. C O N F I D E N T I A L

CLINICAL TRIAL PROTOCOL, R01 HL121510

Effect of KNO_3 Compared to KCl on Oxygen UpTake in Heart Failure with Preserved Ejection Fraction (KNO_3CK OUT HFpEF)

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Sponsor: University of Pennsylvania

Sponsor Representative: Emma A. Meagher, MD

Sponsor email: psom-ind-ide@pobox.upenn.edu

Funding Sponsor: National Heart, Lung and Blood Institute (NHLBI)

Study Chair / Grant Principal Investigator:

Julio A. Chirinos, M.D., Ph.D.

Site Principal Investigators (alphabetical order)

Sanjiv Shah, M.D. (P.I., Northwestern Site)

Sujith Kuruvilla (P.I., Corporal Michael J. Crescenz VAMC Site)

Payman Zamani, M.D. (P.I., Penn Site)

Sub-Investigators (alphabetical order)

Thomas Cappola, M.D.

Jesse Chittams, M.Sc.

Alexandra Hanlon., Ph.D.

Kenneth Margulies, M.D.

Ravinder Reddy, MD, PhD

Srinath Adusumalli, MD, M.Sc.

Data Coordinating Center:

Alexandra Hanlon, Ph.D. (Director) and Jesse Chittams, M.Sc.

NIH Program Officer:

Patrice Desvigne-Nickens, M.D.

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1. SPECIFIC AIMS

Heart failure (HF) affects ~2% of the population and ~10% of adults aged >75 years. Approximately 50% of HF patients demonstrate a preserved left ventricular (LV) ejection fraction (EF), and thus have HF with preserved EF (HFpEF). Many therapeutic interventions are available for HF with reduced EF, but there are currently no proven effective pharmacologic interventions for HFpEF. Given its enormous public health, clinical and societal burden, finding effective therapies for HFpEF is a top priority. Accumulating evidence implicates various peripheral abnormalities in the pathophysiology of HFpEF.

Exercise intolerance with reduced aerobic capacity is the hallmark of HFpEF and greatly impairs quality of life (QOL). Effective O_2 extraction requires cardiac output redistribution towards and within exercising muscle, mediated by vasodilation in active muscle tissue. Dietary nitrate increases plasma nitrite, an endothelium-independent source of the potent vasodilator nitric oxide (NO). Importantly, the conversion of nitrite to NO occurs preferentially in the setting of hypoxia, as occurs in exercising muscle. Furthermore, nitrate-nitrite-derived NO has been shown to enhance mitochondrial function, representing an additional pathway to improve exercise tolerance. Therefore, nitrate supplementation represents a logical intervention to improve exercise tolerance in HFpEF. Our preliminary data from a double-blinded cross-over placebo-controlled trial demonstrates that a single dose of inorganic nitrate increases aerobic capacity (peak O_2 consumption during a symptom-limited maximal effort exercise test), enhances the peripheral vasodilator response to exercise, and improves skeletal muscle mitochondrial oxidative function.

Late systolic LV load is an important determinant of LV dysfunction and remodeling. 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. Our preliminary data demonstrates that a single oral dose of inorganic nitrate reduces late systolic load in HFpEF. Thus, in addition to an exercise-enhancing effects, augmentation of the nitrate-nitrite-NO pathway has the potential for chronic “disease modifying” benefits in HFpEF.

Based on preliminary data demonstrating the effects of a single oral dose of inorganic nitrate in HFpEF, the current trial was designed to assess the efficacy of sustained oral administration of inorganic nitrate in a more diverse and representative sample of HFpEF patients and to further assess the mechanisms by which inorganic nitrate enhances oxygen uptake and exercise capacity. This is a randomized double-blind crossover clinical trial, in which 84 subjects with HFpEF 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 approximately 6 weeks, or; (2) Potassium chloride (KCl), at a dose of 12-18 mmol/d by mouth for approximately 6 weeks. A 1-week washout period will be introduced between the 2 interventions. The purpose of the trial is to test the efficacy of KNO_3 on a number of clinical and physiologic endpoints in subjects with HFpEF.

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.

Hypothesis 2b: KNO_3 improves muscle blood flow during exercise, measured with flowsensitive PC-MRI during a standardized plantar flexion exercise test.

Hypothesis 2c: KNO_3 improves muscle Cr recovery kinetics (a marker of oxidative capacity), measured with MRI. We will also assess the exercise-induced spatial matching (i.e., within muscle) of blood flow and oxidative capacity using a highly novel method (chemical exchange saturation transfer) that allows, for the first time, for highresolution spatial mapping of Cr in muscle.

Hypotheses 2d-e: 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).

We will explicitly ensure that a balanced sex distribution is present in our sample, and that African-Americans are adequately represented. We will pre-specify stratified analyses of men vs. women and African American vs. non-African American subjects. We will also perform analyses to assess whether sex or race modify the effects of KNO_3 on our endpoints. This is important, because sex differences have been demonstrated in responses to inorganic nitrate in other populations.[1, 2] Similarly, African Americans have been shown to have more pronounced wave reflections,[3] and have been shown to demonstrate differential responses to other NO donors (organic nitrates) in the presence of HF with reduced ejection fraction (HFrEF).[4, 5]

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

At 2 enrolling centers (Corporal Michael J. Crescenz Veterans Affairs Medical Center and University of Pennsylvania), we will perform an ancillary study ("Cardiac MRI sub-study") to assess the effect of KNO_3 on myocardial perfusion (MP) and myocardial perfusion reserve (MPR). This ancillary study will be funded by a grant from the Department of Veterans Affairs VISN-4 Competitive Pilot Project Fund (CPPF) obtained by Dr. Sujith Kuruvilla (VA site PI and co-Investigator in the ancillary studytrial). We will assess myocardial perfusion using novel MRI techniques.

The sub-study will test the hypothesis that KNO_3 administered over a period of ~4-6 weeks, will improve MP and MPR in subjects with HFpEF as compared to KCl. We will also explore whether MPR (measured during the placebo intervention, KCl) correlates with increased myocardial fibrosis (measured as the extracellular volume [ECV]) using cardiac MRI), and with subclinical myocardial dysfunction (measured by impaired systolic/diastolic strain) in this population.

We plan to enroll up to 20 subjects in this ancillary study. Subjects participating in the ancillary study will undergo all procedures per the parent trial, but in addition, will undergo a stress perfusion cardiac MRI study during the last 2 weeks of phase 1 and phase 2, during which MP, MPR values, myocardial fibrosis and myocardial strain measurements will be obtained. Of note, this may occur either before or after the exercise endpoint assessment, based on schedule availability. We anticipate that this substudy will contribute to a better characterization of the effects of inorganic nitrate therapy on the myocardium.

2. TRIAL SUMMARY TABLE (OBJECTIVES AND ENDPOINTS)

Trial Title / Acronym	Effects of KNO_3 Compared to KCl on Oxygen UpTake in Heart Failure with Preserved Ejection Fraction (KNO_3CK OUT HFpEFp)
Funding Agency	National Heart, Lung and Blood Institute, National Institutes of Health
Phase	Phase IIb Randomized Clinical Trial (RCT)
Number of subjects	84
Study Sites	University of Pennsylvania, Northwestern University, and the Corporal Michael J. Crescenz Veterans Affairs Medical Center
Study Design	Randomized, double-blinded, placebo-controlled crossover study
Randomized Intervention	Potassium Nitrate (KNO_3) capsules, at a dose of 6 mEq (1 capsule) three times daily for approximately 6 weeks, versus, potassium chloride (KCl) capsules administered at a dose of 6 mEq (1 capsule) three times daily for approximately 6 weeks
Primary Aim	To assess whether KNO_3 therapy improves exercise capacity, quantified as: (1) Total work performed during a maximal-effort exercise test; (2) Peak oxygen consumption (VO_2) during a symptomlimited maximal effort exercise test. These will be the co-primary endpoints of the trial.
Secondary Aims	1. To assess whether KNO_3 improves quality of life 2. To assess whether KNO_3 improves the systemic vasodilator response to exercise 3. To assess whether KNO_3 improves muscle blood flow during exercise. 4. To assess whether KNO_3 improves muscle PCr recovery kinetics (a marker of oxidative capacity) 5. To assess whether KNO_3 improves LV diastolic function and myocardial systolic strain 6. To assess whether KNO_3 reduces late systolic LV load from wave reflections
Exploratory Aims	1. To assess whether KNO_3 improves the exercise-induced spatial matching (i.e., within muscle) of blood flow and oxidative capacity (assessed by chemical exchange saturation transfer). 2. To assess whether KNO_3 has different effects in men vs. women and in African-American vs. non-African American subjects 3. To assess whether KNO_3 improves: • Ventilatory threshold, VO_2 kinetics • Natriuretic peptide levels

	• Physical activity (measured via accelerometry) • 24-hour late systolic pressure augmentation, measured with the Aurora device
	4. To assess the relationship between nitrate and nitrite levels achieved and the therapeutic response achieved in each subject (improvement in endpoints in response to KNO_3 vs. placebo)
Primary Endpoint	Total work performed and peak oxygen uptake (VO_2) during a symptom-limited maximal effort exercise test after approximately 6 weeks of KNO_3 vs. KCl.
Secondary Endpoints	1. Quality of life score, assessed using the Kansas City Cardiomyopathy Questionnaire. 2. The change in systemic vascular resistance reserve during exercise during a symptom-limited maximal effort exercise test. 3. Muscle blood flow during exercise, measured with flow sensitive PC-MRI during a standardized plantar flexion exercise test. 4. The time constant of Cr recovery after a plantar flexor exercise protocol. 5. Measures of LV diastolic function (E/e', left atrial volume index) and peak global systolic myocardial longitudinal and circumferential strain. 6. Augmentation index, late systolic wall stress and aortic input impedance.
Ancillary Study	Up to 20 subjects will be enrolled at the CMC VA Medical Center and the University of Pennsylvania into an ancillary study. The sub-study will test the hypothesis that KNO_3 administered over a period of ~4-6 weeks will improve myocardial perfusion and myocardial perfusion reserve in subjects with HFpEF as compared to KCl. Participation in the ancillary study will not change any of the main trial procedures.

3. BACKGROUND AND SIGNIFICANCE

Heart failure (HF) affects ~2% of the western population and 10% of adults age 75 years or older.[6] HF is the most common cause of hospitalization in adults >65 years of age.[6] Approximately 46-54% of patients with HF have HFpEF.[6-18] The burden of HF has increased dramatically over the last several years.[6, 19] The number of new HF cases increased from 348,000 in 2000 to 670,000 in 2007, a 93% increase over this time period. Not only is HF already an epidemic, but with the aging of the population, a dramatic further increase in its prevalence is expected. Furthermore, the relative prevalence of HFpEF (as a proportion of the total burden of HF) appears to be increasing as the population ages.[1, 14] Therefore, although already a huge epidemic, a further dramatic increase in the prevalence of HFpEF is expected.[6] HFpEF is a malignant disease with a high mortality rate, high hospitalization risk and poor quality of life (QOL). Studies consistently demonstrate higher mortality rates in HFpEF patients compared with age- and sex-matched controls. Annual mortality rates ranged from ~3.5-6% in randomized trials[20-22] to ~15% in

community-based studies.[23] Approximately 50% of such deaths occur from cardiovascular causes.[24] In addition to its high morbidity and mortality, HFpEF has been shown to be associated with an impaired QOL.[25, 26]

Multiple therapies that provide substantial clinical benefit in HF with reduced ejection fraction are available. In contrast, there are currently no effective pharmacologic interventions that improve outcomes in patients with HFpEF.[27] Thus, there is an urgent need to develop novel treatment strategies. Our trial will test a novel intervention (inorganic nitrate) to modify key physiologic abnormalities (arterial vasodilator reserve, muscle O_2 delivery and utilization, mitochondrial function and late systolic LV load from arterial wave reflections), which have the potential for both immediate-term improvements in exercise tolerance and long-term "diseasemodifying" effects. The trial is designed to address the potential clinical benefit of this approach in HFpEF using endpoints with direct clinical relevance, and also to characterize the specific physiologic mechanisms involved. This would identify a new therapeutic paradigm and a readily implementable therapeutic intervention in HFpEF.

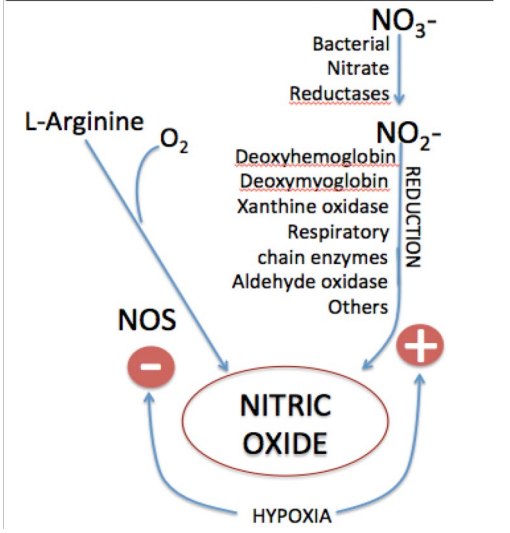
3.1. The nitrate–nitrite–NO activation pathway

NO formation occurs via 2 pathways in mammals (Figure 1): (1) NO synthases (NOS) catalyze the formation of NO from L-arginine and O_2;[28] (2) Circulating nitrate (previously considered an inert product of NO metabolism) can be converted to NO through the nitrate-nitrite-NO pathway, which is independent of NOS.[28-33]

The nitrate-nitrite-NO pathway is dependent primarily on dietary inorganic nitrate intake. Vegetables (leafy green vegetables and beetroot, in particular)[28, 33-36] are the main source of inorganic nitrate in the diet (>80%). Oral cavity commensal bacteria reduce nitrate to nitrite, which has a high oral bioavailability(>90%).[33, 35, 36] Nitrite present in the blood stream is reduced directly to NO, a reaction catalyzed by several molecules, particularly deoxygenated myoglobin,[37-40] and deoxygenated hemoglobin,[41] but also xanthine oxidoreductase,[42] mitochondrial respiratory chain enzymes[43], aldehyde oxidase[44], carbonic anhydrase,[45] vitamin C[46], polyphenols[47, 48] and even endothelial NO synthase.[49, 50] Nitrate is also directly absorbed in the gastrointestinal tract (without requiring conversion to nitrite), with high bioavailability (>90%)[51] and circulates in plasma with a half-life of ~6-8 hours, in contrast to nitrite, which has a very short half-life (~30-40 minutes).[52-54] Circulating nitrate is taken up by the salivary glands and secreted in the saliva (thus being susceptible to both direct reabsorption as nitrate or to conversion to nitrite followed by absorption). This entero-salivary cycle provides a natural, "slow" and sustained source of nitrite from ingested nitrate, thus allowing for favorable pharmacokinetics and for safe oral administration of "pharmacologic" doses in HFpEF (as discussed further in this application). Most (65%) inorganic nitrate eventually undergoes renal excretion.

