The sponsor will submit a voluntary CIOMS form. See Section 7.4 for reporting procedures.

A copy of this report should be kept at the site and also forwarded to the sponsor regulatory lead within the same timeline used for reporting to regulatory authorities.

7.6. Pregnancy

Pregnancy is a contraindication to enrollment in the study. Pregnancy occurring during the study period, although not considered an SAE, must be reported to the NIH, local IRB and Sponsor within the same timelines as an SAE. The pregnancy will be recorded on the appropriate case report form. The drug will be discontinued immediately and subject discontinued from the trial, but the pregnancy will be followed until final outcome. Any associated AEs or SAEs that occur to the

mother or fetus/child will be recorded in the AE case report form or CIOMS form for SAEs.

8. STUDY MEASUREMENTS AND DATA COLLECTION

8.1. Assessment of exercise capacity

We will use a supine bicycle exercise protocol in conjunction with expired gas analysis to assess oxygen consumption (VO_2) during exercise. Subjects will perform a maximal exertion-limited exercise test using a graded-exercise protocol. We will use a supine cycle ergometer designed for stress echocardiography (Stress Echo Ergometer 1505, Medical Positioning, Inc, Kansas City, MO). Subjects will undergo expired gas analysis with a Parvo Medics True One 2400 device (Parvo Medics, Sandy, UT), an Innocor device (Innovision Inc) or equivalent. Resistance began at 15 W for 3 minutes, increasing to 25 W for 3 minutes, and then increasing by 25 W every 3 minutes thereafter. Breath-by-breath information will be recorded. We will use custom-designed software already developed in Matlab (MathWorks, Natick, MA) at our lab for offline processing and quantification of all exercise data.[85] All data quantification will be blinded to treatment. Total work performed will be computed and exercise efficiency will be defined as (total work/total oxygen consumed). Exercise efficiency is not a formal endpoint of the trial, but will be used to interpret changes in the co-primary outcomes. After these exercises, we will use a device that uses Near Infrared Spectroscopy [NIRS] to measure the oxygen levels in the left calf and forearm muscles (Portamon and Portalite devices, Artemis, Netherlands).

8.2. Quality of life

Quality of life will be assessed with the Kansas City Cardiomyopathy Questionnaire (KCCQ).[86] We will administer the KCCQ to subjects prior to randomization and at the end of each intervention phase (6-week time point in both phases). This is a validated 23-item questionnaire that assess physical function, symptoms, social function, self-efficacy and knowledge, and quality of life. It has been used extensively in heart failure studies (http://cvoutcomes.org/pages/3214)

8.3. Doppler Echocardiography

This procedure is similar to clinical cardiac echocardiograms. An ultrasound probe will be placed on the surface of the skin to obtain images of the heart. This takes approximately 20 minutes. This will occur at all three visits.

Measurement of LV filling and myocardial strain: given that inorganic nitrate may also induce venodilation and myocardial effects, we will also assess the effects of potassium nitrate on (1) Early mitral annular early diastolic tissue velocity; (2) The ratio of early diastolic mitral inflow velocity to mitral annular tissue velocity (a surrogate of LV filling pressures); (3) Peak early diastolic intraventricular pressure gradient, a marker of ventricular relaxation, assessed with color M-mode interrogations of mitral inflow.[87,88] We note that the latter is not simply based on the inflow propagation velocity, but rather on solving Euler momentum equation.[87] This method is able to accurately assess LV relaxation[87]. Systolic function will be assessed via systolic

myocardial strain (using speckle tracking echocardiography), which we have successfully applied in previous studies.[66,69,89]

