**CLINICAL RESEARCH PROTOCOL**

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|---|---|---|
| **INVESTIGATIONAL PRODUCT(S):** | | Potassium Nitrate<br>Nicotinamide Riboside<br>Propionyl-L-Carnitine<br>Potassium Chloride |
| **STUDY NUMBER(S):** | **IRB Number** | 844627 |
| | **Other Protocol Identifiers** | CAMRIS: 735<br>CHPS: |
| **PROTOCOL(S) TITLE:** | | Matching Perfusion to Metabolic Activity in HFpEF |
| **REGULATORY SPONSOR:** | | University of Pennsylvania |
| **FUNDING SPONSOR(S):** | | National Institutes of Health |
| **PRINCIPAL INVESTIGATOR** | | Payman Zamani, MD, MTR |
| **ORIGINAL PROTOCOL DATE:** | | |
| **VERSION NUMBER:** | | v1.0 |
| **VERSION DATE:** | | 29 January 2021 |

**PRINCIPAL INVESTIGATOR SIGNATURE**

| | |
|---|---|
| STUDY SPONSOR: | National Institutes of Health |
| STUDY TITLE: | Matching Perfusion to Metabolic Activity in HFpEF – The MPMA HFpEF Study |
| STUDY ID | 84467 |
| PROTOCOL VERSION | v1.0 |

I have read the referenced protocol. I agree to conduct the study in accordance to this protocol, in compliance with the Declaration of Helsinki, Good Clinical Practices (GCP), and all applicable regulatory requirements and guidelines.

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| Principal Investigator Name | Payman Zamani, MD, MTR | Signature | |
| Affiliation: | University of Pennsylvania | Date | |

## Abbreviations

AE – Adverse events
ATP – Adenosine triphosphate
BID – Twice daily
Cr – Creatine
CrAT – Carnitine acetyltransferase
CrCEST – Creatine chemical exchange saturation transfer
DSMP – Data safety and monitoring plan
DSMB – Data safety and monitoring board
GCP – Good clinical practice
HIPAA – Heath Insurance Portability and Accountability Act
HFpEF – Heart failure with preserved ejection fraction
HFrEF – Heart failure with reduced ejection fraction
HTN - Hypertension
IDS – Investigational Drug Service
IRB – Institutional Review Board
KCCQ – Kansas City Cardiomyopathy Questionnaire
KCl – Potassium chloride
$KNO_3$ – Potassium nitrate
LG – Lateral gastrocnemius
MRI – Magnetic resonance imaging
$NAD^+$ – Nicotinamide adenine dinucleotide
NIRS – Near infrared spectroscopy
NO – Nitric oxide
NR – Nicotinamide riboside
OxPhos – Oxidative phosphorylation capacity
$O_2$ – Oxygen
PB – Placebo
PLC – Propionyl-L-Carnitine
SOP – Standard operating procedure
QD – Daily
SAE – Serious adverse events
SkM – Skeletal muscle
SVR – Systemic vascular resistance
$t_{1/2,Cr}$ – Half-time of creatine recovery
TCA – Tricarboxylic acid cycle
TID – Three times daily
ULN – Upper limit of normal
UP – Unanticipated problem
UPenn – University of Pennsylvania
VT – Ventilatory threshold
$VO_2$ – Oxygen uptake
$VO_{2,peak}$ – Peak oxygen uptake
vPIVOT – Velocity and Perfusion, Intravascular Venous Oxygen saturation, and $T_2$*
$\Delta AVO_2$ – Arteriovenous $O_2$ content difference
$^{31}P$ – 31-Phosphate

# 1 STUDY SUMMARY

## 1.1 Synopsis

| | |
|---|---|
| **Title:** | Matching Perfusion to Metabolic Activity in HFpEF – The MPMA HFpEF Study |
| **Short Title:** | Perfusion to Metabolic Activity in H FpEF |
| **Study Description:** | This study will test whether Potassium Nitrate ($KNO_3$), with and without Propionyl-L-Carnitine (PLC) and Nicotinamide Riboside (NR), improves submaximal exercise endurance and skeletal muscle oxidative phosphorylation capacity (SkM OxPhos) in participants with Heart Failure with Preserved Ejection Fraction (HFpEF). This study will also test whether the response to supplemental oxygen identifies HFpEF individuals who derive additional benefit from combination therapy with $KNO_3$ + PLC + NR, as opposed to $KNO_3$ alone. |
| **Objectives:** | 1. Compare the impact on submaximal exercise endurance of pharmacologic therapy aimed primarily at increasing intramuscular perfusion ($KNO_3$) versus combination therapy to increase intramuscular perfusion and mitochondrial reserve ($KNO_3$ + NR + PLC).<br>2. Assess the relationship between the individual response of submaximal exercise endurance to our pharmacologic interventions and the individual response of SkM OxPhos to supplemental oxygen ($O_2$). |
| **Primary Endpoint:** | 1. The primary endpoint will be the change in submaximal exercise endurance (time to fatigue at 75% of peak workload) between the interventional therapies and active control (KCl) |
| **Secondary Endpoints:** | 1. Change in SkM OxPhos following plantar flexion on MRI<br>2. Change in exercise vasodilatory reserve<br>3. Change in KCCQ overall summary score<br>4. Change in steps per day<br>5. Change in the kinetics of $O_2$-consumption<br>6. Peak oxygen consumption during submaximal exercise |

| | |
|---|---|
| | 7. Change in intramuscular perfusion following plantar flexion on MRI |
| **Exploratory Endpoints:** | Assess the impact of our interventions on:<br>(1) Arterio-venous concentration gradients for plasma substrates at baseline, 4 minutes of exercise, and at the time of exhaustion<br>(2) Respiratory exchange ratio, a non-invasive metric of whole-body substrate oxidation obtained from expired gas analysis, assessed at 4 minutes of exercise<br>(3) Tissue-based mitochondrial respiration<br>(4) Tissue-based metabolomics<br>(5) Muscle proteome |
| **Study Population:** | This study will be performed in approximately 53 stable outpatient participants with symptomatic Heart Failure with Preserved Ejection Fraction. |
| **Phase:** | Phase II |
| **Description of Sites/Facilities** | Center for Human Phenomic Sciences<br>Presbyterian Medical Center<br>University of Pennsylvania |
| **Enrolling Participants:** | This will be a single center study |
| **Description of Study Intervention:** | Investigational Medications:<br>• Potassium Nitrate ($KNO_3$) 6 mmol three times daily<br>• Propionyl-L-Carnitine (PLC) 1000 mg twice daily<br>• Nicotinamide Riboside (NR) 300 mg three times daily<br>• Potassium Chloride (KCl) 6 mmol three times daily<br>The order in which subjects will receive the following 3 interventions will be randomized and administered in a double-blind fashion:<br>1. $KNO_3$ alone |

2. $KNO_3$ + PLC + NR
3. KCl

**Study Duration:** 60 months

**Participant Duration:** Approximately 7 months

## 1.2 Schema

Total anticipated enrollment: 53 participants

3 period cross-over study during which all participants will receive the following interventions in randomized double-blind order, with a 2-week washout period in between phases:

- Potassium Nitrate ($KNO_3$)
- Potassium Nitrate + Nicotinamide Riboside + Propionyl-L-Carnitine ($KNO_3$ + PLC + NR)
- Potassium Chloride (KCl), as an active control

**Study Overview:**

| Visit | Procedures | Primary Objective |
|---|---|---|
| **Visit 1:** Baseline Testing | a) Informed Consent and Randomization<br>b) Max Effort CPET<br>c) Submax verification study<br>d) KCCQ | - Determine Peak $VO_2$<br>- Determine peak workload (PW) and 75%PW to be used in subsequent submaximal tests<br>- Submax workload titration to target ex duration between 3-6 min |
| ***6 weeks of Period 1*** | | |
| **Visit 2:** Period 1 Endpoint Assessment 1 | a) Muscle Biopsy<br>b) Submax (75%PW) Exercise test<br>c) KCCQ | - Determine Phase A submax exercise time<br>- Determine Phase A submax $VO_2$ on/off-kinetics<br>- Assess muscle metabolome, citrate synthase activity, rates of carbohydrate and fatty acid oxidation |
| **Visit 3:** *(3-7d later)* Period 1 Endpoint Assessment 2 | Skeletal muscle calf MRI with plantar flexion exercise | - SkM OxPhos – CrCEST<br>- Intramuscular perfusion – vPIVOT |
| ***2-Week Washout, followed by 6 weeks of Period 2*** | | |
| **Visit 4:** Period 2 Endpoint Assessment 1 | a) Muscle Biopsy<br>b) Submax (75%PW) Exercise test<br>c) KCCQ | - Determine Phase B submax exercise time<br>- Determine Phase B submax $VO_2$ on/off-kinetics<br>- Assess muscle metabolome, citrate synthase activity, rates of carbohydrate and fatty acid oxidation |
| **Visit 5:** *(3-7d later)* Period 2 Endpoint Assessment 2 | Skeletal muscle calf MRI with plantar flexion exercise | - SkM OxPhos – CrCEST<br>- Intramuscular perfusion – vPIVOT |
| ***2-Week Washout, followed by 6 weeks of Period 3*** | | |
| **Visit 6:** Period 3 Endpoint Assessment 1 | a) Muscle Biopsy<br>b) Submax (75%PW) Exercise test<br>c) KCCQ | - Determine Phase C submax exercise time<br>- Determine Phase C submax $VO_2$ on/off-kinetics<br>- Assess muscle metabolome, citrate synthase activity, rates of carbohydrate and fatty acid oxidation |
| **Visit 7:** *(3-7d later)* Period 3 Endpoint Assessment 2 | Skeletal muscle calf MRI with plantar flexion exercise | - SkM OxPhos – CrCEST<br>- Intramuscular perfusion – vPIVOT |
| ***One-month washout*** | | |
| **Visit 8:** (~1 month later) | Skeletal muscle calf MRI with plantar flexion exercise:<br>• Room Air<br>• 100% $O_2$ | - Determine the change in SkM OxPhos with 100% oxygen<br>- Compare the change in SkM OxPhos with 100% oxygen to the difference in submax exercise time between the $KNO_3$ and $KNO_3$+NR+PLC arm |