NO production by the nitrate-nitrite-NO pathway is enhanced under hypoxic conditions: exercising muscle is characterized by a low pO_2,[33] which favors the formation of NO from circulating nitrite.

Figure 1. Pathways for NO formation in mammals: The L-Arginine pathway (left) and the nitrate-nitrite-NO pathway (right)



Deoxyhemoglobin supports the reduction of nitrite to NO and is thought to play a key role in modulating small resistance vessels (particularly of skeletal muscle), where O_2 extraction from the circulation to the tissues is most marked. Here, the O_2 saturation of hemoglobin approaches the P_{50} (the O_2 concentration at which half the heme is saturated), an optimum balance point between the greater reductive potential of heme in the R (oxy) state tetramer and the number of un-ligated deoxy-heme sites necessary for nitrite binding (which are more plentiful in the T-state tetramer). This results in near-maximal conversion rates of nitrite to NO and hence vasodilatation.[55, 56] Similarly, NO by deoxy-myoglobin enhances blood flow to skeletal muscle and matches O_2 supply to increased metabolic demands under hypoxic conditions.[37] Xanthine oxido-reductase also converts nitrite to NO when O_2 levels are low.[42] In sharp contrast to NO production from the nitrate-nitrite-NO pathway, which is enhanced in the presence of hypoxia, NO production via the classic L-arginine pathway is strongly inhibited by hypoxia[50] (Figure 1). In summary, the endogenous nitrate-nitrite pathway is a physiological effector of hypoxic vasodilation via NO release, which is independent of the L-arginine pathway and largely independent of NO synthase.

3.2. Peripheral mechanisms of exercise intolerance in HFpEF

Exercise intolerance is the hallmark of HFpEF and determines a poor quality of life.[25, 26, 57, 58] Therefore, enhancing exercise capacity in this population is a key objective with immediate clinical relevance. We believe that new attempts to achieve this unmet goal should be based on careful considerations regarding the pathophysiology of exercise intolerance in HFpEF. The early pathophysiologic paradigm was that increases in LV filling pressure during exercise were not accompanied by increases in end-diastolic volume, failing to recruit the Frank-Starling mechanism and to augment stroke volume.[59] Studies also reported the presence of chronotropic incompetence, leading to an abnormal cardiac output reserve.[60-62] However, various studies have not found abnormal end-diastolic LV volume during exercise,[60, 63] stroke volume reserve (i.e.,

increase during exercise),[60-62] chronotropic incompetence[63] or cardiac output reserve.[61] Thus, rather than resulting exclusively from cardiac abnormalities, HFpEF is now seen a complex disease and there is a need to address not only cardiac, but also peripheral abnormalities in this condition.[64, 65]

Reduced O_2 utilization is a key abnormality in the pathophysiology of exercise intolerance in HFpEF. Peak O_2 uptake (VO_2) during exercise, the most widely accepted index of aerobic capacity, is consistently reduced in HFpEF.[59-62, 66] Peripheral O_2 utilization requires not only an adequate cardiac output during exercise, but also an adequate flow distribution, characterized by blood flow being preferentially directed to exercising skeletal muscle, in order to achieve an adequate matching of perfusion with metabolic demands. This preferential flow distribution is dependent on the exercise-induced reduction in the local resistance of skeletal muscle arterioles (i.e., exercise-induced vasodilation). Therefore, as discussed below, the normal exercise induced vasodilation in exercising muscle serves 2 important roles: (1) To reduce the "total" (i.e., systemic) vascular resistance during exercise, which reduces LV afterload, promoting a greater cardiac output for any given contractile state; (2) To preferentially divert flow to highly oxidative muscle, promoting matching between metabolic needs and oxygenated blood delivery, thus allowing the "periphery" to optimally "utilize" the cardiac output to maximize O_2 consumption.

Exercise arterial vasodilatory reserve is abnormal in HFpEF, leading to excessive LV afterload. During exercise, LV afterload must decrease to accommodate increases in cardiac output without excessive rise in arterial pressure. In several studies, compared to age-matched hypertensive subjects during exercise allows the local vasculature to overcome humoral and reflex-mediated vasoconstriction.[67] NO bioavailability and release is a key mechanism mediating this response.[67, 68] Importantly, impaired vascular responses within skeletal muscle can have dramatic consequences for O_2 extraction, creating a marked imbalance between O_2 delivery and requirement in muscle which results in a large O_2 deficit, accentuated intracellular metabolic perturbations and enhanced glycogenolysis even at low levels of activity (reviewed in [67]). The vasodilator response in exercising muscle is dependent on both endothelium and endotheliumindependent pathways.[67] As will be discussed below, nitrite-derived NO is a potent endotheliumindependent mediator of hypoxic vasodilation.[37, 68, 69] and increase muscle blood flow during exercise.[29, 70] Of note, HFpEF patients exhibit endothelial dysfunction[71, 72] and may be particularly dependent on endothelium-independent NO to enhance skeletal muscle flow during exercise.

Importance of flow distribution within muscle is also important. Although blood flow toward locomotive muscle groups is clearly important during exercise, blood flow (Q) within active muscles is not homogeneous, being greater in highly oxidative muscles, which normally demonstrate greater vasodilatation.[73, 74] Hypoxic vasodilation favors preferential vasodilation in tissues with high oxidative capacity, which optimizes supply-demand matching (reviewed in [67, 68]). Dysregulation of these control processes provides an excess flow and therefore O_2 delivery to muscles with less oxidative capacity, reducing muscle and whole-body fractional O_2 extraction.[74] Nitrite-derived NO-mediated hypoxic vasodilation[37, 68, 69] thus may not only enhance overall muscle bulk flow, but also optimize flow distribution within muscle. Therefore, we believe that it is important for a study that targets muscle hypoxic vasodilation to not only address "overall" bulk muscle flow as a mechanistic endpoint, but also flow distribution within muscle. This important

and elusive goal, although well characterized in animal models,[67] has been limited in human studies by the lack of techniques to spatially map (i.e., image) oxidative capacity. Our group has recently developed and validated an ad hoc technique[75, 76] which, for the first time, allows for non-invasive high-resolution spatial mapping (i.e., producing an anatomic image) of phosphocreatine kinetics (a marker of oxidative capacity). Therefore, our study will not only assess the effects of our intervention on exercise-induced bulk muscle flow and muscle oxidative capacity , but also on the matching of blood flow distribution and oxidative capacity within locomotive muscle.

3.3. Importance of wave reflections and late systolic load: beyond improvements in exercise capacity

While addressing exercise intolerance in HFpEF is a key therapeutic goal, there is also a need to address long-term underlying abnormalities that contribute to chronic LV remodeling and the long-term course of the disease. As discussed below, a large body of evidence now indicates that late systolic pulsatile load from wave reflections (which are increased in HFpEF)[77], have adverse long-term consequences on LV remodeling and function. Our preliminary data suggests that enhancing nitrate-nitrite-pathway-derived NO production favorably impacts these systemic arterial abnormalities. Therefore, in addition to their exercise-enhancing mechanistic effects, interventions that enhance the nitrate-nitrite-NO pathway exert peripheral arterial effects with a potential for chronic “disease-modifying” benefits in HFpEF.

The pulse wave generated by the LV travels forward in arteries and is partially reflected at sites of impedance mismatch (i.e., bifurcations, points of change in arterial size or wall stiffness, predominantly in middle-sized conduit arteries).[78-80] Wave reflections travel back to the heart, merging into a discrete reflected wave and arrive while the LV is still ejecting blood in mid-to-late systole.[80, 81] Wave reflections increase the late systolic workload of the LV and profoundly impact the LV loading sequence (late relative to early systolic load).[78, 79, 82-84]

Late systolic load from wave reflections leads to LV hypertrophy. For any given level of systolic pressure, late-systolic load exerts deleterious effects on the LV.[80, 85, 86] Kobayashi et al[85] used a Wistar rat model and performed constriction of either the ascending aorta (which increased LV early systolic load) or suprarenal abdominal aorta (which caused prominent late systolic loading from a reflected wave at the distal aortic constriction site arriving at the heart in mid-to-late systole).[85] Despite an identical peak LV pressure in rats that underwent ascending vs. descending aortic constriction, rats that underwent descending aortic banding (and were thus exposed to greater late systolic load) demonstrated much greater LV hypertrophy and fibrosis than those undergoing ascending aortic banding (which were exposed to increased early systolic load).[85] These animal causal findings are supported by our observational human data demonstrating an association between reflected wave amplitude an LV hypertrophy in the general population[87] and by Hashimoto’s study demonstrating that changes in reflection magnitude during antihypertensive therapy strongly predicted regression of LV mass independent of blood pressure reduction.[88] Of note, effects of standard antihypertensive therapy on wave reflections are unpredictable, with reflections actually increasing in some patients.[88]

Late systolic load promotes diastolic dysfunction. Gillebert et al studied the effect of the timing of systolic load on LV relaxation in dogs by inflating balloons in the ascending aorta during either early or late systole. They found that late systolic inflation of an aortic balloon increases the LV relaxation constant tau much more than early systolic inflation, demonstrating a cause-effect relationship between late systolic load and diastolic dysfunction.[89] In support of these causal findings, wave reflections have been shown to be independently associated with diastolic dysfunction in clinical cohorts.[90, 91] We have developed a novel technique to assess timeresolved LV wall stress during ejection,[92] and more recently showed in the population-based Asklepios study [83, 92-95] that wave reflections selectively increase late LV myocardial wall stress[83, 96] and that late systolic wall stress was associated with lower mitral annular relaxation velocities, in sharp contrast to early systolic wall stress which was associated with greater relaxation velocities.[95] Therefore, available data implicates the loading sequence as an independent correlate of myocardial relaxation in humans. Wave reflection magnitude was a strong predictor of incident heart failure among 5,934 participants in the Multiethnic Study of Atherosclerosis, who were free of clinically apparent cardiovascular disease[84, 97] during 7.61 years of follow-up (and after adjustment for multiple confounders, including blood pressure), reflection magnitude strongly predicted incident HF (Hazard ratio per 10%-increase=2.69; 95%CI=1.79-4.04; P<0.0001), and was a stronger predictor of HF than blood pressure and any other standard modifiable risk factor. Similarly, for any given level of systolic and diastolic blood pressure, a greater area under the pressure curve in late systole (relative to early systole) is strongly predictive of incident HF.[98] Our findings implicate late systolic load from arterial wave reflections as a novel strong risk factor for HF, strongly supporting animal and human mechanistic findings from previous studies and demonstrating the relevance of late systolic load in humans.

3.4. Rationale behind the use of inorganic nitrate as a therapeutic approach in HFpEF

The arterial hemodynamic characteristics of HFpEF patients (stiff arteries with wide pulse pressure,77 reduced exercise-induced vasodilation[62, 63, 66], enhanced wave reflections[77] dictate a set of “ideal” characteristics for a vasoactive intervention in this condition, namely, one that: (1) Does not significantly reduce mean arterial pressure at rest, avoiding hypotension; (2) Enhances exercise-induced vasodilation to reduce LV afterload during exercise; (3) Has selectivity for enhancing local vasodilation in hypoxic/acidotic environments, in order to match blood flow to metabolic demands (i.e., directing blood flow to exercising muscle); (4) Reduces wave reflections and late systolic load, which likely contribute to LV diastolic dysfunction and maladaptive remodeling.[80, 85, 86, 88-91, 95] Inorganic nitrate precisely satisfies these characteristics. Inorganic nitrate exerts weak vasodilator effects in the absence of hypoxia but acts as a potent arterial vasodilator in the presence of local hypoxemia. Concentrations of nitrite (for which nitrate is a precursor) as high as 100-1000 µmol/L are typically required to induce relaxation of arterial rings in vitro.[99] Similarly, a lack of vasodilator activity was seen with a concentration of 200 µmol/L of nitrite in the forearms of healthy volunteers.[100] This situation is, however, different under hypoxic conditions. Maher et al studied 40 volunteers and demonstrated that, during normoxia, forearm arterial blood flow during local infusions of nitrite at incremental doses (from 40 nmol/min to 7.84 µmol/min) increased by a maximum of 64% during normoxia, but it increased by as much as 121% during hypoxia.[101] The nitrate-nitrite pathway thus seems ideal for enhancing vasodilation, O_2 extraction and utilization in HFpEF. By virtue of enhancing hypoxia-mediated vasodilation, the

vasodilatory reserve would be increased, reducing LV afterload during exercise and directing blood flow "where and when it is needed", enhancing O_2 delivery and this utilization. Indeed, inorganic nitrate improves muscle blood flow during exercise.[29, 70] Our preliminary data support the promise of inorganic nitrate in HFpEF, via promotion of exercise-induced vasodilation, ultimately leading to enhanced aerobic capacity, in the absence of blood pressure reductions at rest, while at the same time reducing LV late systolic load. This represents an optimal "tailored" approach to abnormal hemodynamics in HFpEF.