8.4. Measurement of late systolic load and arterial wave reflections:

We will use a high-fidelity Millar applanation tonometer[54] to record carotid pressure waveforms, which will be calibrated using brachial artery pressures. Central arterial tonometric recordings and Doppler flow velocity files will be processed off-line using custom-designed software written in Matlab (The Mathworks, Natick, MA) as previously described.[66,90] We have successfully implemented this method in multiple previous studies[8,57,66,67,91] and have published a tutorial detailing our analysis methods.[8,53] After signal-averaging of pressure and flow, time alignment of carotid pressure and LV outflow curves will be performed to maximize concordance of the rapid systolic upstroke of pressure and flow, concordance of the dicrotic notch and cessation of flow, zero value of the phase angle of higher-frequency harmonics (7^{th} to 10^{th}) of input impedance, and linearity of the early systolic pressure-flow relationship.[90] After computation of aortic input impedance, proximal aortic characteristic impedance (Zc) will be computed in the frequency domain as previously described.[90] Pressure and flow harmonics were separated into forward and backward components using standard wave separation analysis.[8,53,90,92] The sum of forward and backward pressure harmonics yields the forward and backward waves, respectively. We will assess the reflection coefficient in the first 3 harmonics. A reflection coefficient (Γ) is derived from the ratio of two sine waves, it is a complex number with an amplitude and phase-angle, which can correspond to different degrees of destructive or constructive interference between forward and backward waves. Therefore, the net-effect of reflections will be expressed as the real part of Γ, which becomes increasingly positive as pressure from wave reflections increases (constructive interference), and negative when destructive interference leads to a net decrease in pressure by wave reflections at a given harmonic.[93] Since all harmonics of wave reflection contribute variably to systolic LV load, our primary measure of late systolic load will be the net pressure related to wave reflections during ejection in the time domain, which better represents the impact of reflections on LV afterload. We will first compute the product of flow and aortic Zc (QZc product), which represents the pressure resulting from the interaction of blood flow with aortic root Zc.[8,54] The relation between QZc and measured pressure reveals the direct effect of wave reflections on the arterial system.[94] We will therefore quantify the additional ejection-phase pressure load from wave reflections arising distal to the root (i.e., reflection-related pressure time integral during ejection, as the difference between measured pressure and the QZc product). Carotid-femoral pulse wave velocity (PWV), an index of large artery stiffness,[95,96] will also be measured[54,97] using a Sphygmocor device (Atcor Medical).[95] We will also measure carotid-radial PWV using the Sphygmocor device. PWV is not a formal endpoint of this trial, since our pilot trial did not demonstrate an effect of nitrate-rich beetroot juice on this endpoint (unpublished data). However, it may aid in interpreting changes in pulsatile hemodynamics in the trial. Similarly, when available, arterial tonometry may be performed during and immediately after exercise for exploratory purposes.

8.5. Blood nitrate/nitrite level measurements

We will document an intervention-related change in blood nitrate and nitrite. Nitrate and nitrite

levels will be sampled before and approximately 2 hours after the last oral dose on the morning of the exercise test, after the patient has been on each intervention for 4 ± 1 weeks. Measurements will be performed at Dr. Harry Ischiropoulos' lab at the University of Pennsylvania. Venous blood samples will be drawn into lithium-heparin tubes (which have very low levels of nitrate/nitrite) and centrifuged at 4,000 rpm for 10 min within 3 min of collection and frozen at -80°C for later analysis. Blood will also be obtained at peak exercise. After thawing at room temperature, blood samples will be deproteinized using cold ethanol precipitation as previously described.[98] The nitrate and nitrite content of deproteinized blood will be determined using a modified detection chemiluminescence technique using a Ionics/Sievers nitric oxide analyzer (NOA 280), as first described by Dr. Ischiropoulos' lab[99] and later adapted by Allen et al for human blood.[100]

8.6. Flow-mediated brachial artery dilation

Endothelial function will be assessed using flow-mediated dilation of the brachial artery (FMD) according to published guidelines.[101] The protocol is described in greater detail elsewhere.[102-104] Briefly, brachial artery diameter will be measured using a 10-Mhz ultrasound transducer (Unex Co. Ltd., Nagoya, Japan) at baseline after 5 minutes of supine rest. A stereotactic arm will be used for optimal transducer position. A pneumatic cuff will be inflated over the right forearm to 50 mmHg above the systolic pressure and deflated after 5 minutes. The diastolic brachial artery diameter will be measured continuously using the Unex device beginning at 30 seconds prior to cuff inflation and ending 2 minutes after cuff release. Maximal arterial vasodilation will be evaluated by assessment of change in diameter following cuff release.

8.7. Other assessments

Weight and Height: Weight will be measured to the nearest 0.1 kg with the use of calibrated scales, while the subjects are wearing light clothing and no shoes. Height will be measured at baseline to the nearest 0.1 cm using a wall-mounted stadiometer.

Genetic Expression Analysis: Blood RNA samples will be collected using BD PAXgene blood RNA tubes (BD Biosciences) in order to explore changes in genetic expression in response to the study medication (potassium nitrate) and whether patterns of genetic expression predict response to the study medication. Two 2.5 mL samples (total of 5 mL of blood) will be collected at each 6-week endpoint assessment visit.

9. STUDY CENTER MANAGEMENT PLAN

9.1. Principal Investigator

Dr. Chirinos will serve as the Principal Investigator for this protocol. In this role, he will lead operations at enrollment site and will be responsible for various aspects of the study, including data collection, adherence to all policies and procedures, and maintenance of study protocol to ensure that the specific objectives are being met. The PI will oversee the safety of study

participants. The PI will also interact with the data management team and the IND sponsor as needed. He will also establish subcommittees and working groups to complete specific activities, monitor study implementation, and hold regular investigator calls/meetings. Dr. Chirinos will supervise the investigative team to develop and coordinate procedures and generate reports and presentations. Other roles of Dr. Chirinos will include directing the echocardiography and arterial hemodynamics core laboratory for this trial.