# 2 INTRODUCTION AND RATIONALE

## 2.1 Study Rationale

**The incidence of HFpEF is increasing, leading to more patients who are functionally limited and with a poor quality of life.** While the incidence of heart failure with reduced ejection fraction (**HFrEF**) has decreased, that of heart failure with preserved ejection fraction (**HFpEF**) continues to rise,[1-5] accounting for ~50% of HF hospitalizations,[4, 6, 7] and disproportionately affecting females.[2, 8] Patients with HFpEF suffer mentally and physically,[9, 10] similar to their HFrEF counterparts.[11-16] **Yet in contrast to HFrEF, there are no effective therapies for HFpEF,[17] despite numerous clinical trials.[18-20]** One reason trials have failed may be the significant heterogeneity found across HFpEF patients,[21, 22] precluding a "one-size-fits-all" approach to treatment. Indeed, this type of approach, where phenotypic differences between subjects are ignored and all subjects are treated similarly, may have masked clinically-significant benefit in some.[23-25] Yet no study has prospectively evaluated a strategy to determine which HFpEF patients are likely to respond to a specific drug intervention. In this protocol, we will test novel therapies that specifically target abnormalities identified in HFpEF patients and that are associated with impaired exercise capacity. We will also test an MRI-based strategy to determine which HFpEF participants are more likely to benefit from our different therapeutic approaches.

## 2.2 Background

**The determinants of exercise capacity among HFpEF patients are heterogenous.** Reduced aerobic capacity is the hallmark of HFpEF, yet exactly what limits an individual patient may be varied between individuals. In an in-depth analysis of oxygen ($O_2$) transport during exercise by Houstis et al., 79 subjects with HFpEF and 55 controls underwent cardiopulmonary exercise testing with invasive hemodynamic measurements.[20] The authors found significant heterogeneity across patients with regard to the site(s) of impairment in $O_2$ transport, even across participants with similar peak oxygen uptake (**$VO_{2,peak}$**).[20] Specifically, nearly all of the participants studied had ≥ 2 significant impairments (<80% of control values) in the steps of $O_2$ transport and utilization (alveolar ventilation, lung diffusion capacity, cardiac output, hemoglobin concentration, skeletal muscle diffusion capacity, and mitochondrial oxidative capacity). These data suggest that although exertional intolerance is the common final result, exactly which factors along the $O_2$ transport pathway are impaired may be different among HFpEF patients, **suggesting that there is marked heterogeneity in the patient population regarding the factors that limit exercise.**

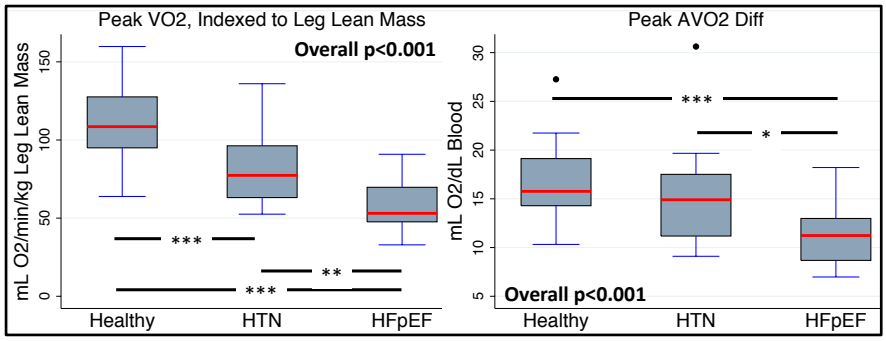


**Figure 1 – *Peak $VO_2$ and the arteriovenous $O_2$ content difference are reduced in HFpEF.*** Healthy (n=20), HTN (n=19), and HFpEF (n=20) individuals performed a maximal effort cardiopulmonary exercise test on a supine cycle ergometer. Echocardiography was used to determine cardiac output, and the ΔAVO2, a marker of SM oxygen utilization, was solved using the direct Fick equation. *** adjusted p≤0.001, ** adjusted p≤0.01, * adjusted p<0.05

**The skeletal muscle of HFpEF patients may be an important site of exercise limitation.** The study by Houstis et al. also identified that the arteriovenous $O_2$ content difference (**ΔAVO2**) at peak exercise, a marker of skeletal muscle (**SkM**) oxygen utilization, is consistently impaired in HFpEF patients across several studies.[20, 26, 27] In our preliminary studies, we evaluated exercise capacity in healthy individuals (n=20), HFpEF

subjects (n=20), and age-matched hypertensive subjects (**HTN**, n=19) during cycle ergometry.[28] HFpEF subjects had markedly lower peak $VO_2$, compared to either control group. **Importantly, HFpEF subjects had a diminished $\Delta AVO_2$ (Fig. 1)**, while peak cardiac output was no different, suggesting to us that the SkM may be an important site of exercise limitation in HFpEF.

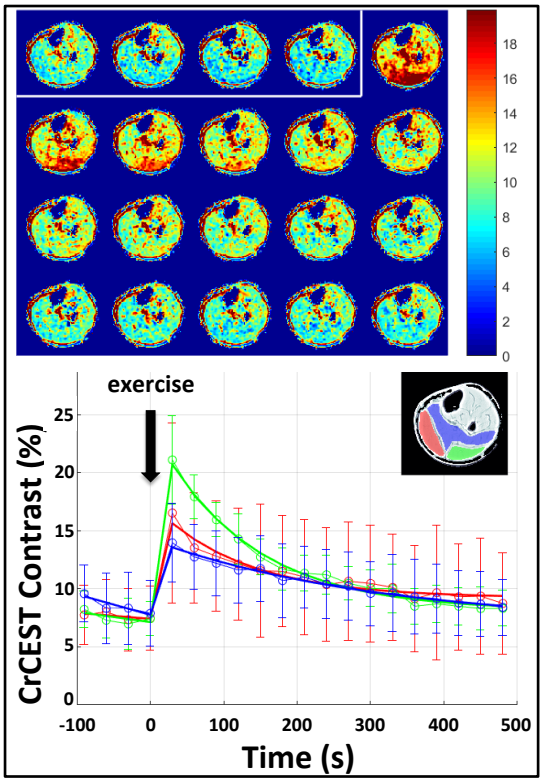


**Figure 2 – Example of exercise-induced changes in Free Cr, measured by CrCEST.** One healthy 36-year-old male performed plantar flexion exercise for 2 minutes (0.75 Hz). Baseline Cr maps are shown in the upper left (white box). Serial Cr maps following exercise are displayed at 30s intervals, with relative Cr concentrations displayed on the bar on the upper right.

**The rate of skeletal muscle oxidative phosphorylation (OxPhos) is reduced in HFpEF.** Focusing our attention on the skeletal muscle, we then assessed SkM OxPhos (Healthy=20, HTN=17, HFpEF=14) using a novel MRI-based technique developed by our collaborators at UPenn: Creatine Chemical Exchange Saturation Transfer (**CrCEST**, **Fig. 2**).[29-33] CrCEST measures free Cr concentration, which increases during exercise as it releases from phosphocreatine (PCr) to generate ATP, and decreases during recovery as the mitochondria generate ATP and reform PCr.[31, 34] CrCEST imaging has the ability to interrogate oxidative capacity in an anatomically-resolved manner, allowing for recovery parameters to be determined for individual muscle groups, without the confounding influence of non-activated intervening tissue (e.g. fat, inactivate muscle) which contributes to $^{31}P$ measurements.[29, 31-33]

Using CrCEST, we found that the half-time of Cr recovery ($t_{1/2,Cr}$) of the lateral gastrocnemius muscle, the muscle most activated by our plantar flexion protocol, was markedly prolonged in HFpEF participants, compared to both healthy and age-matched hypertensive controls (**Fig. 3**).

Moreover, among all study participants, $t_{1/2,Cr}$ correlated with Peak $VO_2$, indexed to leg lean mass (Spearman's rho:-0.28, p=0.047) and the ventilatory threshold ($VT_{leg\ lean}$, Spearman's rho: -0.34, p=0.01), demonstrating an association between SkM OxPhos and a patient's aerobic capacity.

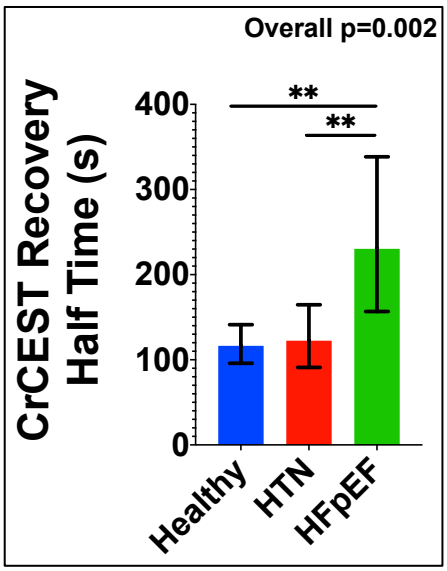


***Figure 3 – SkM OxPhos is prolonged in HFpEF.*** The linear half-time of Cr recovery was calculated following a standardized plantar flexion exercise protocol. Data are displayed as the geometric mean with the 95% CI. ** Bonferroni-adjusted p<0.01

**Mitochondrial function is normal in HFpEF.** The prolonged SkM OxPhos seen in our HFpEF subjects (**Fig. 3**) may be attributable to several different abnormalities: (1) impaired mitochondrial function; (2) diminished mitochondrial volume; or (3) diminished $O_2$ availability to respiring mitochondria.[35] To investigate these possibilities further, we invited participants back to undergo a muscle biopsy of the vastus lateralis (Healthy=12, HTN=9, HFpEF=11). To examine mitochondrial function, we measured $O_2$ flux from freshly isolated mitochondria that were given a saturating level of ADP, pyruvate, and malate, finding no difference among groups. We also subjected muscle homogenates to tritiated palmitate and assessed rates of palmitate flux,[36, 37] again finding no difference among groups. We then assessed mitochondrial morphology (n=50 mitochondria/subject) on electron microscopy, finding no difference in

individual mitochondrial size, Feret's diameter, or electron density between groups. While there are limitations to these techniques, including the use of isolated mitochondria as opposed to permeabilized fibers,[38] these findings suggested to us that, at least at rest, isolated mitochondria in HFpEF and controls are morphologically and functionally similar.