3.5. Preliminary data: effects of a single-dose of inorganic nitrate in HFpEF

We performed a randomized double-blind cross-over trial (clinicaltrials.gov NCT01919177) of a single dose of 12.9 mmol of inorganic nitrate given as 140 mL of concentrated nitrate-rich beetroot juice (NR-BR; Beet it; Sport, James White Drinks Ltd., Ipswich, UK) versus an otherwise identical beetroot juice which had been nitrate-depleted (used as the control or "placebo" juice, PB), allowing for a genuine double-blinded design.[102] The intervention was given before a maximal-effort cardiopulmonary exercise test, with both experiments separated by a wash out period of at least 5 days (mean=11.8 days). We administered NR-BR 3 hours prior to testing because this period of time has been shown to correspond to peak circulating nitrite levels after oral nitrate administration. We tested the hypothesis that a single dose of inorganic nitrate would increase exercise capacity in HFpEF and assessed key physiologic adaptations to exercise. Specifically, we assessed: (1) Peak oxygen consumption (VO_2); (2) Total work performed; (3) Exercise efficiency, defined as the ratio of total work performed to total O_2 consumed (efficiency has been improved by inorganic nitrate in young athletes)[70, 103-107]; (4) Exercise vasodilatory reserve (the change in peripheral vascular resistance from rest to peak exercise, normalized to the resting value); (5) Skeletal muscle mitochondrial oxidative function; (6) Aortic augmentation index, a metric of late systolic load.

We included subjects with symptomatic HF who had a preserved LV EF (>50%) and who were required to have a ratio of the early mitral inflow velocity (E) to septal tissue Doppler velocity (e') >8 and at least one other sign of chronically-elevated filling pressures including: (1) left atrial volume index >34 mL/m^2; (2) elevated NT-pro-BNP level; or (3) elevated filling pressures (pulmonary capillary wedge pressure > 12 mmHg) on prior cardiac catheterization. Subjects had to be on stable medical therapy. Exclusion criteria included non-cardiac conditions limiting exercise tolerance (i.e. orthopedic issues, peripheral arterial disease with claudication, neuromuscular disorders); gait instability; rhythm other than sinus; infiltrative or hypertrophic cardiomyopathy; pericardial disease; primary pulmonary arteriopathy; coronary revascularization within 60 days; clinically significant valvular disease; clinically significant lung disease felt to contribute to exercise intolerance; significant ischemia seen on stress testing within the past year and not revascularized.

After each intervention, we performed a resting echocardiogram and arterial tonometry, followed by a maximal-effort supine-bicycle cardiopulmonary stress test using a graded exercise protocol, beginning at 15 Watts (W) for 3 minutes, increasing to 25 W for 3 minutes, then increasing by 25 W every 3 minutes thereafter.[108] Measurements during exercise included expired gas analyses (ParvoMedics True One 2400; Parvomedics, Utah, USA), echocardiographic assessments of stroke volume and continuous interrogation of muscle oxygenation of the left gastrocnemius

muscle with near-infrared spectroscopy (Portamon MkII device, Artinis medical systems; The Netherlands). We also assessed mitochondrial oxidative function measured via the recovery of oxygen consumption (measured with near-infrared spectroscopy) in forearm skeletal muscle after a bout of handgrip exercise, which corresponds to the phosphocreatine recovery kinetics, as previously described and validated against ^{31}PMRI.[109, 110] Details of the procedure may be found in previous publications. [108,109-111] In brief, the procedure measures the rate of oxyhemoglobin decline during brief high pressure inflations (at 200 mHg using a rapid inflation system), during which the decline in local muscle O_2 is due exclusively to consumption, as the arterial occlusion removes the confounding impact of arterial inflow. After a brief bout of forearm exercise, several of such brief occlusions are performed, allowing for assessments of the rate of O_2 consumption at various time points after exercise. Exercise induces increases in the rate of O_2 consumption, which upon cessation of exercise, declines towards baseline in a mono-exponential fashion. This is characterized by a time constant (τ, tau) that corresponds to the time constant of PCr recovery kinetics.[109, 110] Although we used this relatively inexpensive near-infrared spectroscopy procedure in our pilot trial, we will use more informative MRI techniques in this proposal.

Participants' mean age was 66±9 years. The population was enrolled from the Philadelphia VA Medical Center and was 88% male and 82% African-American (we will explicitly assure adequate representation of women in the KNO_3CK OUT HFpEF trial). Subjects were obese (BMI 35.4±5.4), had a high prevalence of hypertension (100%). Median NT-pro-BNP was 326 (IQR=290-352) pg/mL. Mean LA volume index was 35.7±10.9 mL/m^2. Serum NO metabolites were markedly greater after NO_3^- supplementation (median 326.0 [IQR 290.0-352.0] versus 10.0 [IQR 9.0-13.0] μM, *P*=0.0003).

3.5.1. Changes in exercise capacity and oxygen uptake: Nitrate supplementation resulted in a greater peak VO_2 (12.6±3.7 versus 11.6±3.1 mL O_2/kg/min; *P*=0.005) and total work performed (55.6±35.3 vs. 49.2±28.9 kJ; *P*=0.04). Ventilatory threshold (a marker of the cellular anaerobic threshold) was greater following NO_3- supplementation (7.6±1.8 versus 7.0±1.4 mL O_2/kg/min, P=0.03). Because total work performed and O_2 consumption increased in tandem, exercise efficiency was no different. This is interestingly, different than the effect seen in athletes and younger subjects, among whom exercise efficiency increases[70, 103-107], demonstrating the importance of characterizing specific adaptations to exercise in this particular patient population.

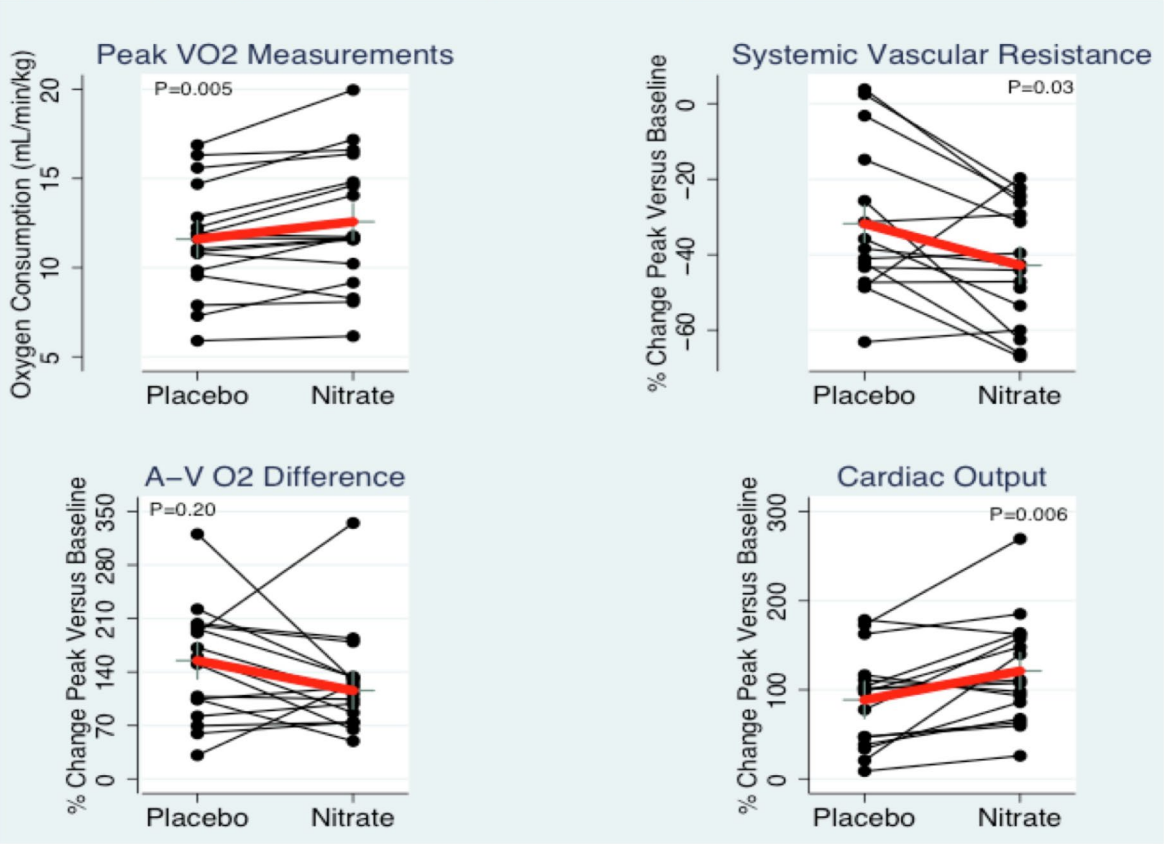
3.5.2. Exercise vasodilatory reserve: NR-BRJ led to a significant fall in systemic vascular resistance (SVR) at peak exercise (-42±17%) compared to PB (-32±20%; P=0.03). This was accompanied by an increase in cardiac output (% change in NR-BRJ 122±60 vs PB: 89±53%, P=0.006), increased heart rate (78.0±24.1 versus 65.6±21.0%, P=0.001) and a trend towards greater stroke volume (22.6±22.4 versus 12.7±25.4%, P=0.13).

3.5.3. Effects on filling parameters: Interestingly, we found no evidence of reduced preload, since end-diastolic volume (EDV) significantly increased with beetroot supplementation (112±12 mL vs 93±6 mL; P=0.04), whereas the E/E' ratio did not change (NR-BRJ: 12.1±0.9 vs. PB: 11.5±0.8; P=0.90). The increased LV EDV may suggest improvements in diastolic function, which we plan to further assess in this proposal using measurements of intraventricular pressure gradients.

3.5.4. Augmentation index: The aortic AIx (derived from radial tonometry) was significantly decreased by NO_3- supplementation (NO_3- 132.2±16.7 versus PB 141.4±21.9%, P=0.03).

3.5.5. Changes in resting blood pressure: There was no significant change in resting BP

Figure 2. Physiologic effects of a single dose of inorganic nitrate



(mean arterial pressure change=0.17 mmHg; systolic BP change=0.7 mmHg; both *P*>0.85) with NO_3^- supplementation. This is in contrast to BP-lowering effect in healthy volunteers and drug naïve hypertensive subjects[112, 113] and likely explained by recent data indicating that the BPlowering effect of inorganic nitrate is mediated by its renal effects in pathways downstream of the AT1-receptor.[114] These effects are likely attenuated in patients who are treated with ACE inhibitors/angiotensin receptor blockers for BP control (such as occurs in HFpEF patients).

In summary, a single dose of inorganic nitrate increased peak VO_2 and total work during a symptom-limited maximal effort exercise test. It enhanced the exercise vasodilatory reserve, without affecting SVR or blood pressure at rest. It also reduced late systolic LV load, which is implicated in diastolic dysfunction and LV remodeling. Nitrate also led to increased cardiac output during exercise, likely from a reduced afterload, but potentially also mediated by increased EDV (suggesting effects on diastolic function). Finally, in accordance with other studies, our pilot data suggest an improvement in mitochondrial oxidative function, suggesting improved ATP production.[115, 116] However, nitrate did not affect the O_2 cost of exercise, as has been demonstrated in younger and/or healthier populations.[70, 105, 106, 117, 118] This demonstrates the importance of having disease-specific and agent-specific data to design our phase IIb trial. Our pilot trial was limited by its small sample size, inclusion of mostly men (we will explicitly assure

adequate representation of women in this trial). However, it does support the need for a larger phase IIb randomized trial of prolonged nitrate supplementation in HFpEFp.

3.6 Effect on Myocardial Perfusion Reserve in HFpEF ("Cardiac MRI sub-study")

Myocardial biopsy studies in HFpEF patients have revealed cardiomyocyte hypertrophy, interstitial myocardial fibrosis, incomplete myocardial strip relaxation, increased cardiomyocyte stiffness, increased production of reactive oxygen species at the coronary endothelial cell level, along with increased expression of adhesion molecules and production of pro-inflammatory cytokines.[119-122] In view of these findings, a new paradigm has been suggested in the pathophysiology of HFpEF (Figure 3). The paradigm proposes that the co-morbidities seen in HFpEF patients induce a proinflammatory state which activates coronary microvascular endothelial cells to produce reactive oxygen species and induces cardiomyocyte hypertrophy resulting in concentric LV remodeling and increased interstitial collagen deposition proceeding to diastolic dysfunction.

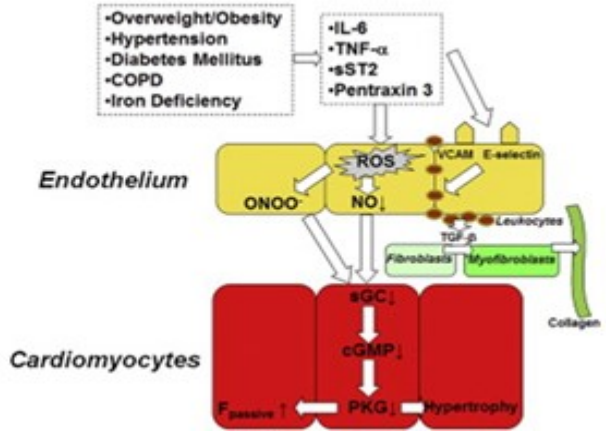


Figure 3: Adapted from Paulus et al. Novel paradigm in HFpEF patients. JACC Volume 62, Issue4 (236-271)

MRI is an ideal tool to assess these pathophysiological phenotypes and assess the effect of KNO_3 on microvascular myocardial function. Coronary microvascular dysfunction which is proposed to underlay this disease process can be estimated by measuring MPR by means of quantitative vasodilator stress cardiac MRI. MPR is calculated by a ratio of stress coronary perfusion over rest perfusion. Myocardial fibrosis can be quantitatively assessed by the measurement of ECV by myocardial T1 mapping. [123]

The ancillary study of the KNO_3CK OUT HFpEF trial represents a unique opportunity to assess the effect of inorganic nitrates on MPR values in this patient population.

4. STUDY DESIGN AND POPULATION

4.1. Overview of study design

In this phase IIb, double-blind, cross-over placebo-controlled trial, we will assign 84 subjects with HFpEF to: (A) Potassium nitrate administered by mouth at a dose of 6 mEq three times daily for approximately 6 weeks, or (B) Potassium chloride (KCl) at identical doses. The order of the interventions (AB-BA design) will be randomized, with a 1-week 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. placebo (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 three sites for this trial will be the Hospital of the University of Pennsylvania, Northwestern University and the Corporal Michael J. Crescenz VA Medical Center.