9.2. Investigators Committee

An Investigators Committee will hold a monthly call and discuss aspects of the study, including enrollment, retention, data management, quality assurance, and other issues as they arise. Dr. Chirinos will chair the committee, which will include the following members: Julio Chirinos, Anupam Kumar, Howard Hermann, Frank Silvestry, Saif Anwaruddin. Minutes of these meetings will be distributed and kept on file. The PI will have authority to make decisions that require immediate attention when the committee is unavailable to meet. The PI will adjudicate disagreements within the committee. The sponsor has the final responsibility for all study decisions.

9.3. Operational Affairs Committee

This committee will be responsible for oversight and coordination of the day-to-day activities of the project. Committee members will include the site PI, the study coordinators, and the echocardiography/tonometry quantification technician. This group, along with all of the members of the Investigators Committee, will hold an in-person meeting at the beginning of the project to review the study protocol and the policies and procedures for its implementation. Members of the Operational Affairs Committee will participate in the Investigators Committee, but may hold additional meetings/calls throughout the project to review subject recruitment, retention, data transfer, quality of data, etc. Minutes of these meetings will be distributed and kept on file.

9.4. Data Management

The data for this trial will be collected in ad hoc source documents by the research staff during each study visit, using trial-specific and visit-specific data collection forms. All source documents collected in this trial will be housed inside of a locked cabinet in the offices of the research staff. Data capture and storage will be accomplished within the framework of the Research Electronic Data Capture (REDCap) project. REDCap is a secure, web-based application designed exclusively to support data capture for research studies. It provides an intuitive interface for data entry with data validation, audit trails for tracking data manipulation and export procedures, automated export procedures for seamless data downloads to common statistical packages, including SAS, and procedures for importing data from external sources. As of March 2015, REDCap was in production use or prototype build-status for more than 99,000 studies spanning numerous research focus areas across a consortium of 940 active institutional partners, including the University of Pennsylvania. Once the REDCap database is launched into production mode, the study coordinators will be responsible for transmitting the participant coded electronic data using standardized data collection instruments to the database manager who will check the data

for accuracy and completeness and follow-up with the research assistants as needed. The research coordinators will perform manual data entry from source documents and self-administered participant questionnaires (such as quality of life questionnaires).

9.5. Quantification Core Laboratories

Various physiologic core laboratory units will be in charge of analyzing physiologic data from the trial.

Echocardiography Core Laboratory: The echocardiography core lab, directed by Dr. Chirinos, has extensive experience in analyzing echocardiographic data for multicenter trials. The lab functions as the core lab for 2 ongoing international multicenter trials in heart failure (CVRx HOPE4HF and CVRx NeoHF) and one US-based multicenter study focused on HFPEF and HFREF (BMS CV002004). The core lab has all the capabilities required for echocardiographic data management and quantification in this study. The core lab has well-established standard procedures for receiving and logging imaging data, quantitative analyses, quality control, audit trails, and communication with study sites (queries about individual issues, feedback for quality control). The core lab will be responsible for generating and updating manuals for echocardiographic data acquisition, which will standardize procedures across the sites. The core lab will certify individual technicians performing measurements for the study after reviewing of sample scans (from normal volunteers) across the 2 sites, and will review and update certification as needed. The core lab will have the capacity to veto individual technicians from performing studies for the trial, based on data quality. The echocardiographic core lab will hold monthly calls with a representative of the data management core to discuss issues regarding core lab data management.

Arterial hemodynamics and exercise physiology core laboratory: The arterial hemodynamics core lab, directed by Dr. Chirinos, has experience in analyzing hemodynamic data for various large cohort studies (MESA, Asklepios)(57,67,69,71,106-109) and currently functions as the core lab for physiologic data for several ongoing multicenter studies (ACRIN 4008, BMS CV002004, iCAP study, CREST study). The core lab has specific experience in assessing specific exercise-related phenotypes relevant to inorganic nitrate effects(82) and has adequate capabilities required for physiologic data management and quantification in this study. The physiology core lab will hold monthly calls with a representative of the data management core to discuss issues regarding core lab data management.

Imaging data management: All imaging electronic data will be stored within a secure HIPAA-compliant network-attached storage server, and is accessible only to those who are specifically given access by the core lab director. The secure server has mechanisms for redundant storage and data backup at different physical locations, to prevent data loss from disasters (such as a fire).

10. STATISTICAL CONSIDERATIONS

10.1. Power calculations

We will randomize 22 subjects to one of 2 sequences (i), each of which consists of 2 periods (AB/BA design). We now have pilot data regarding the distribution of the changes in peak VO_2 (the primary endpoint of the study) as well as various secondary endpoints, among patients with HFpEF. We considered an increase in peak VO_2 of ~0.6 ml/kg/min to be the minimum clinically-significant change. This was based on the fact that even "modest" changes of comparable magnitude in peak VO_2 have been associated with improved outcomes.[110] The standard deviation of the change in peak VO_2 in our pilot trial was 1.23 ml/kg/min. Therefore, the standardized effect size is 0.49 or greater. Assuming a 90% retention rate, enrolling 22 subjects in this cross-over trial will have 80% power to detect an effect size as low as 0.66 in the intervention-induced change of our study endpoints, with a two-sided α=0.05.