However, it could be that HFpEF patients have a lower mitochondrial volume density.[35, 39] We therefore assessed SkM mitochondrial content, both by measuring citrate synthase activity and by quantifying mitochondria volume density on our electron microscopy images. We did not find evidence for decreased mitochondrial volume density in our HFpEF samples using either technique. However, a report (Healthy=17, HFpEF=20) by Dr. Kitzman's group at Wake Forest demonstrated reduced citrate synthase activity in HFpEF,[40] suggesting *reduced* mitochondrial content. We considered whether the discrepancy between the findings represents a Type II error, or **alternatively, that decreased mitochondrial content, and hence mitochondrial reserve capacity, is a HFpEF phenotype not present in all patients.** Yet whether this is the case, and determining which HFpEF patients have impaired mitochondrial reserve and thus may benefit from a mitochondrially-directed intervention, can currently only be done with a muscle biopsy.

**Intramuscular perfusion may be different than bulk blood flow.** Finally, impaired blood flow to the lateral gastrocnemius muscle during exercise and recovery could also explain the impaired SkM OxPhos in HFpEF. In fact, numerous studies,[41-43] including ours,[28] demonstrate an impaired vasodilatory reserve during cycle ergometry exercise in HFpEF subjects, suggesting that HFpEF patients cannot efficiently redistribute blood flow to locomotive exercising muscle. In our initial efforts to investigate this possibility, we measured popliteal artery bulk blood flow using the same plantar flexion exercise protocol (Healthy=7, HTN=8, HFpEF=5), repeated immediately after the CrCEST studies. Although popliteal artery blood flow increased with exercise, we found no difference in peak popliteal artery blood flow between groups (p=0.26), suggesting that **bulk arterial blood flow** could not explain the differences in SkM OxPhos seen among the groups.

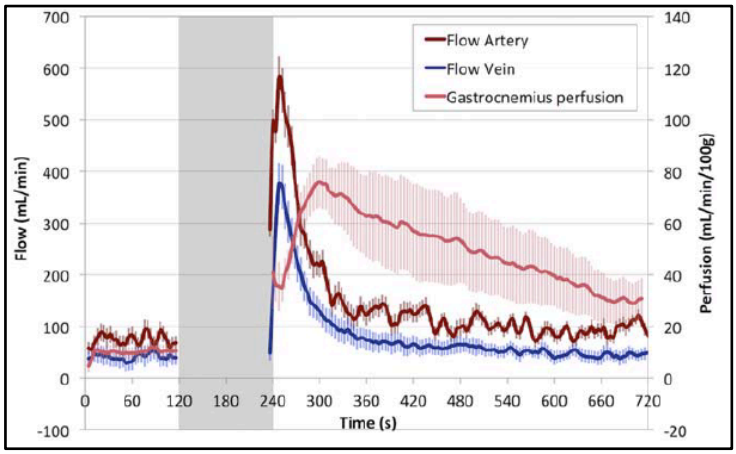


***Figure 4 – Conduit artery blood flow characteristics, such as peak timing, are different than those of directly measured intramuscular perfusion following plantar flexion exercise.*** From Englund, 2018.

**Novel MRI technique to assess intramuscular perfusion: beyond bulk blood flow.** Our collaborators have developed a novel technique to simultaneously quantify conduit artery flow velocity and *intramuscular* perfusion following plantar flexion exercise with high temporal resolution (4s): Velocity and Perfusion, Intravascular Venous Oxygen saturation, and $T_2$* (**vPIVOT**).[44-46] In a prior study in healthy individuals, conduit artery blood flow rate peaked 8±3 seconds after the cessation of plantar flexion exercise, yet intramuscular gastrocnemius perfusion, the muscle most activated by plantar flexion, did not peak until 101±53s (**Fig. 4**). The physiologic concept of importance is noting that oxygen can only be consumed after it has reached the mitochondria. **To this end, measuring intramuscular perfusion, as we propose to do here using vPIVOT, is more physiologically relevant than conduit artery bulk blood flow in interpreting SkM OxPhos**.

**$VO_2$ kinetics are an important determinant of exercise capacity and are impaired in HFpEF.** In our preliminary studies, we measured the half-time of $VO_2$ recovery following our maximal effort cycle ergometry studies. We found that $VO_2$ recovery was prolonged in HFpEF subjects (Half-time of $VO_2$ recovery: Healthy

53.5±7.3, HTN 63.4±16.5, HFpEF 69.4±17.3 s, p=0.004). Importantly, across all subjects, the half-time of $VO_2$ recovery correlated with SkM OxPhos on CrCEST (Spearman's rho: 0.29, p=0.045) and the ventilatory threshold (Spearman's rho: -0.29, p=0.03), reinforcing the notion that recovery kinetics are related to SkM OxPhos and portend important relationships to functional capacity.[47, 48] Our results are in line with other studies that found slowed $VO_2$ kinetics in HFpEF patients.[49, 50] In healthy individuals, speeding $VO_2$ kinetics has been shown to improve exercise endurance.[51-53] However, in HFpEF, the interplay between submaximal exercise endurance, SkM OxPhos, intramuscular perfusion, and $VO_2$ kinetics is not well-understood. **We seek to fill this gap and test the hypothesis that submaximal exercise tolerance in HFpEF patients can be increased by improving SkM OxPhos. Improved exercise tolerance will allow HFpEF patients to perform his/her activities of daily living more comfortably and enhance the likelihood that a patient may engage in prescribed exercise training.[54]**

We will test this hypothesis by evaluating pharmacologic strategies to improve submaximal exercise in HFpEF patients, while also determining the impact of our interventions on SkM OxPhos and $VO_2$ kinetics. The therapies to be tested in this study are affordable and expected to be well-tolerated, based on many prior studies that utilized these agents. Importantly, even a small improvement in exercise tolerance is expected to allow patients to perform more activities of daily living, increasing their quality of life and independence, while potentially reducing the burden on the healthcare system. Thus, our study has the potential to have a major impact on the lives of millions of patients who suffer from this debilitating condition and who currently are without any pharmacologic treatment options.

### *2.2.1 Pharmacokinetics, Pharmacodynamics and Toxicology – Rationale for Dose Selection*

#### *a) Rationale and drug dosing for inorganic nitrate, an intervention primarily targeting SkM perfusion*

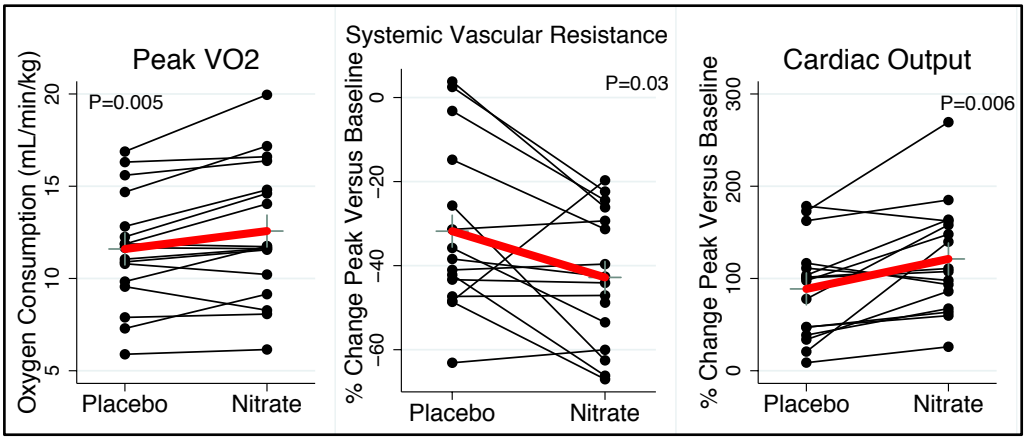


***Figure 5 – Inorganic nitrate enhances the vasodilatory reserve in HFpEF, allowing for higher cardiac output and peak $VO_2$ in HFpEF subjects (n=17).*** Note: Four HFpEF subjects experienced a decline in peak $VO_2$ with inorganic nitrate compared to PB (non-responders). *Red line indicates mean difference.*