4.3. Study Population

We will enroll 84 subjects meeting the following criteria. Eligibility will be determined by the site PI according to the criteria outlined below.

4.3.1. Inclusion Criteria:

1. Adults aged 18-90 years of age
2. A diagnosis of heart failure with NYHA Class II-III symptoms
3. LV ejection fraction >50% during baseline echocardiography
4. Stable medical therapy: no addition/removal/changes in antihypertensive medications, or beta-blockers in the preceding 30 days
5. Elevated filling pressures as evidenced by at least 1 of the following:
    a. Mitral E/e' ratio > 8 (either lateral or septal), with low e' velocity (septal e'<7 cm/sec or lateral e'< 10 cm/sec), in addition to one of the following:
        i Enlarged left atrium (LA volume index >34 ml/m^2)
        ii Chronic loop diuretic use for control of symptoms
        iii Elevated natriuretic peptides (BNP levels >100 ng/L or NT-proBNP levels >300 ng/L)
    b. Mitral E/e' ratio > 14 (either lateral or septal)
    c. Elevated invasively-determined filling pressures previously (resting LVEDP>16 mmHg or mean pulmonary capillary wedge pressure [PCWP] > 12 mmHg; or PCWP/LVEDP≥25 mmHg with exercise)
    d. Acute heart failure decompensation requiring IV diuretics

4.3.2. Exclusion Criteria:

1. Supine systolic blood pressure <100 mm Hg
2. Pregnancy: Women of childbearing potential will undergo a pregnancy test during the screening visit
3. Orthostatic hypotension defined as >20 mm Hg decrease in systolic blood pressure 3-5 minutes following the transition from the supine to standing position
4. Uncontrolled atrial fibrillation, as defined by a resting heart rate>100 beats per minute
5. Hemoglobin < 10 g/dL
6. Inability/unwillingness to exercise
7. Moderate or greater left sided valvular disease (mitral regurgitation, aortic stenosis, aortic regurgitation), any degree of mitral stenosis, severe right-sided valvular disease, or presence of a prosthetic valve in the mitral position

8. Hypertrophic, infiltrative, or inflammatory cardiomyopathy
9. Clinically significant pericardial disease, as per investigator judgement.
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 either (1) subsequent revascularization, or; (2) a subsequent angiogram demonstrating the absence of clinically significant epicardial coronary artery disease, as per investigator judgement.
15. Left ventricular ejection fraction <45% in any prior echocardiogram or cardiac MRI, unless this was in the setting of uncontrolled atrial fibrillation.
16. Treatment with phosphodiesterase inhibitors that cannot be withheld
17. Treatment with organic nitrates
18. Significant liver disease impacting synthetic function or volume control (ALT/AST > 3x ULN, Albumin <3.0 g/dL)
19. eGFR < 30 mL/min/1.73m^2
20. G6PD deficiency. In males of African, Asian or Mediterranean decent, this will be formally evaluated by enzyme testing prior to drug administration. A negative screening test for G6PD will be required in these subjects for inclusion in the study. If a quantitative test is being performed, a clinically significant reduction in G6PD activity (<60% of normal) will exclude subjects.
21. Methemoglobinemia – baseline methemoglobin level >5%
22. Serum K>5.0 mEq/L
23. Severe right ventricular dysfunction
24. Any medical condition that, in the opinion of the investigator, will interfere with the safe completion of the study.
25. Contraindications to MRI (except as noted below), including the presence of a pacemaker, metal implants, claustrophobia, or that have known medical conditions which can be exacerbated by stress such as anxiety or panic attacks. Inability to lie flat in the MRI scanner for 90 minutes is also an exclusion criterion.

Participants who have contraindications for MRI but are otherwise good candidates for the study, may be enrolled. In this case, participants will undergo all study procedures except for the MRI scans.** *(See paragraph below for more detail)*

In addition, specific exclusion criteria for undergoing scanning in the 7-Telsa MRI scanning system include (these will only apply when the 7T scanner is used. In addition, a 3T scanner is also available at Penn, allowing the choice of the scanner depending on availability (to maximize safety, exclusion criteria for 7T will be applied at Penn for all participants, even if they ultimately are scanned at 3T):**

– ANY intra-luminal implant, filter, stent, or valve replacement
– ANY type of life assist device, pump, or prosthetic
– ANY vascular clip or clamp

– ANY surgically placed clips or clamps or bands on visceral organs
– ANY intracranial implants of any type other than dental fillings
– ANY non-removable piercings, jewelry, or medicinal patch
– ANY personal history of intraocular injury or fragment in or around the orbit that cannot be cleared through radiologic examination
– ANY personal history of bullet, shrapnel, or stabbing wounds that cannot be cleared through radiologic evaluation.

** If any of the above apply, we may investigate the issue further to ensure subject safety. This may include obtaining X-rays or obtaining reports from prior radiographic studies. We may also discuss the case with our radiologists and MRI technicians. In these circumstances, an MRI will only be performed if deemed safe by an attending radiologist on a case-by-case basis.

Subjects that qualify for a 3T or 7T scanner (for the leg MRI study) also qualify for scanning in a 1.5T scanner (for the cardiac MRI substudy). However, the opposite is not necessarily true. Subjects who are determined eligible to undergo scanning in a 3T or 7T scanner (in the primary study) are eligible to undergo scanning in the 1.5T scanner (for the cardiac MRI study). Some situations (such as many intraluminal stents and prosthetic valves) which would exclude scanning at high field (7T or 3T) are considered safe for 1.5T scanning. Subjects who are deemed ineligible to undergo 7T or 3T scanning in the parent trial, may still be enrolled in the ancillary myocardial perfusion study and undergo a cardiac MRI study (at 1.5T). In all cases, participation in the myocardial perfusion / cardiac MRI ancillary study will be approved by Dr. Kuruvilla, who has appropriate clinical expertise to assess the safety of this test, according to current clinical standards. Dr. Kuruvilla is the Director of Advanced Cardiac Imaging at the CMJC Philadelphia VA Medical Center and Assistant Professor at the University of Pennsylvania.

4.3.3. Criteria that will prompt exclusion from the trial at the 6 week visit

Subjects will be discontinued from study participation if they meet any of the following criteria at the 6-week study 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, including clinically significant reversible ischemia in a coronary distribution identified during the cardiac MRI substudy.
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. Although the ultimate authority/decision making will rely on each site PI, these ad-hoc discontinuations will be discussed with the other site PIs, the chair of the trial and the medical director of the IND for input.

Subjects who meet the criteria below will not be immediately discontinued, but will be scheduled for an ad hoc additional 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.

4.3.4 Conditions that will preclude participation in the myocardial perfusion ancillary study include:

1. Prior allergic reactions to gadolinium-based contrast agents.
2. Allergy, or prior severe adverse reactions to regadenoson or aminophylline.
3. 2nd degree AV block, 3rd degree AV block or high-grade AV block.

4.4 Randomized intervention

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

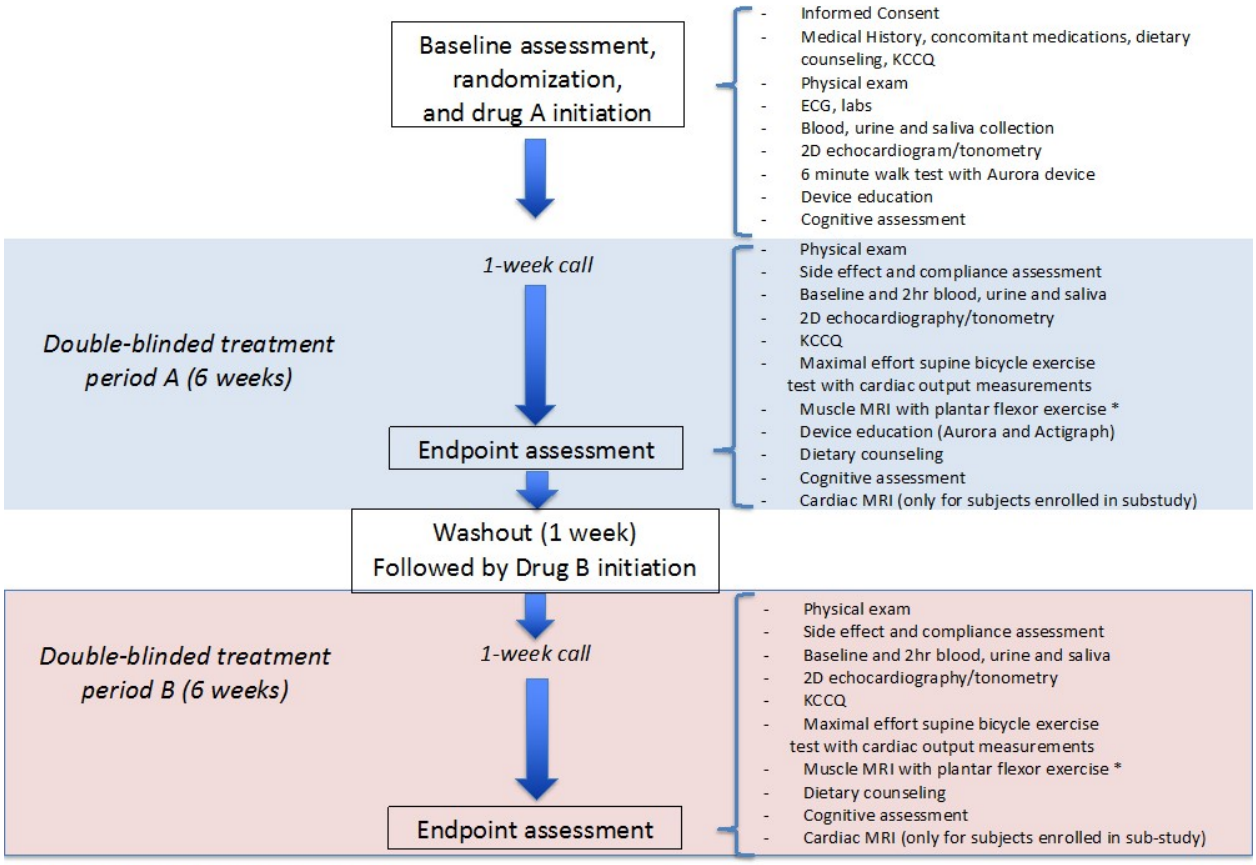
(A) Potassium nitrate: Potassium nitrate crystals will be purchased and placed into oral gelatin capsules with an inert filler (lactose monohydrate). Capsules will be prepared at the University of Pennsylvania Investigational Drug Service [3600 Spruce Street, Ground floor Maloney Building, Philadelphia, PA 19104]. Each capsule will contain 610 mg KNO_3-, corresponding to 6.03 mmoles of NO_3-, plus 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.

(B) Control drug: For the purpose of conducting this clinical trial, a control capsule will be made consisting of potassium chloride, granular, USP (450mg) plus lactose monohydrate, spray dried, NF (300mg) combined and packed into an identical capsule shell. We chose potassium chloride 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

A letter may be sent to potential subjects prior to initial contact. An overview of the study design and flow of study visits is presented in Figure 4.

Figure 4. Overview of the study design and procedures.



KCCQ – Kansas City Cardiomyopathy Questionnaire.
- *Not performed for subjects enrolled at the NW site*

The following investigational devices will be used during the study:

- Aurora device: wearable pulse collection device (watch)
- 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 subject eligibility is 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. Informed consent may be obtained in-person or virtually.

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 childbearing

potential. Blood will be collected for measurement of (a) comprehensive metabolic panel; (b) complete blood count; (c) NT-pro-BNP; and (d) methemoglobin. A pregnancy test (women of childbearing potential) and Glucose-6-phosphate dehydrogenase (G6PD) deficiency screening (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 level determination. Following the collection of baseline labs and biomarker samples, subjects will be provided with a standard low nitrate breakfast.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 will be instructed to avoid such foods while participating in the study.

Computerized cognitive assessment testing will be performed using cogstate software (Cogstate; New Haven, CT). The assessment includes a customized battery of seven tests that measure various cognitive domains. The battery includes the following tests:

- International Shopping List Test
- Gorton Maze Learning Test
- Detection Test
- Identification Test
- One Card Learning Test
- One Back Test
- International Shopping List Test- Delayed Recall (memory)

The system works with an internet server administered and maintained by Cogstate, which quantifies the subject's performance/responses and calculates cognitive scores. No personal identifiable information will be entered/uploaded into the server.

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 excluded from the study

Initial Visit Procedures
• Informed Consent [a] • Eligibility assessment • Medical History, Concomitant Medications • Dietary counseling • KCCQ •Physical Exam (including orthostatic vital signs and anthropometric measurements) •ECG, Laboratory tests (CBC, comprehensive metabolic panel, NTproBNP or proBNP, methemoglobin; pregnancy test and G6PD deficiency screening as needed) • Blood, urine and saliva collection • Cognitive assessment • 2D echocardiogram, arterial tonometry • Device (Aurora and Actigraph) education • 6 minute walk test with O_2 saturation and Aurora device
[a] Informed consent will be obtained before any study procedures

5.2. Randomization procedure

A stratified blocked randomization will be performed based on enrollment site (UPenn/Northwestern/ VAMC), sex, and ethnicity. A sufficient number of complete blocks will be generated for each stratum so that randomized assignments are available for every eligible subject. Strata-specific treatment order assignments and will be provided by a member of the data management team. 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.

To preserve blinding, the strata-specific treatment order assignments and the associated studyspecific patient identification number will be provided to each study IDS representative site in electronic format (excel spreadsheet) pre-sealed envelopes.

Upon determination of subject eligibility into the trial, the IDS representative will break the seal of the next envelope for the appropriate stratum to determine the treatment group assignment based on the appropriate stratum-specific scheme and will record the patient identification number for that study subject.

5.3. Intervention Phase 1

Subjects will be randomized to either active drug (KNO_3) or placebo (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 the shipment 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 for side-effect assessment. 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) will prompt exclusion of patients from the trial.