Based on the distribution of the changes in study endpoints from repeated measurements of the Kansas City Cardiomyopathy Questionnaire in AS patients, we estimated that this trial study is powered (80%) to detect the following changes in other endpoints: 4.6 points for the Kansas City Cardiomyopathy Questionnaire, 8.2% for exercise vasodilatory reserve, 19% for cardiac output reserve and 4.2% change in reflection magnitude. We note that these differences are well below what is considered a clinically significant change in KCCQ (10 points or more, http://cvoutcomes.org/pages/3217) or the effect size that has been observed for these endpoints in our pilot trial.[85] Therefore, we are confident that we will achieve adequate power not only for our clinical endpoints, but also for our physiologic measures. PASS11[111] was used to perform power analyses.

10.2. Data Analysis Plan

The co-primary outcome variables will be peak VO_2 and KCCQ score. All secondary outcome measures are continuous variables. The predictor of interest for all aims will be intervention (KNO_3 therapy vs. control), with analyses based upon the total number of subjects randomized. Initial descriptive estimates of all measures will be generated for study participants at each time point by treatment group. Statistics will include estimates of central tendency, measures of variability, and derived moments of skewness and kurtosis. Analyses of distributional properties will be performed to determine if variance stabilizing or normalizing transformations should be applied. Outliers will be assessed via visual inspection of distributions and checked for accuracy. Aim 1 will assess the effects of potassium nitrate therapy on peak O2 / work performed and quality of life score (co-primary outcomes). An initial assessment of the treatment effect will be performed using the paired t-test and the non-parametric Wilcoxon sign-rank test on the difference between the paired within subject outcome measures. This will be followed by a more comprehensive linear mixed-effects model analysis[112] allowing for assessments of the treatment effect on each continuous outcome of interest while controlling for effects of other covariates such as period, sequence, and a random subject effect nested within sequence. For non-normal distributed outcomes, we will utilize non-parametric methods or consider distribution-stabilizing transformations. The intervention groups will *initially* be compared within each period (time invariant covariates will only be compared for period 1) according to continuous covariates

described above using parametric or non-parametric one-way ANOVA models, depending upon whether or not normality appears to be in question. Levine's tests will be used to assess homogeneity of variance. Additionally, the intervention groups will be compared within period according to categorical covariates using Fisher's Exact tests. Significant differences between groups on these variables will result in their use as control variables in the modeling of outcome. Separate models will be generated for each of the two outcome measures, with each outcome measure regressed on intervention group assignment, along with baseline outcome and any other covariates deemed prognostic in preliminary analyses. The linear mixed-effects models will incorporate adjustments for any period effect and include data from dropouts.[113-115] The model will include subject-specific intercepts as random effects, and assumes independent and identically distributed random errors within subject. Restricted maximum likelihood estimation will be used, and an appropriate covariance matrix will be specified. Model assumptions will be examined (e.g., QQ plots to assess normally distributed residuals for valid Wald tests). We will make every possible effort to minimize missing data and ensure final assessments for participants opting to discontinue study participation. Missing data, however, is an inevitable problem in a longitudinal study. The mechanism for missingness-missing completely at random (MCAR), missing at random (MAR), nonignorable or not missing at random (NMAR)-will be evaluated prior to implementing methodology intended to minimize bias from missing data.[116] We anticipate that ~10% of randomized subjects will not complete the study.

The intent-to-treat principle of including all randomized participants in the outcome models will be followed. SAS software procedures will be used for creating and analyzing multiple imputed data sets for incomplete multivariate data. Instead of filling in a single value for each missing value, Rubin's[117] multiple imputation procedure replaces each missing value with a set of plausible values that represent the uncertainty about the correct value to impute. These multiple imputed data sets are then analyzed using standard procedures for complete data and combining the results from this analysis. Assuming monotone missing data patterns emerge, either a parametric regression method that assumes multivariate normality or a nonparametric method that uses propensity scores will be implemented, depending upon distributional patterns.