While nitric oxide (**NO**) has many functions, including contributing to fuel metabolism[36] and myocardial stiffness,[55] NO also contributes to the exercise vasodilatory response.[48, 56-59] This is particularly important for HFpEF patients, in whom numerous studies document an impaired ability to decrease systemic vascular resistance with exercise.[42] In a pilot study, we tested the hypothesis that supplementation with inorganic nitrate, which can be reduced to NO in the hypoxic and acidic environment of exercising muscle,[60-63] improves exercise capacity in HFpEF.[64] In a 2x2 cross-over trial of 17 HFpEF subjects, we found that a single dose of 12.9 mmol of inorganic nitrate led to significant improvements in the vasodilatory reserve during exercise, allowing for greater cardiac output, higher peak $VO_2$, and higher ventilatory thresholds (**Fig. 5**).[64] Using near infrared spectroscopy (**NIRS**) on the calf muscle during exercise, we found a tendency for oxyhemoglobin to fall to a lesser degree during exercise following inorganic

nitrate than during exercise following placebo (-11.3% vs. -15.8%, p=0.07), suggesting that intramuscular perfusion was improved with inorganic nitrate. Notably, other reports have identified a similar vasodilatory effect of inorganic nitrate, including a reduction in blood pressure[65-74] and vascular stiffness,[71, 75] and an increase in vascular conductance during exercise.[48, 76, 77] **These data suggest to us that the predominant effect of inorganic nitrate is to improve intramuscular perfusion during exercise.**[58, 78]

Relevant to this proposal, we also found a trend towards improved SkM OxPhos using NIRS and a forearm exercise paradigm (p=0.083),[79, 80] and a trend towards improved $VO_2$ kinetics following submaximal exercise (p=0.089). Unfortunately, however, our experimental design precluded an assessment of whether these physiologic changes reflected improved flow or improved mitochondrial oxidative capacity, a topic to be addressed in the current proposal. One additional study also identified an ergogenic benefit to inorganic nitrate specifically in HFpEF. In a cross-over trial, Eggebeen et al. treated 20 subjects with HFpEF with 6 mmol of inorganic nitrate daily for 7 days. Submaximal exercise tolerance, defined as the exercise time at 75% peak workload, was significantly increased by inorganic nitrate (inorganic nitrate: 449±180 vs. placebo: 363±125 s, 24% increase, p=0.02).[74] Other studies have also shown an improvement in $VO_2$ kinetics with inorganic nitrate,[51, 52, 81] yet none of these studies provided mechanistic data to explain the benefit.

**$KNO_3$ dosing regimen.** We then performed a dose finding study of potassium nitrate ($KNO_3$) capsules specifically in HFpEF.[82] We demonstrated that 6 mmol of $KNO_3$ given three times daily appeared to be well tolerated in HFpEF patients, without clinically significant hypotension or methemoglobinemia. Additionally, we found an improvement in exercise time and quality of life.[82] Based on our data, we will use a 6 mmol dose of $KNO_3$ three times daily in this study.

### *b. Rationale and drug dosing of nicotinamide riboside (NR) and propionyl-L-carnitine (PLC), as the intervention targeting mitochondrial reserve*

As shown in **Fig. 5**, we found several (n=4, 24%) non-responders in our study of acute inorganic nitrate, despite their having increased blood levels of nitric oxide metabolites (e.g. nitrate and nitrite).[64] Similarly, in the Eggebeen et al. study, ~25% of the HFpEF subjects did not experience an increase in exercise endurance with inorganic nitrate.[74] ***To realize the salutary benefits of inorganic nitrate, the skeletal muscle mitochondria must have the reserve capacity to utilize the additional oxygen delivered; therefore,* we hypothesized that individuals who did not respond to inorganic nitrate might have impaired mitochondrial reserve.** This led us to consider whether we could identify individuals who have a skeletal muscle metabolic signature consistent with an impairment in mitochondrial reserve. We executed a prospective skeletal muscle biopsy study to answer this question. As above, we did not find a decrease in mitochondrial content. However, we did find **reductions in intramuscular $NAD^+$, propionyl-CoA, and acetylcarnitine in our HFpEF muscle biopsy samples compared to controls** (**Fig. 6**), indicating metabolic perturbations that may lead to energetic deficits and impair mitochondrial reserve.

**Rationale for $NAD^+$ supplementation.** Nicotinamide adenine dinucleotide ($NAD^+$) is essential for energy production given its role in carrying electrons to the electron transport chain, and it is also a necessary co-factor for the sirtuins, which deacetylate a host of proteins, including those involved in fuel metabolism[83-91] and mitochondrial biogenesis (e.g. PGC-1α and HIF-1).[91-93] $NAD^+$ declines with aging,[91, 94-98] and is lower in murine models of diabetes and obesity,[86] conditions of great importance to HFpEF. Additionally, deficiencies in $NAD^+$ limit mitochondrial ATP production from oxidative phosphorylation and render muscle more prone to weakness,[97] as seen in HFpEF patients.[99]

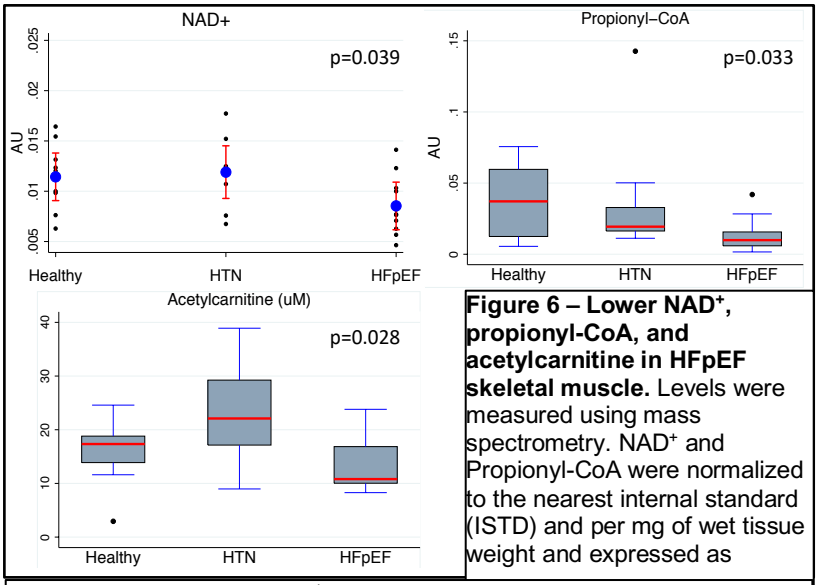


**Figure 6 – Lower NAD⁺, propionyl-CoA, and acetylcarnitine in HFpEF skeletal muscle.** Levels were measured using mass spectrometry. NAD⁺ and Propionyl-CoA were normalized to the nearest internal standard (ISTD) and per mg of wet tissue weight and expressed as arbitrary units (AU). For NAD⁺, data are presented as means (blue) with 95% CI (red whiskers); black dots are individual data points. For propionyl-CoA and acetylcarnitine: box represents 25th-75th percentile; median is the red line; black dots indicate outliers.

**NAD⁺ is reduced in HFpEF SkM.** We found NAD⁺ concentrations to be significantly reduced in resting muscle biopsy tissue of HFpEF patients compared to controls (Healthy: 0.01±0.003, HTN: 0.01±0.003, HFpEF: 0.009±0.003 AU, p=0.039, **Fig 6**). Concentrations of NAD⁺ correlated with our CrCEST metrics of SkM OxPhos (Spearman's rho: -0.47, p=0.01), suggesting that NAD⁺ supplementation has the potential to improve SkM OxPhos, perhaps by increasing mitochondrial reserve. In line with this, prior studies demonstrated several beneficial effects of increased NAD⁺ on the mitochondria: improved SkM OxPhos in models of aging;[91, 92, 97] improved SkM oxidative metabolism and glucose tolerance in high-fat diet induced obesity;[89, 93, 100] improved SkM OxPhos and mitochondrial biogenesis in mitochondrial disease models;[100-105] improved exercise endurance in transgenic mouse models of increased NAD⁺,[106, 107] increased mitochondrial respiration in humans with diabetes[108] or mitochondrial myopathy[104], and an increased capacity to generate acetylcarnitine in overweight/obese humans.[109] Finally, supplementation with NR enhanced peripheral blood mononuclear cell mitochondrial respiration, isolated from adults with HFrEF.[110]

***Table Examining NR Supplementation in Human Subjects (compiled Sept 2020)***

| Author | Population, n | Dose/Duration | Primary Outcome | Results | Adverse Events |
|---|---|---|---|---|---|
| **Airhart, 2017**[111] | Healthy volunteers<br><br>n=8 | 250 mg BID escalating to 1000 mg BID<br><br>9 days | Whole blood [NR] and [NAD⁺] | 1. Whole blood NAD+ and NR increased | None<br><br>Slight decrease in [K⁺] (-0.4 mEq) and [Hgb] (-0.4 g/dL) |
| **Dellinger, 2017**[112] | Healthy adults (60-80 years old)<br><br>n=120 | NR + Pterostilbene (NRPT): randomized, double blind, placebo (PB) controlled parallel design<br>1. 250 mg NR + 50 mg PT daily<br>2. 500 mg NR + 100 mg PT daily<br>3. PB<br><br>8 weeks | Whole blood [NAD⁺] | 1. Whole blood NAD⁺ increased in dose dependent manner<br>2. Increased 6 min walk distance and 30s chair stand test | No serious AEs<br><br>AE's possible related to NR+PT<br>1. Nausea<br>2. Fatigue<br>3. Headache<br>4. GI upset<br>5. Diarrhea<br><br>Increased total cholesterol and |