Subjects may experience symptoms upon dose uptitration that do not prompt exclusion from the trial, as per the criteria noted above (for example, but not limited to, mild dizziness, gastrointestinal symptoms, fatigue). In these instances, the Site Principal Investigator can reduce the dose back to 6-mmol capsule taken twice per day, for the remainder of the treatment phase. For subjects who have a baseline serum K 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/1.73m^2, 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 approximately 5 weeks into each interventional phase, the Aurora and Actigraph devices will be given to the subjects. The goal will be for the subjects to wear these devices 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 his/her next visit.

6-week endpoint assessment: Following ~6-weeks of therapy with KNO_3/KCl, subjects will return for endpoint assessments. Physical examination with measurement of orthostatic blood pressure, pill count, KCCQ, dietary counseling, cognitive assessment, 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 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. Exercise cardiac outputs will also be obtained using the same technique. An additional blood sample will be obtained at peak exercise. The Aurora watch will be worn during exercise when possible. Subjects enrolled at Penn or the VA will undergo an assessment of skeletal muscle mitochondrial oxidative capacity using MRI either before, or at least 3 hours after, peak VO_2 testing. Based on scheduling and availability, the MRI may be performed on a different day than the exercise study. The Kansas City Cardiomyopathy Questionnaire (KCCQ) will be administered.

Phase 1: 6-week Visit Procedures

- Interim medical history, concomitant medications

- Physical exam (including orthostatic vital signs)
- Pill count
- Blood, urine and sputum collection, blinded drug administration
- ECG, labs (CBC, comprehensive metabolic panel, methemoglobin, pregnancy test as needed, BD PAXgene RNA samples)
- KCCQ
- Blood, urine, and saliva collection approximately 2 hours post-administration
- Cognitive assessment
- 2D echocardiogram, arterial tonometry
- Cardiopulmonary test with resting and exercise cardiac output measurements, blood draw at peak exercise
- If feasible, plantar flexor exercise / MRI (fat/water imaging / Creatine CEST / PC-MRI); these measurements are not performed at the Northwestern site
- Data download of the Aurora device and Actigraph

***Additional visit for subjects participating in the Cardiac MRI sub-study*:** Subjects who participate in the MPR ancillary study will undergo a cardiac MRI with regadenoson administration. This will be scheduled during the last 2 weeks of the study intervention (4-6 weeks after initiation of drug administration in Phase 1), such that it will not interfere with the scheduling of the main trial visits. The cardiac MRI may take place on a different day than the exercise study or the lower extremity MRI scan and may occur either before or after the exercise endpoint study visit.

5.4. 1-week washout period

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

5.5. Intervention Phase 2

Following the washout period, subjects will receive either active drug (KNO_3) or placebo (KCl) during phase 2 of the trial. Subjects will receive the intervention (KNO_3 or KCl) that was not administered in phase 1, such that each subject will receive both study interventions in this cross-over design. The ~1-week call and the ~6-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 the shipment 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 for side-effect assessment. 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 6mmol 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) will prompt exclusion of patients from the trial.

Subjects may experience symptoms upon dose uptitration that do not prompt exclusion from the trial, as per the criteria noted above (for example, but not limited to, mild dizziness, gastrointestinal

symptoms, fatigue). In these instances, the Site Principal Investigator can reduce the dose back to 6-mmol capsule taken twice per day, for the remainder of the treatment phase.

At approximately 5 weeks into each interventional phase, the Aurora and Actigraph devices will be given to the subjects. The goal will be for the subjects to wear these devices 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 his/her next visit.

6-week endpoint assessment: Following ~6-weeks of therapy with KNO_3/KCl, subjects will return for a repeat endpoint assessment. Physical examination with measurement of orthostatic blood pressure, pill count, KCCQ, dietary counseling, cognitive assessment, 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. Exercise cardiac outputs will also be obtained using the same technique. An additional blood sample will be obtained at peak exercise. The Aurora watch will be worn during exercise when possible. Subjects enrolled at Penn or the VA will undergo repeat assessment of skeletal muscle mitochondrial oxidative capacity using MRI either before, or at least 3 hours after, peak VO_2 testing. Based on scheduling and availability, the MRI may be performed on a different day than the exercise study. The Kansas City Cardiomyopathy Questionnaire (KCCQ) will be administered. Following the final endpoint assessment, subjects' participation in the study will be over.

Phase 2: 6-Week Visit (final visit) Procedures

- Interim medical history, concomitant medications
- Physical exam (including orthostatic vital signs)
- Pill count and side effect assessment
- Blood, urine, and sputum measurements, followed by blinded drug administration
- ECG, labs (CBC, comprehensive metabolic panel, methemoglobin, pregnancy test as needed, BD PAXgene RNA samples)
- KCCQ
- Blood, urine and, saliva collection approximately 2 hours post-administration
- Cognitive assessment
- 2D echocardiogram, arterial tonometry
- Cardiopulmonary test with resting and exercise cardiac output measurements, blood draw at peak exercise
- If feasible, plantar flexor exercise / MRI (fat/water imaging / Creatine CEST / PC-MRI); these measurements are not performed at the Northwestern site
- Data download of the Aurora device and Actigraph

Additional visit for subjects participating in the Cardiac MRI Sub-study: Subjects who are to participate in cardiac MRI sub-study will undergo a Stress Cardiac MRI with regadenoson administration. This will be scheduled during the last 2 weeks of the study intervention (4-6 weeks after initiation of drug administration in phase 2), such that it will not interfere with the scheduling of the main trial visits. The cardiac MRI may take place on a different day than the exercise study or the lower extremity MRI scan and may be performed either after or before the exercise assessment.

5.6 Adverse Event Reporting

All adverse events 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 per Section 7 in 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 Data Safety Monitoring Board, the Sponsor andand 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, including pill count; (3) adverse effects, (4) specific reason for withdrawal.

Early Termination Visit Procedures
• Vital signs and physical exam
• Pill count and medication adherence log
• Safety labs
• Adverse event assessment
• Documentation of reason for withdrawal
• Retrieval of Aurora and Actigraph devices and data download

5.8. Concomitant Medication

Subjects should be treated with standard of care medications for HFpEF or associated comorbidities. As per inclusion criteria, subjects should be on a stable medical regimen for HFpEF prior to entry. Further adjustment of diuretics or 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 contra-indicated during the study period.

6. DRUG DISPENSING, ACCOUNTABILITY, AND DESTRUCTION

Drug dispensing will be managed by the Penn Investigational Drug Pharmacy, in collaboration with the Northwestern Investigational Drug Pharmacy. KNO_3 and KCl capsules will be compounded at the Penn IDS. Penn IDS will bottle, label, and dispense product for the Penn and VA sites. Penn IDS will ship product to Northwestern IDS for bottling, labeling, and dispensing of product at the Northwestern site. Prior to initiation of each phase, participants will receive a sufficient supply of KNO_3 or placebo 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) and ±1 week for the 6-week 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 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 and the Sponsor has authorized it.

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 respective 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 ofemergency 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 (i.e., if any one of these 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

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 is unlikely to be implicated, but there is a low likelihood that a causal relationship exists.
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 with HF with preserved ejection fraction (HFpEF).

1. Unplanned hospitalization, ER visit or clinic visit for worsening HF
2. Arrhythmias, particularly atrial fibrillation
3. Sudden cardiac death
4. Acute coronary syndrome
5. Cerebrovascular event
6. Venous thromboembolism
7. Lightheadedness
8. Worsening renal function
9. Shortness of breath at rest or during/after exercise
10. Fatigue at rest or during/after exercise

The following are potential expected side effects of KNO_3:

1. Stomach discomfort
2. Slight headache
3. Dizziness

4. New onset or worsening lightheadedness
5. Low blood pressure
6. Stomach ache, diarrhea, nausea, or vomiting
7. Worsening shortness of breath
8. Worsening fatigue
9. Flushing
10. Rash
11. Mild orthostatic hypotension

Clinically significant methemoglobinemia or significant symptomatic orthostatic hypotension (20 mmHg or greater reduction in systolic blood pressure with associated symptoms such as dizziness upon standing) are potential expected side effects to KNO_3, although we will consider them unexpected given prior data in this patient population with this particular drug and dosing scheme.

The following are potential expected side effects of regadenoson administration (for subjects participating in the cardiac MRI sub-study):

1. Headache
2. Dizziness
3. Nausea or stomach discomfort
4. Decreased sense of taste
5. Mild chest discomfort
6. Shortness of breath
7. Flushing (warmth, redness, or tingly feeling under the skin)

These side effects are transient and generally resolve upon clearance of the drug and/or reversal of drug effects with dypiridamole.

The following are potential expected side effects of gadolinium-based contrast administration (for subjects participating in the cardiac MRI sub-study):

1. Injection site pain (from the needle)
2. Burning at injection site (if the dye contacts the skin)
3. Headache
4. Dizziness or faintness
5. A decrease in blood pressure
6. Nausea
7. Vomiting
8. Sweating
9. Paresthesias

These side effects occur in a small minority of patients, but if they do occur they will be noticed within minutes of the injection and are transient in nature.

7.3. Recording and Reporting of Adverse Events

The site PIs 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 PIs will be responsible

for ensuring that all adverse events are noted, followed and reported to the Sponsor database and IRB as per local requirements.

SAEs occurring from the time of *signed informed consent* to the Week 15 phone visit will be captured on a CIOMS form. AEs will be classified according to the guidelines/definitions specified in section 7 of the protocol. Any AE rated >=3 in severity and all SAEs 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 (at psom-ind-ide@pobox.upenn.edu), and the medical director (Dr. Townsend), with CC to the study chair (Dr. Chirinos). The IRB should be notified as per institutional guidelines at each respective site. The medical director 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 investigator or qualified designee will enter the required information regarding the AE into the appropriate module of the eCRF, All study procedures and cumulative adverse events are subject to full IRB review at least yearly and DSMB review every 6 months.

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 a modification to study drug is appropriate, the PI must obtain written approval from the Sponsor to proceed with this exception to the protocol.

7.4. Follow-up

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

The DSMB will review detailed safety data approximately every 6 months throughout the study, as detailed in the Data Safety Monitoring Section of this protocol and in the Data Safety Monitoring Charter.

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 occurring at his/her site and evaluate for "unexpectedness" and relationship to study drug. The Site Investigator is required to complete a report for any event identified as serious, study drug related and unexpected, using the CIOMS format, and to submit it to the Sponsor within 1 working day of the PI or site personnel being aware of it. The sponsor will submit a safety report according to regulations.

A copy of the report sent by the PI to the Sponsor should be kept at the site Regulatory Binder

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 Sponsor within the same

timelines as an SAE, and to the IRB per local requirements . The pregnancy will be recorded on the appropriate note to file. The drug will be discontinued immediately and subject terminated 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 or SAE case report form.

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.[111] 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.

8.2. Quality of life assessment

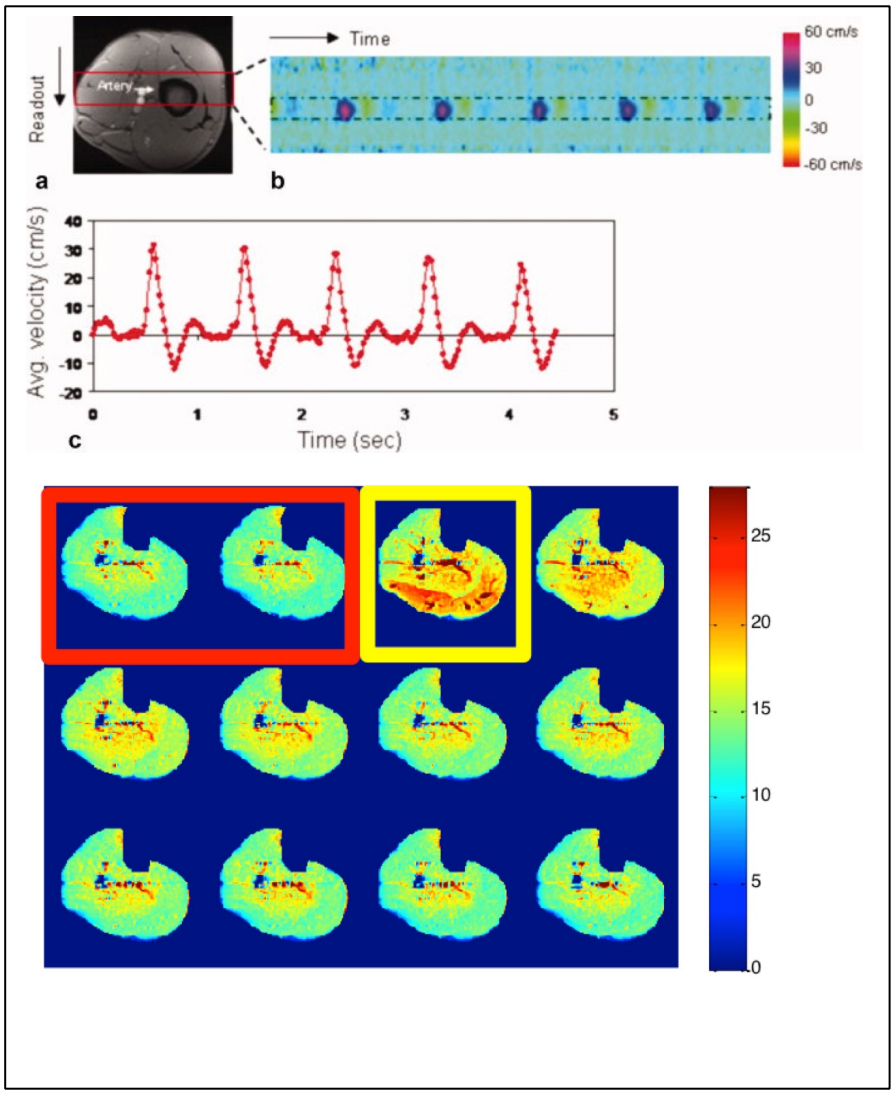
This will be assessed with the Kansas City Cardiomyopathy Questionnaire (KCCQ).[124] We will administer the KCCQ to subjects prior to randomization and at the end of each intervention phase (6-week time point in both phases). This is a validated 23-item questionnaire that assess physical function, symptoms, 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. Assessment of the systemic vasodilatory response to exercise

Blood pressure will be measured with a validated oscillometric device at rest, at each stage of exercise and after exercise. Cardiac output will be measured at rest and at each stage of exercise using echocardiography. Systemic vascular resistance (SVR) will be calculated at rest and at peak exercise as mean arterial pressure / cardiac output. Systemic vasodilatory reserve will be measured as the reduction in SVR during exercise, relative to SVR at rest ([rest SVR– peak exercise SVR] / rest SVR).[111] Depending on equipment availability at each site, we may also monitor and record muscle oxygenation in the calf (lateral aspect of left gastrocnemius) and forearm (flexor digitorum superficialis) during exercise non-invasively using a Portamon or Portalite near-infrared spectroscopy device (Artinis Medical Systems; The Netherlands). Both the Portamon and Portalite are investigational devices and not currently approved medical devices in the U.S.