Analytic methods described above will take advantage of all available data. The possibility of systematic bias in the outcomes for those who withdraw exists. Baseline characteristics will be compared among subjects with and without complete follow-up, recognizing that statistical power associated with finding true statistical differences may be limited. To assess potential biases, a comparison of withdrawal rates and or/time to withdrawal will be included. If the number of subjects lost to follow-up is small and the missing observations can be documented as being MAR or MCAR, then the primary hypotheses will be tested using the complete observed data. If missing observations cannot be assumed to be MAR or MCAR, more complex approaches will be considered. Sensitivity analyses for these models will be performed should they be implemented.[118]

Our secondary aims involve exploratory analyses investigating mechanisms of action. In addition to assessing the effect of our randomized intervention on each mechanistic endpoint, exploratory structural equation modeling will be used to evaluate associations between outcomes and biologic mechanistic pathways. The modeling will be carried out in three sequential steps: (1) exploratory factor analysis, (2) confirmatory factor analysis, and (3) structural equation modeling. The exploratory factor analysis will be based on principal axis factoring to decrease the number of

variables. For both theoretical and empirical reasons, it will be assumed that retained factors are correlated and thus an oblique rotation method will be used. Measured and latent variables will be examined for co-linearity, and related variables will be combined into single factors with individual factor loadings. To determine the number of factors, the model will be evaluated against the following four rules: (1) eigenvalues greater than 1.0[119]; (2) Glorfeld's[120] extension of parallel analysis, where a large number of random correlation matrices are generated to compare the number of eigenvalues that are significant by chance[121]; (3) high internal consistency (an alpha coefficient of ≥ 0.70) for unit-weighted factors,[122] and (4) interpretability.[123] The heaviest weight will be placed on the Minimum Average Partial and parallel analysis methods, with the scree test as a visual adjunct.[124] The next step involves incorporating the factors into a model using confirmatory factor analysis. The model will be tested using goodness of fit tests to assess the overall fit of the model to the data. Various models will be tested and compared prior to arriving at the best fitting model. And finally, the best fitting model obtained from confirmatory factor analysis will incorporate a structural equation model designed to examine the links between the randomized intervention, the mechanistic variables and the clinical variables assessed in the trial.

11. PROTECTION OF HUMAN SUBJECTS

11.1. Potential benefits of the proposed research to the study subjects and importance of the knowledge to be gained, and risk/benefit ratio

Potential benefits: There are no anticipated direct benefits to the subjects as a result of their participation in this study nor will this be implied when obtaining consent. However, if our hypothesis is correct, subjects in the active medication groups may experience improvements in their functional class and quality of life, although this will not be implied in any way during informed consent or enrollment.

Importance of the knowledge to be gained: If our hypothesis is correct, this study may identify an effective specific intervention to treat mechanistic abnormalities in AS patients. Furthermore, if improving these abnormalities leads to an enhanced exercise capacity in this patient population, this would lead to a new paradigm in the field and a better understanding of the mechanisms that lead or contribute to AS, which will accelerate the discovery of new treatments for this condition. In addition, if potassium nitrate proves effective in enhancing exercise capacity and/or quality of life in this trial, this would identify a readily implementable, inexpensive therapy for this condition.

Risk/benefit ratio: the results of this study may ultimately lead to an effective treatment for persistent symptoms of AS after AV. Since there is minimal risk and potential benefits to medical knowledge and society, the risk / benefit ratio is acceptable.

11.2. Risks to study subjects

The conduct of these studies will involve 22 human subjects, each exposed to both the active drug (potassium nitrate) and control (potassium chloride) intervention (cross-over design), and therefore studied as their own controls. All subjects will be adults able to give informed consent. Participants will be recruited from the Hospital of the University of Pennsylvania. The ethnic distribution of the population in our center will favor adequate representation of minorities (particularly, African-Americans) in the sample.

The study involves various tests (arterial tonometry, Doppler echocardiography, a cardiopulmonary exercise stress, blood draws) and the administration of randomized therapy (KNO_3 vs. control).

11.2.1. Potential Risks of study intervention (potassium nitrate)

The main potential risks of nitrate administration are related to its potential effect on (1) blood pressure; and (2) methemoglobin levels.

Effects on blood pressure: Regarding the blood pressure reduction, as described above, many studies have demonstrated a reduction in blood pressure in both hypertensive and normal subjects following nitrate supplementation. This was summarized in a recent meta-analysis, demonstrating an approximate 4 mm Hg reduction in systolic blood pressure and 1 mm Hg reduction in diastolic blood pressure.[43] Importantly, in a study by our group of a single dose of inorganic nitrate in subjects with HFpEF, we did not observe any change in blood pressure following nitrate ingestion. Importantly, the lack of a blood pressure response to nitrate has been shown in both elderly[125] and diabetic individuals,[126] suggesting that there may be important differences in nitrate effect by subgroups. In our pharmacokinetics study, we found a mild (~12 mm Hg) asymptomatic reduction in blood pressure recorded at the time of our study visits during 2-week drug administration, which occurred in the absence of any hypotensive symptoms.[127] We did not find a reduction in ambulatory blood pressure. Therefore, although we do not anticipate major changes in BP or significant hypotension with this dose, we will implement exclusion criteria and safety blood pressure checks throughout the conduct of the study to ensure subject safety (see next section).