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| | | | | | LDL in treatment groups |
| **Martens, 2018[113]** | Lean healthy, middle-aged and older men and women<br><br>n= 30 | Crossover: PB vs. NR 500 mg BID<br><br>6 weeks duration/phase | Peripheral blood mononuclear cells (PBMC) [$NAD^+$] | 1. Oral NR increased PBMC [$NAD^+$]<br>2. NAAD sensitive biomarker of $NAD^+$ metabolism<br>3. Greater $NAD^+$ increase in subjects with lower baseline PBMC $NAD^+$<br>4. Trend for reduced pulse-wave velocity, a marker of aortic stiffness | No serious AEs.<br><br>Possible NR AEs:<br>• Nausea<br>• rash/flushing<br>• leg cramp<br>• bruising |
| **Dollerup, 2018[114] and 2020[115]** | Healthy, sedentary, obese insulin-resistant men (40-70 yo)<br><br>n=40 | Randomized, double blind, parallel design: PB vs. NR 1000 mg po BID<br><br>12 weeks | 1. Insulin sensitivity<br><br>2. Muscle $NAD^+$ metabolome<br><br>3. Muscle mitochondrial respiration | 1. No change in insulin sensitivity<br>2. No change in muscle $NAD^+$ with NR<br>3. No change in global protein acetylation with NR<br>4. No change in mitochondrial content or respiration with NR | No serious AEs due to NR<br><br>Possible AEs in NR:<br>• Pruritus<br>• Excessive sweating<br>• Bloating<br>• Transient changes in stool |
| **Conze, 2019[116]** | Healthy overweight adults between 40-60 years old<br><br>n=140 | Randomized double-blind, parallel design<br>1. Placebo<br>2. NR 100 mg/d<br>3. NR 300 mg/d<br>4. NR 1000 mg/d<br><br>8 weeks | Urinary MeNAM levels (NAD metabolite) | 1. Urinary MeNAM increased in dose dependent fashion<br>2. PBMC $NAD^+$ levels increased in dose dependent manner<br>3. 1000 mg/day concluded to be upper limit for NR administration | No dose dependent AEs.<br><br>Possible AE: nausea, muscle pain/soreness |
| **El Hassan, 2019[117]** | Healthy elderly (70-80) men<br><br>n=12 | Double-blind, placebo controlled, cross-over: NR 500 mg twice daily<br><br>21 days/phase; no washout period | | 1. Increased muscle NAAD and MeNAM<br>2. Muscle NR and NAD were not increased by NR<br>3. Whole blood $NAD^+$, NAAD, and MeNAM increased with NR<br>4. Genes associated with energy metabolism (TCA cycle/glycolysis/mito genes) downregulated by NR, but no change | No AEs with NR |

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|---|---|---|---|---|---|
| | | | | in glycolytic protein expression, mitochondrial bioenergetics or metabolism noted<br>5. No change in mitochondrial respiration or content<br>6. NR decreased inflammatory proteins (e.g. IL-6, IL-2, TNF-a) | |
| **Dolopikou, 2019**[118] | Twelve young (~22 yo) and twelve old (~72 yo) healthy men<br><br>n=24 | Randomized placebo-controlled single-dose cross-over trial of NR 500 mg versus PB, with a 10d washout<br><br>Single dose | Peak $VO_2$<br><br>Concentric peak torque (leg)<br><br>Isometric peak torque (leg)<br><br>Fatigue Index | 1. NR increased RBC glutathione and reduced urine $F_2$-isoprostane in older patients<br>2. NR increased RBC NADPH levels in both young and old<br>3. Older subjects exhibited higher isometric peak torque and less fatigue following NR | Not reported |
| **Remie, 2020**[109] | 45-65 years old, overweight/obese, sedentary men and post-menopausal women<br><br>n=13 | Double-blind randomized placebo-controlled crossover: 1000 mg NR/d<br><br>6 weeks/phase | Insulin sensitivity | 1. Intramuscular $NAD^+$ not different between NR and PB; however, skeletal muscle NAAD and MeNAM increased<br>2. No change in skeletal muscle mitochondrial respiration or mitochondrial complex proteins<br>3. Intramuscular acetylcarnitine (C2) increased<br>4. Slight increase in fat-free mass with NR | No AEs or side effects related to NR |

**$NAD^+$ precursor (NR) dosing.** Most cells, except for neurons, cannot import $NAD^+$ directly, instead relying on its synthesis from tryptophan or Vitamin $B_3$ analogs. $NAD^+$ can also be recycled intracellularly from its byproducts (e.g. nicotinamide, nicotinamide mononucleotide [NMN], or nicotinamide riboside [NR]) in what is known as the "salvage pathway" (**Fig. 7**).[87, 119] Both NR and NMN are orally bioavailable,[87] and could be used to bolster $NAD^+$ levels;[119] however, very little data has been generated in humans with NMN supplementation.[120, 121] On the other hand, substantial evidence supports using NR to increase $NAD^+$ metabolism. Our co-investigators demonstrated that the SkM preferentially uses NR for $NAD^+$ regeneration,[85] and that NR supplementation increases mitochondrial $NAD^+$,[122] supporting our planned use of NR in this

proposal that is focused on the SkM. Importantly, while several studies have shown that NR increases whole blood $NAD^+$ *(see table above)*, more recent studies have additionally demonstrated that NR supplementation leads to increased markers of $NAD^+$ metabolism specifically within human skeletal muscle.[109, 117]

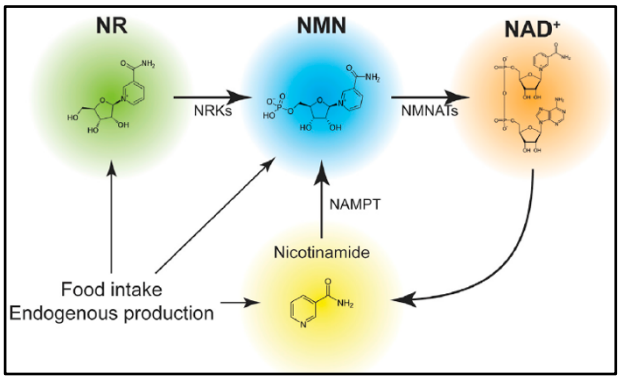


**Figure 7 – $NAD^+$ can be synthesized from nicotinamide riboside (NR).** From Yoshino, 2018.

*Safety of NR Supplementation:* Several studies have demonstrated the safety of NR supplementation in humans.[109, 111, 113-118, 123] In a study of 8 healthy individuals, increasing doses of up to 1000 mg po BID for 2 days were well tolerated, increasing whole blood $NAD^+$ levels by nearly 100%.[111] In another study, 40 obese sedentary men were randomized to 12 weeks of NR 1000 mg po BID versus placebo. NR was well-tolerated, and there was no increase in adverse events.[114] In 12 elderly men, 1000 mg of NR daily for 21 days did not lead to any adverse events.[117] One additional study tested combined NR + Pterostilbene (NRPT) in 120 older adults (age 60-80) for 8 weeks. No serious adverse events were noted; however, a small increase in cholesterol (LDL and total cholesterol) was noted in the combined NR+PT groups.[112] In the largest study to date,140 obese middle-aged individuals were treated with increasing doses of NR up to 1000 mg/day. This maximal dose was shown to be safe, suggesting that 1000 mg/day was consistent with the tolerable upper limit of intake,[116] and in line with the findings of the European Scientific Committee that suggested an upper limit of 900 mg/day for nicotinamide in adults.[124] No change in LDL/Total cholesterol was seen in the NR group. Based on these data, we will use a dose of NR of 300 mg po TID.

**Rationale and dosing for propionyl-L-carnitine (PLC).** Our preliminary data demonstrate a reduction in propionyl-CoA and acetylcarnitine in HFpEF (**Fig. 6**). Acetylcarnitine acts as a supply depot for acetyl-CoA when acetyl-CoA generation is limited.[125-127] It is produced by the reaction of acetyl-CoA with carnitine, via carnitine acetyltransferase (CrAT), and functions to maintain a low intra-mitochondrial acetyl-CoA/CoA ratio and adequate availability of CoA for ongoing fat metabolism. Because increasing acetyl-CoA can also reduce flux through the PDH complex, maintaining a low acetyl-CoA/CoA ratio by shuttling acetyl-CoA into acetylcarnitine may also improve carbohydrate metabolism.[128] In fact, L-carnitine has been shown to improve insulin sensitivity and carbohydrate metabolism in both murine and human subjects.[126, 129, 130] Oral L-carnitine supplementation has been shown to increase muscle carnitine levels in athletes, along with increases in PDH complex activity, demonstrating the potential utility of oral supplementation.[131, 132] In our biopsy studies, muscle acetylcarnitine negatively correlated with the half-time of CrCEST recovery (Spearman's rho: -0.42, p=0.02), suggesting that increasing acetylcarnitine could speed SkM OxPhos.

In one small study of 31 HFpEF subjects, improvements in diastolic function and shortness of breath were seen following 3 months of treatment with 1500 mg of L-carnitine daily.[133] In a small study of HFrEF patients, 3 grams/day of L-carnitine for 120 days led to significant increases in the maximal workload achieved during cycle ergometry.[134] In another study, 2 grams/day of L-carnitine improved exercise capacity at 3 months in a randomized trial of 80 HFrEF subjects. Following this blinded period, subjects were continued in their groups for 3 years, noting an improvement in survival with L-carnitine. The only adverse effect noted was minor gastrointestinal upset in 3 subjects assigned to L-carnitine, though this did not require withdrawal.[135]

Propionyl-CoA is generated via the breakdown of odd chain fatty acids and branched chain amino acids (BCAA). It is then converted to succinate, providing carbons to be used in the tricarboxylic acid (TCA) cycle.[136-140] During exercise, TCA intermediate concentrations increase,[141, 142] perhaps in order to sustain high rates of acetyl-CoA oxidation and NADH production.[143] This suggests that reductions in propionyl-CoA may slow TCA cycling by impairing the expansion of its intermediates. In our preliminary data, muscle propionyl-CoA

concentration correlated positively and strongly with both submaximal (ventilatory threshold indexed to leg lean mass: spearman's rho: 0.55, p=0.002) and maximal oxygen uptake (peak $VO_2$ indexed to leg lean mass: spearman's rho: 0.59, p=0.0005). As propionic acid is toxic,[139, 144, 145] prior investigation has focused on giving propionyl-L-carnitine (**PLC**), which is acted upon by CrAT to yield propionyl-CoA and L-carnitine.[127, 138, 144, 146] As an additional benefit, PLC may have better penetration into the cell due to its more lipophilic properties than L-carnitine alone.[138, 139, 147, 148]

When given exogenously, PLC is taken up by the heart and skeletal muscle.[149] PLC has been studied previously in HFrEF, where smaller studies demonstrated improvements in exercise capacity and left ventricular performance, using a dose of 1.5 g/day.[150, 151] However, in a larger study of over 500 HFrEF subjects randomized to 1 gram PLC twice daily for 6 months, PLC did not improve exercise tolerance, though supplementation was safe.[152] Interestingly, in the subgroup of patients with higher LV ejection fractions (>30% vs <30%), an increase in exercise time was seen.