8.4. Assessment of muscle perfusion and energetics

If available equipment and personnel are available at the time of enrollment, MRI studies will be performed at rest and immediately after a standardized plantar flexion exercise test using a 3T or a 7T scanner equipped with a 15-channel transmit/receive radiofrequency coil and an MR compatible pneumatically controlled foot pedal. Phase contrast MRI using a fast 1D projection technique[125] will be used to image blood flow with high temporal resolution (Figure 5a-c) .



The primary measure of recovery kinetics will be measured with creatine chemical exchange saturation transfer (CrCEST), except for subjects enrolled at the Northwestern site. Sample CrCEST recovery images of the calf are shown in figure 5d. The first 2 acquisitions are pre-exercise (red box), with the remainder representing sequential acquisition immediately after cessation of exercise. Recovery kinetics of creatine levels will be quantified. Phosphorus spectroscopy is limited by the lack of anatomic information, being able only to assess PCr levels in a large voxel of tissue. In contrast, the chemical exchange saturation transfer (CEST) technique, a novel sensitivity enhancement method developed by our group,[75, 126-128] can detect metabolite content based on exchangerelated properties at either 7T[127, 128] or 3T,[126] utilizing hydrogen signal

(thus not depending on the much weaker phosphorus signal). Creatinine exhibits a concentration dependent CEST effect between its amine (-NH_2) and bulk water protons (CrCEST)[75] which allows for the monitoring of Cr concentration changes with high spatial and temporal resolution. In contrast to phosphorus spectroscopy, this technique allows for spatial mapping (creation of an highresolution anatomic image) of PCr levels (figure 3D). This cannot be accomplished with phosphorus spectroscopy. This novel technique can be used to assess PCr recovery kinetics following exercise, allowing for accurate selection of muscle tissue (rather than fat or bone). More importantly, the serial anatomically mapped PCr information can be used to assess oxidative capacity of muscle tissue from specific pixels in the image. We will also assess muscle fat using a 3-point Dixon separation technique and hydrogen spectroscopy. We will exclude fat from CrCEST computations.

8.5. 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 60 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.[129, 130] We note that the latter is not simply based on the inflow propagation velocity, but rather on solving Euler momentum equation.[129] This method is able to accurately assess LV relaxation.[129] Systolic function will be assessed via systolic myocardial strain (using speckle tracking echocardiography), which we have successfully applied in previous studies.[92, 95, 131]

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

We will use a high-fidelity Millar applanation tonometer[80] 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.[92, 132] We have successfully implemented this method in multiple previous studies[79, 83, 92, 93, 133] and have published a tutorial detailing our analysis methods.[78, 79] 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.[132] After computation of aortic input impedance, proximal aortic characteristic impedance (Zc) will be computed in the frequency domain as previously described.[132] Pressure and flow harmonics were separated into forward and backward components using standard wave separation analysis.[78, 79, 132, 134] 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. As reflection coefficient 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.[135] 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.[79, 80] The relation between QZc and measured pressure reveals the direct effect of wave reflections on the arterial system.[136] 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,[137, 138] will also be measured[80, 139] using a Sphygmocor device (Atcor Medical).[137] 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.

8.7. 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 approximately 6 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.[118] 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[140] and later adapted by Allen et al for human blood.[141]

8.8 Cognitive Assessment

Computerized cognitive assessment testing will be performed using cogstate software (Cogstate; New Haven, CT). These tests will be administered to subjects prior to randomization and at the end of each intervention phase (6-week time point in both phases).The assessment includes a customized battery of seven tests that measure various cognitive domains. The battery includes the following tests:

COGNITIVE ASSESSMENT TESTS			
TEST NAME AND INSTRUCTIONS	**COGNITIVE DOMAIN**	**PRIMARY OUTCOME**	**INTERPRETATION**
International Shopping List Test Tell me the items on the shopping list.	Verbal learning	Number of correct responses remembering the word list on three consecutive trials	Higher score = Better performance
Gorton Maze Learning Test Find the hidden pathway.	Executive function	Number of errors learning the same hidden pathway across the consecutive learning trials	Lower score = Better performance
Detection Test Has the card turned over?	Psychomotor function	Speed of performance; mean of the $\log_{10}$ transformed reaction times for correct responses	Lower score = Better performance
Identification Test Is the card red?	Attention	Speed of performance; transformed reaction times for correct responses	Lower score = Better performance
One Card Learning Test Have you seen this card before in this test?	Visual learning	Accuracy of performance	Higher score = Better performance
One Back Test Is the card the same as the previous card?	Working memory	Speed of performance; transformed reaction times for correct responses	Lower score = Better performance
International Shopping List Test - Delayed Recall Tell me the items on the shopping list that you learned earlier.	Memory	Number of correct responses	Higher score = Better performance

8.9. 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.

Physical Activity: We will measure activity level in all subjects with the Actigraph device, which has been previously validated. This device can be worn on the wrist, and measures acceleration each minute in the anterior-posterior, mediolateral, and vertical axis, and summarizes that information as a vector magnitude. The software will use this data to calculate physical activity in METS and steps. The accelerometer will be provided during both phase 1 and phase 2. We will give the devices (Aurora [see below] and Actigraph) to the subjects during approximately the final week of each interventional phase. We will ask the subjects to put the devices on and will provide informational materials to assist with device placement. We will also discuss device placement with the subjects at the study visits as well as over the phone as needed. Subjects will bring the devices to the endpoint assessment visits. Subjects will be fully oriented in the use of this device. The device may be removed for showering or swimming but otherwise should be worn continuously. The specific details in the use of this device will be provided to study subjects. We will use the average METS of activity over this time period as an estimate of physical activity. We will also records steps taken.

Ambulatory Pulse Wave Analysis: Ambulatory pulse wave analysis will be performed, when feasible, with a novel watch research device (Microsoft Aurora device). This device is not commercially available or FDA approved and is investigational. It continuously measures the radial pulse via a watch-mounted tonometer. The device also measures transit time from the heart to the wrist in an ambulatory fashion. The device will be worn outside of the exercise lab, as well as during the exercise studies themselves. The specific details in the use of this device will be provided to study subjects.

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.

Myocardial Perfusion Reserve: Subjects who agree to participate in the cardiac MRI sub-study will undergo a Stress Cardiac MRI during the last 2 weeks of each cross-over treatment phase. Study parameters assessed during this MRI scan will include: assessment of myocardial perfusion, assessment of myocardial fibrosis, and myocardial strain.

Myocardial perfusion: Stress perfusion images will be obtained following IV injection of regadenoson. Three to four short-axis perfusion images will be acquired each heart beat during the injection of the gadolinium-based contrast agent at a rate of 3 to 4 mL/s via a power injector during first pass of the contrast agent. Forty to sixty image frames will be obtained. Following reversal of regadenoson with intravenous aminophylline, cine images and delayed enhancement images (for focal fibrosis) will be obtained. At the end of the study, perfusion images will be obtained at rest using the same imaging protocol. Myocardial perfusion will be assessed from the time-intensity curves of the myocardium and blood pool. Overall MPR will be obtained as the ratio

of myocardial perfusion after vasodilator administration divided by the myocardial perfusion after reversal of the vasodilator ("rest" images).

Myocardial fibrosis: We will use cardiac MRI to assess diffuse myocardial fibrosis by computing the extracellular volume fraction from pre- and post-gadolinium normalized myocardial 1/T1 and blood 1/T1 changes, after correction for hematocrit. T1 mapping pre- and post- administration of a macrocycic gadolinium-based contrast agent (i.e., Gadobutrol 0.1 mmol/kg or an equivalent dose of Dotarem) will be performed using a modified Look-Locker inversion recovery (MOLLI) sequence with image analysis using CMR42 software (Circle CVI, Calgary, AB, Canada).

Myocardial strain: We will assess myocardial longitudinal and circumferential strain using Cine Displacement ENcoding with Stimulated Echoes (DENSE) sequences.

9. MULTICENTER MANAGEMENT PLAN

9.1. Study Chair/Grant Principal Investigator

Dr. Chirinos will serve as the Study Chair and grant Principal Investigator for this protocol. In this role, he will administer the grant, and communicate with the NIH about study progress. He will communicate with site PIs regarding the science and any operational issues that arise during the trial, establish subcommittees and working groups to complete specific activities, monitor study implementation across sites (Penn, Northwestern, and Corporal Michael J. Crescenz VAMC), and hold regular investigator calls/meetings. As DSMB coordinator, he will convene an independent DSMB, communicate with the DSMB, and participate in open DSMB meetings. Dr. Chirinos will supervise the investigative team to develop and coordinate procedures and generate reports and presentations. He will maintain communication with the DSMB and will provide central fiscal management and reporting. Other roles of Dr. Chirinos will include directing the echocardiography and arterial hemodynamics core laboratory for this trial.

9.2. Site Principal Investigators

A principal investigator will lead operations at each enrollment site (Penn, Northwestern, and Corporal Michael J. Crescenz VAMC) 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. In conjunction with the Sponsor, they will oversee the safety of study participants. They will interact with the Sponsor team, including the Medical Director, the data management team, the study chair and the IND sponsor regulatory lead, as needed.

9.3. 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, Payman Zamani, Sanjiv Shah, Sujith Kuruvilla, and Jesse Chittams. Minutes of these meetings will be distributed and kept on file. The study chair and the site PIs will have authority to make

decisions that require immediate attention when the committee is unavailable to meet. The study chair will adjudicate disagreements within the committee. The sponsor has the final responsibility for all study decisions.

9.4. 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 PIs, the study coordinators at both enrollment sites, the post-doctoral researcher in charge of processing the MRI fat/water imaging, creatine-CEST and PC-MRI data, the echocardiography/tonometry quantification technician and staff members of the biostatistical team. 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.

9.5. Data Management

The UPENN site will serve as the data coordinating center (DCC) and will play a fundamental role in overseeing randomization, data entry, data coordination, data transfer, and DSMB meetings. Dr. Alexandra Hanlon will be director of the DCC. Within the UPENN School of Nursing, DCC will have access to state-of-the-art computing resources capable of supporting studies ranging in size from one to more than 100,000 subjects. The equipment is fully supported by the Office of Technology and Information Systems (OTIS), which has complete business continuity and disaster recovery via redundant, distributed data centers and backup processes, in addition to traditional office and project management software.

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, located at each study site. 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 DCC launches the REDCap database 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), and the DCC will perform comprehensive data quality checks.

9.6. Quantification Core Laboratories

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

9.6.1 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.

9.6.2 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)[83, 93, 95, 97, 142-145] 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[102] and has adequate capabilities required for physiologic data management and quantification in this study. The core lab will have ad hoc teleconference calls with Dr. David Poole (consultant in this grant). Dr. Poole will provide input about specific datasets in which there may be questions about physiologic phenomena. In addition to enhancing the interpretation of our human datasets, this will facilitate scientific interactions and will spark new ideas that will facilitate the translation of human observations to animal models and vice-versa. The physiology core lab will hold monthly calls with a representative of the data management core to discuss issues regarding core lab data management.

9.6.3 MRI core laboratory: The MRI quantification core lab will be directed by Dr. Ravinder Reddy and will include a designated post-doctoral researcher (35% effort throughout the grant duration) who will be in charge of implementing the scans, performing QC of data as the study progresses, and performing standardized quantification of MRI images. Dr. Reddy's laboratory has extensive experience in MRI quantification, with >100 publications in quantitative MRI and 14 completed studies using CEST imaging in the last 5 years.[75, 126, 128, 146-155] The MRI core lab will hold monthly calls with a representative of the data management core to discuss issues regarding core lab data management.

9.6.4 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).

9.6.5 Data Safety Monitoring Board: The DSMB will provide independent oversight of the project. The DSMB will be responsible for assessing: 1) baseline comparability between groups; 2) participant accrual rate and retention; 3) resource availability; 4) data quality with special emphasis on eligibility data; and 5) patient safety. The board will make recommendations regarding: study continuation, protocol modification, and review of additional data. The conference call meetings and progress reports will be set by the DSMB.

9.6.6 Cardiac MRI core laboratory: Cardiac MRI quantification will be supervised by Dr. Kuruvilla, with support from Dr. Chirinos. Dr. Kuruvilla will have access to software available in Dr. Chirinos' core laboratory, including CMR 42 software and Matlab software.

9.7 Conflict of Interest Management

Dr. Chirinos is named as inventor on a patent application which has been assigned to the University of Pennsylvania. This constitutes Clinical trial intellectual property (as defined under University of Pennsylvania policy) relevant to this trial. Therefore, the Offices of Research Services and Vice Provost for Research have formulated a management plan for Dr. Chirinos' participation in the trial as Study Chair and Grant PI. Notification of thist management plan will be distributed to DSMB members, site PIs and Sub-Investigators and the operations of the trial will adhere to this plan.

10. STATISTICAL CONSIDERATIONS

10.1. Power calculations

We will randomize 84 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.[156] 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 84 subjects in this cross-over trial will have 80% power to detect such an effect size 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 in our pilot trial or from repeated measurements of the Kansas City Cardiomyopathy Questionnaire in HFpEF 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, 5 seconds for PCr/Cr recovery kinetics, 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.[111] Therefore, we are confident that we will achieve adequate power not only for our clinical endpoints, but also for our physiologic measures. PASS11157 was used to perform power analyses.