Impact of NO_3^- on methemoglobin levels: Nitrate supplementation increases serum nitrite levels through the actions of an enterosalivary circuit and reduction of nitrate to nitrite by anaerobic bacteria in the oral cavity.[20,25-28] Methemoglobin is generated by the reaction of nitrite with hemoglobin. According to Toxnet, doses between 2-9 grams of NO_3^- have been associated with methemoglobinemia. The dose we will use in our study (18 mmol KNO_3^-) will provide only 1.11 grams (18 mmol NO_3^- x 62 grams/mol NO_3^- x 1mol/1000 mmol), which is well below this threshold. Moreover, this level of nitrate supplementation provides less inorganic nitrate than that found in a traditional Japanese diet, which is rich in vegetables (traditional Japanese diet provides 18.8 mg/kg of nitrate per day x 70 kg individual = 1.3 grams of nitrate).[128] Moreover, not a single cases of methemoglobinemia was observed in our pharmacokinetics trial , at the same doses used in this trial and in the same patient population. In the pharmacokinetics study we measured methemoglobin every half hour after the first administration as well as during steady-state continued oral administration at doses of both 12 mmol/d and 18 mmol/d and did not find any clinically-significant elevations in methemoglobin. We don't anticipate any significant issues with methemoglobinemia in this study since this is a problem that has been reported with nitrite, but not with nitrate at the doses used in this study.

Safety data:

In a double-blind randomized controlled trial in HFPEF, in which 12.9 mmol of inorganic were administered to patients with HFpEF, efficacy was demonstrated for the study endpoint (please refer to research plan). In this pilot study, no side effects were noted in any subject, except for pink urine (which was not different between the nitrate-rich and nitrate-poor beetroot juice,

because this is a consequence of other components of beetroot juice). As the body is able to reduce oxidized Fe^{3+} in methemoglobin back to Fe^{2+} at a rate of approximately 15% per hour,[129] the gradual increase in nitrite that occurs after nitrate administration (and the consequent formation of methemoglobin), should occur slowly enough to prevent significant rises in methemoglobin. To the best of our knowledge, methemoglobinemia has not been reported with oral nitrate administration. In our pharmacokinetic study, we did not observe any clinically-relevant elevations in methemoglobin levels[127].

Other potential safety considerations:

There has been concern regarding whether a high nitrate diet may predispose to gastric cancer in humans through conversion to nitrite in the stomach or through increased urinary excretion of nitrate metabolites. This is a controversial issue derived from animal studies[130], although the relationship between nitrate intake and cancer has not been demonstrated in humans.[23,131,132] In fact, many studies show either no relationship or even an inverse relationship between a high intake of nitrate and the occurrence of gastric cancer.[133,134] [135,136] [137,138] The Joint FAO/WHO Expert Committee on Food reviewed all the available evidence, but failed to establish a definite link between nitrate intake and risk of developing cancer.[139,140] Furthermore, The World Cancer Research Fund/American Institute of Cancer Research found no evidence linking ingestion of vegetables which are known to be high in nitrate with the development of cancer[141]. We note that the highly controversial concern regarding cancer risk associated with nitrate intake is related to long-term intake and not a significant issue with our 6-week administration scheme of potassium nitrate.

11.2.2. Potential Risks of study procedures

Potential risks are associated with the study tests, the study interventions (potassium nitrate) and potential breaches in confidentiality.

Cardiopulmonary stress test

This test is used extensively for research purposes with minimal risk to subjects. The most significant risks of the test are dysrhythmias or other cardiovascular complications, which are extremely rare. These procedures will be performed by qualified personnel according to established American Heart Association Guidelines.[142,143] Non-revascularized myocardial ischemia, which may increase the risk of complications during exercise testing, is an exclusion criterion for the study. We note that prior studies in HFpEF also enrolled subjects with atrial fibrillation[144] and that rate-controlled atrial fibrillation is not a contraindication to exercise testing.

Subjects may feel uncomfortable as a result of pushing themselves during the maximal effort exercise test. Subjects will likely feel short of breath and fatigued as a result of the exercise test. Various other complaints, such as nausea, lightheadedness, and other aches and pains are also possible as a result of the maximal effort exercise study. Although exercise testing may result in exhaustion, rarely do people develop abnormal HR or heart complications during exercise tests. The risk of this happening is the same as if the participant would exert themselves during stressful situations or during exercise elsewhere.

We will perform EKG, HR, and blood pressures monitoring during our exercise test. In addition to the blood pressure (generally increases) and heart rate (generally increases) changes during

exercise, we will also monitor arterial saturation. This will be done non-invasively using a pulse oximeter. Of note, oxygen levels can decrease with exercise, even in individuals without significant cardiopulmonary disease.[145,146] If the arterial saturation falls to below 88% ("severe exercise induced hypoxemia"[146]), we will alert the care provider as this may prompt consideration for additional/alternative causes for arterial hypoxemia.