***Table of Human Heart Failure Studies of Oral Propionyl-L-Carnitine Supplementation (Compiled September 2020)***

| Author | Population, n | Dose/Duration | Primary Outcome | Results | Adverse Events |
|---|---|---|---|---|---|
| Mancini, 1992[153] | NYHA II/III HFrEF, randomized double blind placebo controlled, parallel design<br><br>n=60 | 500 mg PLC, three times daily<br><br>180 days | 1. Change in exercise time on incremental cycle ergometry study<br><br>2. Change in ejection fraction | • Exercise time increased in PLC group at 30 days, continuing to increase to 180 days<br><br>• Ejection fraction improved in PLC group | Not reported |
| Caponnetto, 1994[151] | NYHA Class II HFrEF<br><br>Randomized, double blind, parallel design<br><br>n=50 | 500 mg PLC, three times daily<br><br>180 days | 1. Steady-state submaximal exercise endurance (workload corresponding to 70% peak heart rate during incremental test) | • Exercise endurance increased, starting from 30d and sustained at 180d<br><br>• PLC decreased lactate production at the end exercise<br><br>• LV ejection fraction | No adverse events occurred<br><br>One PB patient developed diarrhea which resolved<br><br>No clinically significant biochemical abnormalities developed |

| | | | | | |
|---|---|---|---|---|---|
| | | | | improved in PLC | |
| Anand, 1998[150] | NYHA Class II/III HFrEF<br><br>Randomized, single-blind, parallel design<br><br>n=30<br><br>(18 to PLC, 12 to PB) | 500 mg PLC, three times daily<br><br>30 days | 1. Impact of IV PLC on invasive hemodynamics acutely and following 30d of oral supplementation.<br><br>*Invasive hemo's only in PLC group*<br><br>2. Peak $VO_2$ on incremental treadmill study | 1. Chronic PLC lowered PA pressure<br><br>2. IV PLC acutely reduced PA and PCWP pressure<br><br>3. Chronic oral PLC reduced PA and PCWP after 30d<br><br>4. PLC did not impact neurohormone levels<br><br>5. PLC increased peak $VO_2$ (+45%) and treadmill time (+21%) at 15d, continuing to 30d | No complications.<br><br>PLC did not affect basic laboratory tests (CBC, liver, renal function) |
| Investigators of the Study on PLC in Chronic HF, 1999[152] | NYHA Class II/III HFrEF<br><br>Phase III, double blind randomized parallel multicenter design<br><br>n=537 (271 to PLC, 266 to PB) | 1-gram PLC, twice daily<br><br>6 months | 1. Maximal exercise duration on incremental cycle ergometry protocol | 1. No significant difference in exercise duration in the overall population, nor in the subgroup of individuals who completed the study (n=353, 188 in PLC and 165 in PB) | No difference in adverse events between PB and PLC.<br><br>Patients with any AE:<br><br>PB: 97 (33.7%)<br><br>vs.<br><br>PLC: 98 (34.3%) |

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|---|---|---|---|---|---|
| | | | | 2. *Post-hoc:* Subjects with a baseline LVEF between 30-40% had an improvement in exercise duration with PLC | p=0.882 |

Additionally, several studies have examined the use of PLC for the treatment of claudications in peripheral arterial disease (reviewed in[154]), providing additional safety data. In the largest study, 485 patients with peripheral arterial disease and claudication were randomized to PLC 1000 mg po BID or placebo for 12 months in a parallel design across 38 centers.[155] Five subjects died in each of the PLC and PB arms. Adverse events requiring treatment discontinuation occurred in 27 in the PLC arm and 30 in the PB group, and adverse events not requiring discontinuation occurred in 38 in the PLC group and 98 in the PB group.[155] In the second largest study, 245 patients were studied in a randomized placebo-controlled parallel design study of up to 3 grams/day of PLC based on exercise response for 24 weeks across 13 centers.[156] Eleven adverse events leading to drug discontinuation occurred in the PLC group, as compared to 3 in the placebo group; however, the adverse events requiring drug discontinuation in the PLC group were judged to be unrelated to study medication. There were 7 adverse events not requiring treatment discontinuation in the PB group and 5 in the PLC group – the most common adverse events were nausea and gastric pain. Moreover, minimal benefit was demonstrated for increasing the dose of PLC beyond 2 grams/day. Overall, these studies add to the evidence that PLC supplementation has an acceptable safety profile, with excellent tolerability. Based on all of the above, we will use a dose of PLC 1000 mg po BID.

## *3 Rationale for potassium chloride (KCl), as an active control*

Potassium is known to impact the vasculature and blood pressure.[157] To isolate the benefits of our intervention to either inorganic nitrate, or the combination of inorganic nitrate+NR+PLC, we will use potassium chloride as an active control. KCl will be given in the same doses as $KNO_3$: 6 mmol three times a day.

### *3.1.1 Known Potential Risks*

**a. Potassium Nitrate –** Inorganic nitrate has been tested in several human studies in HFpEF to date and is very well tolerated.[64, 74, 82] In our Dose-Finding study of 12 individuals with HFpEF, 9 were treated with $KNO_3$ as we propose to do in this study.[82] $KNO_3$ did not lead to clinically significant hypotension or methemoglobinemia.[82] There was a systolic blood pressure lowering effect (approximately 12 mmHg),[72, 82] though we note that HFpEF patients generally have elevated blood pressures.[2] Gastrointestinal symptoms and headache were the most common side effects. Anecdotally, the gastrointestinal side effects can be mitigated by taking study medications with a meal,[69] and may be related to the potassium content, as potassium is known to cause GI side effects in clinical practice.[158]

**b. Propionyl-L-Carnitine –** PLC has been used in humans with heart failure previously.[152] In the largest study, 537 patients with HFrEF were randomized to PLC versus placebo (271 received PLC) at the same dose to be used in this protocol. There was no significant difference in adverse

events between subjects receiving PLC versus those receiving PB.[152] Nausea and gastric pain have been reported in other studies.[156]

c. **Nicotinamide Riboside (NR) -** Several studies have reported results on various endpoints using NR supplementation in humans.[111, 113, 114, 116, 118, 123] In a study of 8 healthy individuals, increasing doses of up to 1000 mg po BID for 2 days were well tolerated, increasing $NAD^+$ levels by nearly 100%.[111] More recently, 40 obese sedentary men were randomized to 12 weeks of NR 1000 mg po BID versus placebo. NR was well-tolerated, and there was no increase in adverse events with supplementation.[114] Finally, in the largest study to date,140 obese middle-aged individuals were treated with increasing doses of NR up to 1000 mg/day. This maximal dose was shown to be safe, suggesting that 1000 mg/day was consistent with the tolerable upper limit of intake,[116] and in line with the findings of the European Scientific Committee which suggested an upper limit of 900 mg/day for nicotinamide in adults.[124] We therefore selected this dose (NR 300 mg po three times daily) to be used in this proposal.
    i. **Possible side effects related to NR noted in the above studies include:**
        1. GI upset: nausea, vomiting, diarrhea, transient changes in stool, bloating
        2. Muscle soreness/cramping
        3. Rash/flushing
        4. Headache

d. **Potassium Chloride:** The main side effect of potassium chloride in clinical practice are gastrointestinal symptoms.[69, 158] We will ask subjects to take study medications with food to minimize this. Potassium itself also has an effect on the vasculature, lowering blood pressure (average of ~3/2 mmHg).[158, 159] We do not expect this to lead to clinically-significant hypotension in our HFpEF participants who are generally hypertensive. Subjects with a baseline potassium >5.0 mEq/L will be excluded from the study.

We note that our study seeks to use a combination of therapies in one interventional arm:

- **Potassium nitrate + Propionyl-L-Carnitine + Nicotinamide riboside**

It is possible that there will also be additional/different side effects when the combination of the above medications is administered. For example, a small study in trained individuals demonstrated that glycine propionyl-L-carnitine increased plasma nitric oxide species, suggesting that there *may* be an added effect with potassium nitrate due to additional nitric oxide release from PLC.[160] Another study in HFrEF individuals found that PLC reduced systemic vascular resistance,[139, 151] which is also a target of $KNO_3$.

On the other hand, in a mouse model of diet-induced obesity, the combination of NR + L-carnitine attenuated weight gain and hepatic steatosis to a greater extent than monotherapy with either agent,[161] suggesting the possibility of additional *salutary* benefits to the combination group. Overall, these data suggest that additional risks/benefits may be present in the combination group that will require close monitoring.

As enumerated in the Data Safety Monitoring Plan, we will ensure adequate safeguards are in place for these side effects to be identified, acted upon as necessary, and documented (*please see Data Safety and Monitoring Plan for additional details*).

**Risks and Benefits Assessment:**

The side effect profile of the study drugs, in isolation, demonstrate acceptable tolerability. Our preliminary data, and additional data from other patient populations, support the conclusion that the supplementation strategies to be tested are rationale. At this time, there are no pharmacologic strategies that consistently show a benefit in HFpEF. Even a small improvement in exercise tolerance is expected to allow

HFpEF patients to perform more activities of daily living, increasing their quality of life and independence, while potentially reducing the burden on the healthcare system. Thus, our study has the potential to have a major impact on the lives of millions of patients who suffer from this debilitating condition and who currently are without any pharmacologic treatment options.