10.2. Data Analysis Plan

The co-primary outcome variables will be peak VO_2 and work performed during a symptom-limited maximal effort exercise test. All secondary outcome measures are continuous variables. The predictor of interest for all aims will be intervention (KNO_3 therapy vs. placebo), 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 O_2 / work performed (co-primary outcomes) and quality of life score (secondary outcome). 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[158] 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.[159-161] 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 (eg,QQ plots to assess normally distributed residuals for valid Wald tests). All mixed-effects models will be analyzed using the SAS PROC MIXED procedure[162] in SAS version 9.3 (SAS Institute, Inc., Cary, NC). 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.[163] 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[164] 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.[165]

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[166]; (2) Glorfeld's[167] 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[168]; (3) high internal consistency (an alpha coefficient of $\geq$ .70) for unit-weighted factors,[169] and (4) interpretability.[170] The heaviest weight will be placed on the Minimum Average Partial and parallel analysis methods, with the scree test as a visual adjunct.[171] 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.

10.3. Subgroup analyses and considerations about sex

Although women represent 60% of patients with HFpEF, our sample will include 50% women and 50% men. This is due to the fact that we explicitly wish to perform sex-stratified analyses, in

addition to testing for a potential modifying effect of sex on the effects of KNO_3. Having an equal balance in terms of the number of males and females maximizes the statistical power to test for significant interaction effects between sex and other variables of interest. Furthermore, the slight increase in the proportion of males sampled will help to maximize the statistical power for sub-analyses including only males. Balancing the sample on sex will also reduce the chances of having confounding between the race effect and other variables in the model. Lastly, since the over sampling of males is only slightly above the estimated actual population of 40%, the results of the analysis of the sample data should still be generalizable to the overall HFpEF population.

Additional pre-specified subgroup analyses will include diabetes vs. no diabetes, hemoglobin value above and below median, and use vs. non-use of calcium channel blockers.

10.4 Cardiac MRI sub-study analysis and power calculations

The aim of this ancillary study is to assess the paired difference in myocardial blood flow and perfusion reserve in response to KNO_3 or the control intervention (KCl) in a cross-over design. We will therefore utilize paired-t tests to estimate the difference. The non-parametric Wilcoxon sign-rank test will be considered, as appropriate. For exploration of the relationship between MPR and myocardial fibrosis or myocardial function, we will compute Pearson's correlation coefficients for linear associations, or the Spearman's Rank Correlation coefficient for monotonic non-linear associations. A p-value less than 0.05 will be considered significant.

Power calculations: The standard deviation of the change in MPR in repeated measures in prior studies is 0.5. Assuming a dropout rate of 10% (effective n=18), the ancillary study will have 85% power to detect a standardized effect size of 0.75 or greater. Therefore, our study will be powered to detect an improvement of 0.375 in MPR with KNO_3 vs. KCl.

11. PROTECTION OF HUMAN SUBJECTS

11.1. Potential benefits of the proposed research, 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 peripheral mechanistic abnormalities in HFpEF patients. Furthermore, if improving these peripheral 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 HFpEF, 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 HFpEF, which is urgently needed to help millions of patients. 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 84 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.

Subjects will be enrolled with explicit assurance for adequate representation of women and African Americans. Assuring representation of women is key for the scientific aspects of this disease (high incidence in women). Assuring adequate representation of African-Americans is key for this intervention with a nitric oxide donor (since African Americans with HF and reduced EF have demonstrated differential responses to NO donors compared to Caucasians).[172-175] Children will not be enrolled. Participants will be recruited from the Hospital of the University of Pennsylvania, Northwestern University Medical Center, and the Corporal Michael J. Crescenz VAMC. Assuring this gender and ethnic distribution will not be a problem given the clinical populations in the enrolling medical centers.

The study involves various tests (arterial tonometry, Doppler echocardiography, a cardiopulmonary exercise stress, a lower extremity MRI without gadolinium administration with a standardized plantar flexor exercise protocol, blood draws) and the administration of randomized therapy (potassium nitrate vs. placebo). The ancillary study involes a cardiac MRI with gadolinium and regadenoson administration.

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.[51] Importantly, in our study of a single dose of inorganic nitrate in this specific population (HFpEF), we did not observe any change in blood pressure following nitrate ingestion. This suggests that subjects with HFpEF may have a different response to nitrate than other populations and thus hypotension may be a less pronounced side effect. Importantly, the lack of a blood pressure response to nitrate has been shown in both elderly [176] and diabetic individuals,[177] further 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.[178] We did not find a reduction in ambulatory blood pressure. Therefore, although we don't 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. [28, 33-36] 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).[179] 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 (same dosing scheme used in the KNO_3CK OUT HFpEFp trial) and did not find any clinically-significant elevations in methemoglobin. We don't anticipate any significant issues with methemoglobinemia in the KNO_3CK OUT HFpEFp study since this is a problem that has been reported with nitrite, but not with nitrate at the doses used in this study.

Pilot safety data: In our double-blind randomized controlled trial in HFPEF, in which 12.9 mmol of inorganic nitrate 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,[180] 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.[178]

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[181], although the relationship between nitrate intake and cancer has not been demonstrated in humans.[31, 106, 182] 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.[183, 184185, 186] 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.[189, 190] 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.[191] 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.

Lower extremity MRI: The non-invasive nature and the lack of ionizing radiation make the risks associated with MRI small. A MRI scan requires the participant to be in a partially enclosed space inside the scanner. Some people may find this to be uncomfortable. Claustrophobia is an exclusion criterion for the study. The MRI scanner produces different types of noises during a scan. Participants are given special earplugs to reduce the noise. A MRI scanner has a strong magnet which attracts certain metals. If anyone has these types of metal in their body, the MRI's strong magnetic field can cause them to move which may cause injury. Subjects with be thoroughly screened for the presence of body metal and excluded from the study if they are unable to safely undergo the MRI study. If body metal/device may be present, we may investigate the issue further by obtaining X-rays or prior radiographic reports. We may also discuss the case with our radiologists and MRI technicians. An MRI will only be performed if deemed safe by an attending radiologist.

The greatest risk of MRI scanning is a metallic object flying through the air toward the magnet and hitting the subject. To reduce this risk we require that all people involved with the study remove all metal from their clothing and all metal objects from their pockets. No metal objects are allowed to be brought into the magnet room anytime. In addition, once the subject is in the magnet, the door to the room will be closed so that no one inadvertently walks into the magnet. Some of the imaging sequences and/or RF coils are not FDA approved but are considered nonsignificant risk investigational devices.

Risks of 7.0T MRI: To date no persistent adverse effects have been reported by facilities with magnetic field strengths at 7.0T. However, there has been an increase in the number of people reporting transient dizziness or nausea upon being moved into the field. This dizziness lasts less than 10 minutes and can be reduced by reducing the speed at which the subject is placed into the magnet. Risks are not cumulative and effects are transient. In addition, there has also been an increase in the number of subjects reporting a metallic taste in their mouth over that of 1.5 and 3.0T systems. The FDA has approved the use of 7.0T magnets for research imaging in humans. All subjects will be informed of the possibility of having these sensations during the informed consent process.

If an X-ray is deemed to be necessary to investigate any potential metal in the body, the subject will be exposed to minor doses of radiation:

For this procedure:	* An adult's approximate effective radiation dose is:	Comparable to natural background radiation for:
ABDOMINAL REGION:		
Computed Tomography (CT)-Abdomen and Pelvis	10 mSv	3 years
Computed Tomography (CT)-Abdomen and Pelvis, repeated with and without contrast material	20 mSv	7 years
Computed Tomography (CT)-Colonography	6 mSv	2 years
Intravenous Pyelogram (IVP)	3 mSv	1 year
Radiography (X-ray)-Lower GI Tract	8 mSv	3 years
Radiography (X-ray)-Upper GI Tract	6 mSv	2 years
BONE:		
Radiography (X-ray)-Spine	1.5 mSv	6 months
Radiography (X-ray)-Extremity	0.001 mSv	3 hours
CENTRAL NERVOUS SYSTEM:		
Computed Tomography (CT)-Head	2 mSv	8 months
Computed Tomography (CT)-Head, repeated with and without contrast material	4 mSv	16 months
Computed Tomography (CT)-Spine	6 mSv	2 years
CHEST:		
Computed Tomography (CT)-Chest	7 mSv	2 years
Computed Tomography (CT)-Lung Cancer Screening	1.5 mSv	6 months
Radiography-Chest	0.1 mSv	10 days
DENTAL:		
Intraoral X-ray	0.005 mSv	1 day
HEART:		
Coronary Computed Tomography Angiography (CTA)	12 mSv	4 years
Cardiac CT for Calcium Scoring	3 mSv	1 year
MEN'S IMAGING:		
Bone Densitometry (DEXA)	0.001 mSv	3 hours
NUCLEAR MEDICINE:		
Positron Emission Tomography – Computed Tomography (PET/CT)	25 mSv	8 years
WOMEN'S IMAGING:		
Bone Densitometry (DEXA)	0.001 mSv	3 hours
Mammography	0.4 mSv	7 weeks

(data from http://www.radiologyinfo.org/en/info.cfm?pg=safety-xray, accessed 10/5/2016; this website is maintained by the Radiological Society of North America)

Plain film X-rays expose subjects to very low doses of radiation. From this table, it can be seen that a bone X-ray of an extremity would expose subjects 0.001 mSv, which is less radiation than an intraoral X-ray performed at the dentist's office (0.005 mSv) and less radiation than a chest X-ray (0.1 mSv).

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.[192, 193] Nonrevascularized 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[194] 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.[195, 196] If the arterial saturation falls to below 88% ("severe exercise induced hypoxemia"[196]), 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 23%, 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 noninvasive 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.

Ambulatory pulse wave analysis (Aurora device) may cause mild discomfort in the wrist.

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 HFpEF 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.

11.2.3. Potential Risks of Cardiac MRI sub-study procedures:

Subjects who have agreed to participate in the cardiaccardiac MRI sub-study will undergo a cardiac MRI with IV gadolinium and IV regadenoson administration. The risks entailed with performing a MRI scan are described under lower extremity MRI in the risk section.

Gadolinium-based contrast agent administration: During the MRI examination, subjects will be administered a gadolinium-based contrast agent (GBCA), which allows for the quantification of diffuse interstitial fibrosis and the visualization of focal scars. Gadolinium-based contrast agents have been linked to increased risk of nephrogenic systemic fibrosis (NSF), a disease that causes fibrosis of the skin and connective tissues throughout the body and that can cause death. There is no known treatment for NSF. While there seems no increased risk for NSF in subjects with normal renal function or only moderate renal insufficiency, risk for NSF seems to increase in subjects with severe renal insufficiency (glomerular filtration rate (GFR) <30 mL/min/1.73m2). In a retrospective study with Omniscan (one of the five FDA approved GBCAs), 4% of subjects with severe renal insufficiency were estimated to be at risk for NSF.[197] Allergic reactions, which may include rash, sweating, itching, hives, facial swelling, and anaphylactic shock, may occur with GBCAs independent of renal function. No increased risk of gadolinium administration is anticipated among patients taking the study drug. There is recent data showing that traces of gadolinium may remain in the body long-term after contrast administration. However reviews by the FDA have not identified adverse health effects from gadolinium retained in the brain or bodily tissues after MRI. Furthermore, the gadolinium agent that is used in this study belongs to a group of macrocyclic gadolinium based agents that are thought to minimize or eliminate this risk.

Regadenoson: Regadenoson will be administered intravenously as part of the stress portion of the exam in order to induce coronary vasodilatation. Regadenoson is used as part of both cardiac MRI and cardiac nuclear stress testing protocols on a daily basis for clinical assessment of ischemia. Common side effects of regadenoson include headache, dizziness, nausea, abdominal discomfort, chest discomfort, flushing and shortness of breath.

Venipuncture and IV placement: As described above.

Potential risk of investigational devices: The Aurora device (pulse wave analysis watch) can produce skin irritation at the tonometer site. This usually goes away within few hours of discontinuing use. The version of the device that we will use for exercise studies uses adhesive electrodes attached to the participant's skin to record the electrical signal from the heart. These may occasionally cause skin itching and irritation.

The Portomon and Portalite (near infrared spectroscopy, or NIRS) devices have no known risks. They are non-invasive devices applied on top ofto the skin to measure the oxygenation of underlying tissue.

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, Corporal Michael J. Crescenz VAMC, and the Northwestern University 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 the MRI study:

Subjects will be thoroughly screened for the presence of body metal and excluded from the study if they are unable to safely undergo an MRI study. We will exclude patients who are not suitable candidates for an MRI study by virtue of having the following absolute or relative contraindications: (i) Central nervous system aneurysm clips; (ii) Implanted neural stimulators; (iii) Implanted cardiac pacemaker or defibrillator; (iv) Cochlear implant; (v) Ocular foreign body (e.g. metal shavings); (vi) Other implanted medical devices: (e.g. drug infusion ports); (vii) Insulin pump; (viii) Metal shrapnel or bullet; (ix) Claustrophobia; (x) Extreme obesity rendering the patient unable to fit into narrow-bore scanners; (xi) Unwillingness of the patient to undergo an MRI. All patients with metallic implants will be individually evaluated prior to MRI. Women with child-bearing potential will have a serum pregnancy test to exclude pregnancy before each MRI scan. Pregnant women will be excluded from the study. Screening for MRI-related exclusion criteria will done during a phone interview (pre-screen) and with an on-site questionnaire at the time of the scan.

Once subjects are deemed able to safely undergo an MRI study, these will be performed according to standard safety practices as per our institutional standards. Voice contact with patients will be maintained throughout the scan.

All people involved with the study remove all metal from their clothing and all metal objects from their pockets. No metal objects are allowed to be brought into the magnet room anytime. In addition, once the subject is in the magnet, the door to the room will be closed so that no one inadvertently walks into the magnet.

11.3.3 Protection against risks associated with cardiopulmonary exercise tests:

These tests will be performed by qualified personnel according to established American Heart Association Guidelines[192], 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.

11.3.4 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 approximately 6 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 MRI study and initiation of the second treatment phase) and immediately before the MRI study performed at the end of the second treatment phase. Methemoglobin levels will be measured before initiation of randomized therapy and approximately 6 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 approximately 6 weeks after initiation of randomized therapy.