Venipuncture and IV placement

According to the 2010 WHO guidelines on phlebotomy, major risks associated with blood donations include hematoma at the site of venipuncture in 2-3%, and vasovagal reactions and fainting in 1%. The placement of an intravenous catheter would be anticipated to increase the risk of hematoma and discomfort slightly. Given that the catheter will be in place for a short-period of time, infection is an unlikely complication. Of note, the amount of blood to be drawn in the study is less than the amount of blood drawn at routine blood donation where the risk of syncope of 1% was defined.

Arterial tonometry and assessments of oscillometric arterial pressure waveforms are non-invasive procedures and do not have any known risks.

During various procedures (echocardiography, arterial tonometry), we will use adhesive electrodes attached to the participant's skin to record the electrical signal from the heart. These may occasionally cause skin itching and irritation.

Pregnancy Risks

We will not be enrolling subjects who are pregnant in this study. Right now, there is not enough research on the potassium nitrate to determine the safety of the mother or unborn child. A pregnancy test will be given to women of child-bearing potential prior to enrollment in the study and administration of the supplement. The pregnancy test will be repeated at the 6-week visit (prior to initiation of the second treatment period). If a woman is enrolled of child-bearing potential, we will ask that they use a medically accepted method of birth control (such as an IUD, birth control combination pill, patch, ring, progestin-only pills, Depo Provera Shot, Implanon, complete abstinence, or condoms) while they participate in the study. In addition, we will test for pregnancy at each study visit and discontinue subjects from the study immediately if she becomes pregnant. As subjects with AS are generally older (>55 years old) we do not anticipate this concern to occur with our study population. We will not enroll pregnant women or women who are lactating.

As with any clinical research study, there is a potential for **breach of confidentiality**. Adequate measures will be taken to minimize this risk (below).

11.3. Adequacy of Protection Against Risks

11.3.1. Recruitment and Informed Consent

Written informed consent will be obtained from the subjects by the investigators prior to entry into the research study. This will be performed in accordance with the guidelines and under the supervision of the University of Pennsylvania Institutional Review Boards. The study procedures and interventions and the associated risks will be explained to the subjects during the informed consent process. Only IRB-approved consent forms and related materials will be used.

11.3.2. Protection against risks associated with cardiopulmonary exercise tests

These tests will be performed by qualified personnel according to established American Heart Association Guidelines(142), under ECG monitoring. Personnel with adequate cardiopulmonary resuscitation training and resuscitation equipment (crash cart) will be available during these tests. Similarly, these tests will be performed in a hospital setting where a full code team can be deployed immediately should complications occur.

11.3.3. Protection against risks of potassium nitrate administration:

Subjects will be thoroughly advised regarding the potential risks of the study medication and precautions needed during its administration. We will exclude individuals who are taking sildenafil (Viagra®), tadalafil (Cialis®), or vardenafil (Levitra®) and we will instruct them not to take these medications while participating in the study, since it is likely that these medications may exaggerate the vasoactive effects of potassium nitrate. Similarly, the use of organic nitrate will be contraindicated during participation in this trial.

Supine and orthostatic blood pressure measurements: Supine vital signs will be measured at the screening visit. The subject will rest in a supine position for a minimum of 3 minutes prior to obtaining vital sign measurements. Subjects with a supine systolic blood pressure >110 will be enrolled in the study. The subject will then assume a standing position for 3-5 minutes. Vital signs (BP and pulse rate) will then be measured while the subject is standing. A reduction in systolic blood pressure >20 mmHg will be considered an exclusion criterion for the study. Blood pressure measurements will be repeated after 4 ± 1 weeks of randomized therapy. Measurements will also be prompted by reports of any report of orthostatic symptom by study subjects at any point during the trial. The presence of symptomatic orthostatic hypotension (20 mmHg reduction in systolic blood pressure with associated symptom such as dizziness) will prompt exclusion of patients from the trial.

Safety Clinical Laboratory Tests: Laboratory evaluations will be collected prior to study drug initiation. Among women who are not surgically sterilized or post-menopausal, a urine pregnancy test will be performed at screening (prior to all baseline studies and drug initiation), at the end of the first treatment phase (prior to the initiation of the second treatment phase). Methemoglobin levels will be measured before initiation of randomized therapy and 4 ± 1 weeks after initiation of randomized therapy in both treatment phases (1 and 2). Methemoglobinemia (methemoglobin levels >10%) will prompt discontinuation of patients from the trial. Other tests, including a complete blood count, liver enzymes, a basic metabolic panel (including serum creatinine for assessments of renal function) will also be performed prior to randomization and 4 ± 1 weeks after initiation of randomized therapy.