## 4 STUDY OBJECTIVES AND ENDPOINTS

**Aim 1:**

| OBJECTIVES | ENDPOINTS | JUSTIFICATION FOR ENDPOINTS |
|---|---|---|
| **Primary** | | |
| Compare the impact on submaximal exercise endurance of pharmacologic therapy aimed primarily at increasing intramuscular perfusion ($KNO_3$) *versus* combination therapy to increase intramuscular perfusion and mitochondrial reserve ($KNO_3$+NR+PLC), as compared to active control (KCl) | **Submaximal Exercise Endurance:** Time to exhaustion while exercising at 75% of peak workload | Submaximal exercise endurance is more relevant to a patient's ability to perform activities of daily living than metrics assessed at higher degrees of exertion (e.g. 'peak') that are not routinely achieved during daily life. |
| **Secondary** | | |
| Assess the impact of our interventions on skeletal muscle oxidative phosphorylation capacity (SkM OxPhos) | **MRI-based assessment of SkM OxPhos**: The kinetics of Creatine recovery following exercise, as assessed using CrCEST MRI spectroscopy | SkM OxPhos is a major topic of interest for this proposal. We hypothesize that our interventions may lead to a benefit in this patient population, predominantly by targeting muscle perfusion and oxidative phosphorylation capacity during exercise. |
| Assess the impact of our interventions on intramuscular perfusion following plantar flexion | **MRI-based assessment of SkM intramuscular perfusion**, as assessed using vPIVOT MRI sequences | Mitochondrial oxidative function is intimately associated with oxygen delivery. Any changes in SkM OxPhos induced by our interventions are best viewed in light of changes to intramuscular perfusion, allowing a better understanding the mechanism of improvement. |
| Assess the impact of our intervention on arterial properties during exercise | **Vasodilatory Reserve:** Systemic vascular resistance (SVR) reserve will be calculated as the % change in systemic vascular resistance at baseline | Our interventions, particularly $KNO_3$, may impact exercise arterial properties, which could influence exercise |

| OBJECTIVES | ENDPOINTS | JUSTIFICATION FOR ENDPOINTS |
|---|---|---|
| | compared to the SVR at 4 minutes of exercise. | capacity by altering muscle blood flow during exercise. |
| Assess the impact of our interventions on quality of life | **Kansas City Cardiomyopathy Questionnaire Overall Summary Score** | Patients with HFpEF have an impaired quality of life that is related to their reduced functional capacity |
| Assess the impact of our interventions on the kinetics of oxygen consumption ($VO_2$ kinetics) during exercise and recovery | **$VO_2$ Kinetics:** "On" and "Off" kinetics will be modeled during the submaximal exercise transient | $VO_2$ kinetics relate to skeletal muscle mitochondrial properties and also to exercise endurance. Through their impact on muscle blood flow and SkM OxPhos, our interventions may affect $VO_2$ kinetics. |
| Assess the impact of our interventions on the maximal/peak rate of oxygen consumption (peak $VO_2$) at the time of exhaustion during the submaximal exercise transient | **Peak $VO_2$** – the maximal rate of oxygen consumption determined during the last 30s of exercise | Because 75%PW will likely be above critical power, it is expected that subjects will evince an additional metabolic cost to exercise that will drive oxygen consumption to maximal/peak rates at the time of exhaustion. |
| Assess the impact of our interventions on ambulatory physical activity | **Steps per day** – we will use actigraphy to document the average number of steps taken per day during the final week of each interventional period | In addition to formal measures of quality of life and exercise tolerance, we will measure steps taken per day as a more patient-centric metric of ambulatory exercise tolerance. |

| OBJECTIVES | ENDPOINTS | JUSTIFICATION FOR ENDPOINTS |
|---|---|---|
| **Tertiary** | | |
| Assess the impact of our interventions on substrate metabolism during exercise | (a) We will measure arteriovenous concentration gradients for plasma substrates (e.g. glucose, lactate, non-esterified fatty acids, and triglycerides) at baseline, 4 minutes of exercise, and at the time of exhaustion. (b) We will also assess the respiratory exchange ratio, a metric of whole-body substrate metabolism obtained from expired gas analysis, at these same time points. Comparisons at 4 minutes of exercise will be the focus. | Inorganic nitrate, PLC, and NR all have been shown to impact muscle metabolism and may alter substrate preference during submaximal exercise. |
| Assess the impact of our interventions on muscle metabolomics | **Tissue metabolite concentrations:** Muscle tissue concentrations of $NAD^+$ and the $NAD^+$ metabolome, propionyl-CoA, and the acylcarnitine profile, and inorganic nitrate will be measured. | We will assess the efficacy of our supplementation strategy on altering tissue concentrations of relevant metabolites. |
| Assess the impact of our interventions on muscle tissue respirometry | ***Ex vivo* tissue measures of substrate metabolism.** We will measure tissue rates of substrate (fat and carbohydrate) metabolism in isolated mitochondria and saponin-skinned fibers. Citrate synthase, a marker of mitochondrial content, will also be assessed. | In addition to our CrCEST-based measures of SkM OxPhos, we will also perform tissue-based measurements to complement the *in vivo* findings. |
| Assess the impact of our interventions on the muscle proteome | **Muscle Proteome:** Relative abundances of proteins related to $NAD^+$ metabolism, mitochondrial biogenesis, and mitochondrial OxPhos will be measured. | Our supplementation strategy may alter concentrations of the proteins involved in substrate utilization and mitochondrial biogenesis |

***AIM 2: IMPACT OF SUPPLEMENTAL $O_2$ ON SKELETAL MUSCLE OXIDATIVE PHOSPHORYLATION CAPACITY***

| OBJECTIVES | ENDPOINTS | JUSTIFICATION FOR ENDPOINTS |
|---|---|---|
| To assess the response of SkM OxPhos to supplemental oxygen | We will compare SkM OxPhos, assessed using the half-time CrCEST recovery, under both room air ($FIO_2$=0.21) and 100% Oxygen ($FIO_2$=1.0) conditions | If a mitochondrial reserve is present, supplemental oxygen should speed SkM OxPhos. On the other hand, if no reserve is present, minimal |

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| | | change in SkM OxPhos to 100% oxygen should be observed. This test will identify HFpEF individuals who harbor additional impairments at the mitochondria ($O_2$-non-responsive) who are more likely to benefit from mitochondrially-directed interventions. |
| To assess whether the contrast in the individual response to our pharmacologic interventions ($KNO_3$ versus $KNO_3$+PLC+NR) on submaximal exercise endurance correlates to the response of SkM OxPhos to supplemental $O_2$ on CrCEST | We will assess the correlation between the change in SkM OxPhos to supplemental oxygen and the contrast in submaximal exercise endurance between the $KNO_3$ and the $KNO_3$+PLC+NR arms. | While $KNO_3$ is hypothesized to improve muscle blood flow, NR and PLC are hypothesized to additionally improve mitochondrial reserve. In patients with intact mitochondrial reserve at baseline, the majority of the benefit to our supplementation strategy will be by increasing muscle blood flow, thus there will be little additional benefit to the NR+PLC. On the other hand, in patients with impaired mitochondrial reserve, the addition of NR+PLC to $KNO_3$ will lead to more substantial improvements in exercise endurance. We will use the CrCEST response to 100% oxygen as a marker of mitochondrial reserve, which should track with the increased exercise response to combination therapy over $KNO_3$ alone. |

# 5 STUDY PLAN

## 5.1 Study Design

*Overview:*

This will be a single-center randomized, placebo controlled, double-blind, 3-treatment, 3-period cross-over trial in 48 HFpEF participants: (A) KCl (active control); (B) $KNO_3$; (C) $KNO_3$+NR+PLC. This design was chosen so that each participant receives all interventions in a pre-specified order across 3 periods; thus, within-subject comparisons of each pair of interventions are possible. Each interventional period will be 6 weeks in duration, separated by a 2-week wash-out period. The Investigational Drug Services (IDS) at UPenn will formulate the KCl and $KNO_3$ capsules; the commercially obtained NR and PLC capsules will be over-encapsulated to maintain blinding.

While changes to the mitochondrial proteome occur early after the initiation of an exercise program (1-2 weeks),[162, 163] the timing of mitochondrial biogenesis in humans is less well known, suggesting that the longer duration of therapy chosen in this study is warranted.[164] Similarly, data are unavailable to specify the washout period for a mitochondrial intervention. In a forced bedrest study, mitochondrial content and OxPhos proteins all decreased after 7 days, demonstrating rapid mitochondrial plasticity.[165, 166] This suggests that our 2-week washout period is reasonable, particularly since this washout ensures an 8-week interval for measuring the outcomes after completion of one intervention.

Randomization schemes will be determined by the statistician and transmitted to the pharmacy. We will split the set of 6 sequences into two sub-sequences (ABC: BCA: CAB) or its dual (ACB: BAC: CBA) and use the full sequence and the sub-sequence to create random blocks of 3 (corresponding to one of the two subsequences) or 6 (the complete sequence). To achieve a balanced design, once one sub-sequence is used, it will not be used again until its dual is selected. We will enroll 53 subjects to account for drop out. In the case of medical necessity, the Principal Investigator can request the randomization codes from IDS.

Following completion of the interventional study enumerated above, subjects will return for a one-day MRI visit during which the response of SkM OxPhos to supplemental inspired oxygen (100% $O_2$) will be assessed. The results from this MRI visit will be compared to the results obtained during the exercise tests.