Significant methemoglobinemia was not observed in our pharmacokinetics trial, at the same doses used in this trial, in the same patient population. In this 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 (same dosing scheme used in the KNO_3CK OUT HFpEFp trial).

Side Effect Management Plan: No side effects have been observed in our single-dose study or our ongoing 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.[198] 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.[198]

11.3.5 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, NIRS, MRI studies, and Doppler echocardiographic examinations will only be performed by appropriately trained personnel as per our institutional standards. Subjects will receive instructions in Aurora device use, including written instructions (Appendix 3), including instructions to loosen the wrist strap if there is discomfort and discontinue its use if discomfort persists.

11.3.7 Protection against risks associated with the MRI study:

See above (Section 11.3.2). It is worth noting that the risk of a cardiac MRI at 1.5T is in general, lower than at 3T or 7T, given the lower magnetic field. Subjects that qualify for a 3T or 7T scanner (for the leg MRI study) also qualify for scanning in a 1.5T scanner (for cardiac MRI during the myocardial perfusion ancillary study). However, the opposite is not necessarily true. Some situations (such as many intraluminal stents and prosthetic valves) which exclude scanning at high field (7T or 3T) are considered safe for 1.5T scanning. If subjects are deemed to be ineligible for 7T or 3T scanning in the parent trial, they may still be enrolled in the ancillary myocardial perfusion study and undergo a cardiac MRI study (at 1.5T). In all cases, participation in the myocardial perfusion / cardiac MRI ancillary study will be approved by Dr. Kuruvilla, who has appropriate clinical expertise to assess the safety of this test, according to current clinical standards. Dr. Kuruvilla is the Director of Advanced Cardiac Imaging at the CMC VA Medical Center and Assistant Professor at the University of Pennsylvania.

Protection against risks associated with IV gadolinium administration: The presence of eGFR >30 mL/min/1.73m^2 is an exclusion criterion from the parent trial. In addition, GFR will be assessed within 3 weeks prior to the cardiac MRI. A GBCA will be only administered for the MRI examination if eGFR >40 mL/min/1.73m^2. Subjects with a prior history of allergic reactions to GBCAs will be excluded from participation. There is recent data showing that traces of gadolinium may remain in the body long-term after contrast administration. However reviews by the FDA have not identified adverse health effects from gadolinium retained in the brain or bodily tissues after MRI. Further the gadolinium agent that is used in this study belongs to a group of macrocyclic gadolinium based agents that are thought to minimize or eliminate this risk.

Protection against risks associated with IV regadenoson administration: Regadenoson should be avoided in patients with unstable angina, severe COPD/asthma and patients with advanced cardiac heart block. Subjects with 2nd degree AV block, 3rd degree AV block or highgrade AV block will be excluded from participation in the ancillary study. Unstable coronary syndromes or significant lung disease are exclusion criteria for the parent trial. In all cases, screening will be performed as per clinical guidelines prior to all patients receiving regadenoson. Cardiac resuscitation equipment and trained staff including a cardiologist will be available before administering regadenoson.

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. Informed consent may be obtained in-person or virtually. 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 appropriate IRBs, and will forward to the NHLBI a copy of the written and dated approval by the IRB Chairman and committee. 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. Investigational New Drug (IND) application

This trial will be done under our currently open Food and Drug Administration IND (IND#123785) to study this particular drug and preparation in this particular patient population. The IND Sponsor will be responsible for interactions with the FDA, with support from the Sponsor Support Unit within the Office of Clinical Research at the Perelman School of Medicine, University of Pennsylvania.

12.4. 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:

A data and safety monitoring plan is in place for this study.

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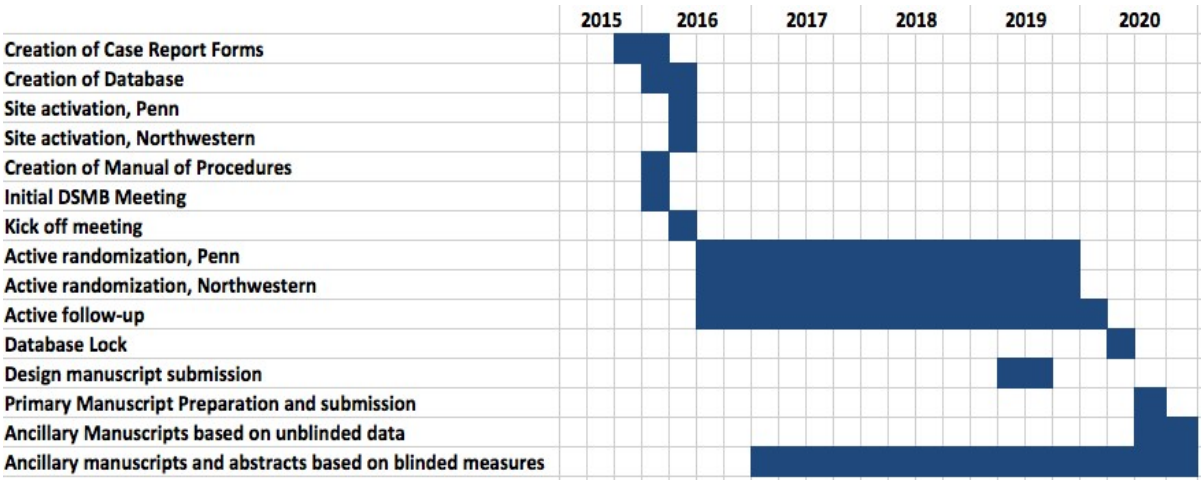
15. APPENDIX 1. SCHEDULE OF EVENTS

SCHEDULE OF EVENTS	Baseline assessment	Phase 1		1 week	1-week washout	Phase 1		2 week
		wk 6				wk 6		
Informed Consent [a]	X							
Eligibility assessment	X							
Medical History	X							
Concomitant Medications	X							
Randomization	X							
Dietary counseling	X							
Kansas City Cardiomyopathy Questionnaire	X							
Physical Exam (including orthostatic vital signs and anthropometric measurements)	X							
12-lead ECG	X							
Laboratory tests (CBC, chem 7, methemoglobin, pregnancy test as needed)	X							
RNA collection with PAXgene tubes								
G6PD test as needed	X							
Cognitive assessment	X							
6 minute walk test with O2 saturation	X			X				
Blood, urine and saliva collection and freezing	X			X				X
								X
2D echocardiogram	X	X		X				
				X		X		X
Arterial tonometry	X			X				X
								X
				X				
Pill count				X				X
								X
Drug accountability				X				
								X
Maximal effort exercise test with expired gas				X				
								X
Exercise cardiac output measurements				X				
				X				X
Plantar flexor exercise / MRI				X				X
				X				X
Assessment of Adverse events				X				X
				X				X
Placement of Aurora device and Actigraph	X			X				X
		X		X			X X	X
Retrieval of Aurora device and Actigraph				X				
				X				X

Note: For subjects participating in the ancillary study, a cardiac MRI will be performed 4-6 weeks after drug initiation in each treatment phase, on a different day than the other study procedures.

16. APPENDIX 2. ANTICIPATED STUDY TIMELINE AND KEY DATES



KEY DATES

Date of 1st patient randomized	**July-Aug, 2016**
Date of last patient randomized	**September 1st, 2019**
Date of last patient follow-up visit	**December 7th, 2019**
Date of data cleaned and ready for analysis	**April 1st, 2020**
Final manuscript preparation	**April-June 2020**
Projected date of publication	**October 1st, 2020**
Patients randomized per year of the study	

	Penn	Northwestern	Cumulative Total	Minimal Enrollment Target
Year 1	6	3	9	7
Year 2	16	12	37	28
Year 3	12	8	57	43
Year 4	12	7	76	57
Year 5	0	0	76*	76*
Total	46	30	76	76

*In the event that enrollment is not completed in Year 4

17. **APPENDIX 3 Instructions for wearing the Aurora Device (Pulse Watch)**

The Aurora device is designed to record your pulse throughout the day. We ask that you wear throughout the day and night, as you may wear a watch. During your first study visit, we will do a demonstration to teach you how to place and secure the device to your wrist.

Use of the Aurora Device is very safe. However, there are a few precautions of which you should be aware:

- The wrist strap will need to be moderately tight for acquisition of good quality pulse signals. It should never be so tight, however, as to restrict circulation in the hand. If you feel a tingling sensation in the hand or wrist where the Aurora device is being worn,

immediately loosen the wrist strap. If the sensation persists, take the device off and discontinue use.

- The device will beep several times during the day. When you hear the beep, you should touch the two contacts on the front of the device with two fingers from the hand that is not wearing the device, as illustrated in the following image:

- The device has been engineered to be robust to normal daily wear conditions, but it is not waterproof. Therefore, avoid submerging the device in water or using it outdoors in heavy rain.
- The Aurora Device can be cleaned using standard isopropyl alcohol wipes. Do not use any other cleaning agents. Do not submerge the device in cleaning agents.
- Do not attempt to open the housing of the device or to dismantle the device.

18. APPENDIX 4. HISTORY OF CHANGES

All changes after version V1 (dated 4/29/16), which was finalized after receiving input from all DSMB members and both IRBs, and before any subject enrollment, are documented below.

Version 2 (Feb 2016): Amended to specifically include

a) The administration of a low-nitrate diet with study medications during Visit 2 and Visit 3.
b) The use of the Aurora watch during the symptom-limited maximal effort exercise test
c) Blood draw at peak exercise
d) Minor grammatical changes and inconsistencies fixed throughout the protocol

Version 3 (July 2016):

a) Changed the language about the randomization procedure (direct communication between IDS and Jesse Chittams, without involvement of the study team.
b) Specified that we will provide subjects with a 14-day supply of study medication at the beginning of each phase.
c) Minor changes in language to meet wording consistent with FDA regulations

d) Added unanticipated device-related effects as potential adverse events.
e) Replaced MEDWATCH format with CIOMS format for SAE reporting
f) Added a section of potential risks of investigational devices.
g) Removed DSMB as an appendix, as it is a stand-alone core document.
h) Removed VaSera measurements to simplify the protocol.

Version 4 (July 2016)

a) Updated the chemistry and manufacturing document
b) Updated the chemistry and manufacturing portion of the protocol

Version 5 (August 2016)

a) Altered the inclusion criteria to remove the stipulation that evidence for elevated filling pressures must have been demonstrated within the last 12 months.

Version 6 (October 2016)

a) Modified the inclusion criteria to include specific criteria for elevated intracardiac filling pressures at rest or with exercise.
b) Modified the exclusion criteria to allow enrollment of individuals with rate-controlled (resting HR<100) atrial fibrillation.
c) Included specific plans to ensure MRI safety for subject who may have metal in his/her body. This includes obtaining X-rays or X-ray reports and discussing the case with an attending radiologist prior the MRI scan.
d) Updated the MRI protocol to include phase contrast imaging instead of ASL.
e) Added the Philadelphia VAMC as an enrollment site, pending VA IRB/ACOS approval and site activation.
f) In response to VA IRB stipulations:
    i. Reworded maximal exercise test to “symptom-limited maximal effort exercise test” throughout the protocol
    ii. Added additional monitoring of serum potassium for participants with a baseline serum K between 4.7 and 5.0 mEq/L in the presence of either: (a) potassiumsparing diuretic use, or (b) Estimated glomerular filtration rate of 30-39 mL/min/1.73m2. In this instance, we will check a serum potassium 1 week after the implementation of the 18 mmol/d dose as a safety measure.
    iii. Added more explicit language about the purpose of KNO_3 in the study, in the specific aims section: “The purpose of the trial is to test the efficacy of KNO_3 on a number of clinical and physiologic endpoints in subjects with HFpEF…”

Version 7 (January 2017)

a. Clarified that the Aurora and Actigraph devices will be given to the subjects at approximately 5 weeks into each interventional phase.
b. Clarified safety monitoring to be performed during the exercise study, including oxygen saturation monitoring.
c. Added a section (4.3.3. Criteria that will prompt exclusion from the trial at the 6 week visit) in response to further VA IRB stipulations
d. Updated figure 3. Overview of study design and procedures

e. Added a sentence describing that a letter may be sent to potential subjects prior to initial contact
f. Added Sujith Kuruvilla, M.D. to the protocol as a sub-investigator

Version 8 (July 20177)

a. Added cognitive assessments via Cogstate tests.
b. Added RNA collection (PAXGene tubes)
c. Added cuff brachial and central BP measurements using BP+ Uscom device.
d. Removed febuxostat/allopurinol as exclusion criteria.
e. Removed NT-proBNP testing for visits 2 and 3.

Version 9 (November 2017)

a. Added ancillary study to perform vasodilatorvasodilator cardiac MRI's in 20 subjects enrolled at CMC VA Medical Center and University of Pennsylvania. The cardiac MRI will be performed ~4-6 weeks into each phase. The ancillary study is designed to assess the effect of KNO_3 on myocardial perfusion (MP) and perfusion reserve (MPR) in HFpEFHFpEF subjects. Stress cardiac MRI's will be performed at 1.5 T scanner. Subjects will be administered GBCA (Gadolinium Based Contrast Agent) and Regandenoson. The potential risks associated with these procedures and adequacy to protect against these risks are also added.
b. Some minor typos were corrected.
c. Ambulatory blood pressure monitoring was removed.
d. The placement of Actigraph (activity monitor) was specified inin the wrist instead of hip.
e. Addition of standardized low nitrate breakfast to baseline visit following the blood draw for labs and biomarker samples.
f. SpecificationSpecification of cutpoints for considering a quantitative G6PD activity test "positive" for clinically significant G6PD deficiency (<60% of normal).
g. Increase of number of enrolled subjects to 84.

Version 10 (October 2018)

a. Added laungage for recent FDA guidance regarding GBCA (Gadolinium Based Contrast Agent) administration. The potential risks associated with these procedures and adequacy to protect against these risks were also updated.
b. Some minor typos and formatting errors were corrected.
c. Clarification of the role of the DSMB and PI reporting requirements
d. Clarification of some exclusion criteria.

Version 11 (March 2020)

a. Added Srinath Adusumalli, MD, M. SC, to protocol as sub-investigator

Version 12 (07-Oct-2021)

a. Added clarification of consent procedure