Significant methemoglobinemia was not observed in our pharmacokinetics trial thus far, at the same doses used in this trial, in a similar patient population. In this study we are measuring methemoglobin every half hour after the first administration as well as during steady-state continued oral administration at doses of both 12 mmol/d and 18 mmol/d. We don't anticipate any significant issues with methemoglobinemia since this is a problem that has been reported with nitrite, but not with nitrate at the doses used in this study. However, a more strict monitoring approach for methemoglobinemia will be implementing should the results of our ongoing pharmacokinetics/safety study raise any concerns.

Side Effect management Plan: No side effects have been observed in our single-dose study or our pharmacokinetics study (in particular, no hypotension or methemoglobinemia). However, a plan for management of side effects will be in place. The use of acetaminophen may be used for headaches during the study. Although hypotension is not expected, it should be managed as per standard clinical practice. In the unlikely event that methemoglobinemia does occur during the trial, we will manage according to best clinical standards.(147) As the endogenous rate of reconversion from methemoglobin to hemoglobin is approximately 15% per hour, most asymptomatic patients can be managed conservatively. Generally, methemoglobin levels >20% with symptoms, or >30% without symptoms, warrant therapy; any patient with levels that are elevated to this range will be referred to the emergency room for management.(147)

11.3.4. Measures to minimize the risk of breach in confidentiality:

All records will be treated with strict confidentiality according to HIPAA guidelines (all study personnel are trained on HIPAA regulations). Blood samples obtained from subjects will be used only for research purposes. Records will be treated with strict confidentiality and stored in a secured, limited access area. A randomly assigned number rather than name will identify all collected samples. A secure database of patient information will be maintained. The investigators and the sponsor will have access to research information and will follow IRB and institutional HIPAA guidelines. Paper files will be saved under lock in a secure IRB-approved area.

11.3.6 Other measures to minimize risk:
Phlebotomy, arterial tonometry, and Doppler echocardiographic examinations will only be performed by appropriately trained personnel as per our institutional standards.

12. REGULATORY STANDARDS

12.1. Informed consent

The site investigator, or a person designated by the site investigator, will fully inform the subject of all pertinent aspects of the clinical trial including the review of the informed consent form approved by an Institutional Review Board (IRB). Prior to a subject's participation in the clinical trial, the Informed Consent Form will be signed and personally dated by the subject or by the subject’s legally acceptable representative. All subjects will receive a copy of the informed consent form.

12.2. Institutional Review Board (IRB)

The site Principal Investigator will submit this protocol to the Penn IRB. The study (study number, protocol title and version number), the document reviewed (protocol, Informed Consent Form, etc.) and the date of the review will be clearly stated on the written IRB approval opinion. During the study, any amendment or modification to the protocol will be sent to the IRB. It will also be informed of any event likely to affect the safety of subjects or the continued conduct of the study, in particular any change in safety and all updates to the protocol will be sent to IRB.

12.3. Source document handling and archiving

Hard copies of subject's study records, including signed informed consent forms, HIPPA forms, source documents, and other study related materials will be stored in the subject binders, in a locked file cabinet in the research coordinator's study office until archived. Research records will be retained for at least 2 years after the completion of this study. All study electronic files will be kept for at least 6 years after IRB acknowledgement of study termination. Files are not be destroyed or deleted without Sponsor approval. Data will be made available to the study sponsor National Institute of Health (NIH) members of the Institutional Review Board, and the Food and Drug administration, if requested by any of these entities.

13. DATA AND SAFETY MONITORING PLAN:

The data and safety monitoring plan is contained in Appendix 3.

14. SUBJECT STIPEND

Subjects will receive financial compensation for participation in this study. We will follow the following reimbursement scheme:

- Completion of Visit #1: $100
- Completion of Visit #2: $150
- Completion of Visit #3: $150

The maximum total amount (if all study visits are completed) will be $400.

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APPENDIX 1. SCHEDULE OF EVENTS

Schedule of Events	Baseline assessment	Phase 1		1-week washout	Phase 2	
		1 week	4 ± 1 week		1 week	4 ± 1 week
Informed consent	X					
Eligibility assessment	X					
Medical History	X		X			X
Concomitant medications	X		X			X
Randomization	X*					
Dietary counseling	X		X			X
KCCQ	X		X			X
Physical Exam	X		X			X
12-lead ECG	X		X			X
Laboratory tests	X		X			X
RNA collection with PAXgene tubes			X			X
G6PD test as needed	X		X			X
6 minute walk test	X					
Blood, urine, saliva collection and freezing	X		X			X
2D Echocardiogram	X		X			X
Arterial tonometry	X		X			X
Drug accountability			X			X
Maximal effort exercise test with expired gas			X			X
Retrieval of actigraph			X			X
Arterial dilation (FMD)			X			X
Assessment of adverse events		X	X		X	X

*performed after baseline screening lab results are available