## 5.2 Scientific Rationale for Study Design

HFpEF subjects suffer from multiple comorbidities that may impact exercise capacity.[167-169] As such, a cross-over design, in which each participant receives all 3 interventions in randomized order, can better account for these phenotypic differences and more efficiently identify any changes in exercise endurance with our interventions, as compared to a parallel design.

The limitations of a cross over study include the need for an adequate washout period, as discussed above, and the more marked impact of dropout on data acquisition.

## 5.3 Justification for Dose

A discussion of the doses to be used in this protocol are enumerated in **Section 2.2.1**. The 3 arms to be tested during the supplementation portion of the study are:

(1) Potassium Nitrate alone

a. $KNO_3$: 6 mmol three times daily

b. Placebo A: Two times daily (Placebo for Propionyl-L-Carnitine)

c. Placebo B: Three times daily (Placebo for Nicotinamide Riboside)

(2) Potassium Nitrate + Nicotinamide Riboside + Propionyl-L-Carnitine

a. $KNO_3$: 6 mmol three times daily

b. Propionyl-L-Carnitine: 1000 mg po two times daily

c. Nicotinamide Riboside: 300 mg po three times daily

(3) Potassium Chloride

a. KCl: 6 mmol three times daily

b. Placebo A: Two times daily (Placebo for Propionyl-L-Carnitine)

c. Placebo B: Three times daily (Placebo for Nicotinamide Riboside)

Because the combination phase includes 3 different compounds, each phase will need to include 3 sets of investigational bottles to maintain blinding. Both the $KNO_3$ and the KCl will be made by the UPenn IDS, as we have done previously,[82] with pharmaceutical grade product obtained from a commercial supplier (e.g. Spectrum Laboratory Products (Gardenia, CA)). Both Nicotinamide Riboside and Propionyl-L-Carnitine will be obtained commercially and delivered to UPenn IDS. UPenn IDS will over encapsulate the pills in order to maintain blinding.

# 6 STUDY POPULATION

## 6.1 Inclusion criteria for patients with HFpEF are:

1. NYHA Class II-III symptoms
2. Left ventricular ejection fraction >= 50%
3. Stable medical therapy for at least 1 month
4. Prior or current evidence for elevated filling pressures as follows:
    a. Mitral early (E)/septal tissue annular (e') velocity ratio > 8, in the context of a septal e' velocity <=7 cm/s or a lateral e' <= 10 cm/s, in addition to one of the following:
        i. Large left atrium (LA volume index > 34 mL/$m^2$)[170, 171]
        ii. Chronic loop diuretic use for control of symptoms
        iii. Elevated natriuretic peptides within the past year (e.g. NTproBNP $\geq$ 125 pg/mL in sinus rhythm or $\geq$ 375 pg/mL if in atrial fibrillation)[172]
    b. Mitral E/e' ratio > 14[171] at rest or during exercise[172]
    c. Elevated invasively-determined filling pressures previously (resting left ventricular end-diastolic pressure >= 16 mm Hg or pulmonary capillary wedge pressure >= 15 mmHg; or PCWP/LVEDP >= 25 mmHg with exercise)[172, 173]
    d. Prior episode of acute heart failure requiring IV diuretics

We note that our inclusion criteria are broadly consistent with the current ESC HFpEF diagnosis algorithm.[172]

## 6.2 Exclusion Criteria

1. Age <18 years old
2. Pregnancy: Women of childbearing potential will undergo a urine pregnancy test during the screening visit. We note that the advanced age of HFpEF subjects (median age of 78 in the Get With the Guidelines-HF program[2]) will make it unlikely that pre-menopausal females will be enrolled.
3. Treatment with organic nitrates or phosphodiesterase inhibitors that cannot be interrupted
4. Uncontrolled atrial fibrillation, as defined by a resting heart rate > 100 beats per minute at the time of the baseline assessment
5. Hemoglobin < 10 g/dL
6. Subject inability/unwillingness to exercise
7. Moderate or greater left sided valvular disease (mitral regurgitation, aortic stenosis, aortic regurgitation), mild or greater mitral stenosis, severe right-sided valvular disease
8. Known hypertrophic, infiltrative, or inflammatory cardiomyopathy
9. Clinically significant pericardial disease, as per investigator judgment
10. Current angina due to clinically significant epicardial coronary disease, as per investigator judgment
11. Acute coronary syndrome or coronary intervention within the past 2 months
12. Primary pulmonary artery hypertension (WHO Group 1 Pulmonary Arterial Hypertension)
13. Clinically significant lung disease as defined by: Chronic Obstructive Pulmonary Disease Stage III or greater GOLD criteria (FEV1<50%), treatment with oral steroids within the past 6 months for an exacerbation of obstructive lung disease, current use of supplemental oxygen aside from nocturnal oxygen for the treatment of obstructive sleep apnea.
    - *Desaturation to <90% on the baseline maximal effort cardiopulmonary exercise test will also be grounds for exclusion*
14. Clinically-significant ischemia, as per investigator's judgement, on stress testing without either (1) subsequent revascularization, (2) an angiogram demonstrating the absence of clinically significant epicardial coronary artery disease, as per investigator judgment; (3) a follow-up 'negative' stress test, particularly when using a more specific technique (i.e., a negative perfusion imaging test following a 'positive' ECG stress test)

- *Exercise-induced regional wall motion abnormalities on the echocardiographic assessment during the baseline maximal effort cardiopulmonary exercise test will also exclusionary*

15. Left ventricular ejection fraction < 45% on a prior echocardiogram or cardiac MRI
16. Significant liver disease impacting synthetic function or volume control (ALT/AST > 3x ULN, Albumin < 3.0 g/dL)
17. eGFR < 30 mL/min/1.73m^2
18. Methemoglobin > 5%
19. Serum potassium > 5.0 mEq/L on baseline testing
20. Severe right ventricular dysfunction
21. Baseline resting seated systolic blood pressure > 180 mmHg or < 100 mmHg
22. Orthostatic blood pressure response to the transition from supine to standing (>20 mmHg reduction in systolic blood pressure 2-3 minutes after standing)
23. Active participation in another study that utilizes an investigational agent (observational studies/registries allowed)
24. Any condition that, in the opinion of the investigator, will interfere with the completion of the study. This may include comorbid or psychiatric conditions that may impede successful completion of the protocol, or logistical concerns (e.g. inability to travel to the exercise unit).

**Additionally, specific exclusion criteria exist for the performance of the MRI studies**:**

- 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 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.

***We note that many contemporary ICD/pacemakers are MRI-compatible. In participants with an implantable device (defibrillator or pacemaker), the specific details of the device will be reviewed and discussed with our radiologists in order to ensure the safety of the participant with that device during scanning at 3T. The participant will only undergo the MRI scan if it is deemed to be safe.*

*If a subject is interested in participation in the study but has contraindications to the MRI portion, the subject can still be enrolled, and the MRI assessments will be omitted.*

### 6.3 Lifestyle Considerations

The following lifestyle changes will be requested during the study:

- Subjects will be asked to follow a low-nitrate diet, avoiding the excessive intake of certain food items such as beets and arugula.
- Subjects will be asked to avoid strenuous exercise for 48 hours prior to each study visit
- A standardized low-nitrate dinner will be provided to each subject prior to each visit
- Subjects will be asked to come to each study visit following an overnight fast
- Subjects will be asked to avoid caffeine for 24 hours prior to each visit due to caffeine's impact on SkM OxPhos and substrate utilization.[174, 175]
- Subjects will be asked to avoid antibacterial mouthwash during the study. Antibacterial mouthwash alters the mouth flora which is a key component in the activation of inorganic nitrate.[176-179]

## 6.4 Screen Failures

Screen failures are defined as participants who consent to participate in the clinical trial but are not subsequently randomly assigned to the study intervention or entered in the study. A minimal set of screen failure information is required to ensure transparent reporting of screen failure participants, to meet the Consolidated Standards of Reporting Trials (CONSORT) publishing requirements and to respond to queries from regulatory authorities. Minimal information includes demography, screen failure details, eligibility criteria, and any serious adverse event (SAE).

Individuals who do not meet the criteria for participation in this trial (screen failure) because of a specific modifiable or transient factor, such as an acute illness which is expected to improve or a recent change in medications, may be rescreened in the future when medically stable. Additionally, if a previously screen-failed subject's clinical status changes or more information becomes subsequently available, a subject can be rescreened for inclusion.

## 6.5 Strategies for Recruitment and Retention

### *6.5.1 Recruitment Plans*

1. *Participants who have participated in prior research studies with our group:* Over the past few years, we have successfully completed several studies in HFpEF subjects.[28, 64, 82, 180] As part of our consent process, we ask for permission to contact participants in the future regarding new studies as they become available. As such, we have a list of HFpEF patients who are interested in participating in research.
2. *Active recruitment of new subjects:* We will leverage an existing recruitment pipeline for heart failure currently used by the Cardiovascular Clinical Research Unit (CCRU) at the University of Pennsylvania. The CCRU team includes clinical research coordinators with extensive experience in the screening of patients with heart failure with preserved ejection fraction for participation in clinical trials (including REDUCE-LAP and various NIH funded Heart Failure trials, such as NEAT-HFpEF, $KNO_3$CKOUT-HFpEF, and INDIE-HFpEF).

**Strategies and Tools to Support Study Recruitment.** Numerous recruitment avenues are in place and may be used in parallel to maximize enrollment.

1. *CCRU recruitment databases*: The CCRU has extensive experience recruiting interested patients for HFpEF trials, including many who have expressed interest in continued engagement with new protocols after having positive experiences with our group and/or participation in Heart Failure Clinical Research Network trials and

others.

2. *Directed advertising*: This may include postings on the Cardiovascular Medicine divisional website, email blasts, clinics, and cardiovascular clinical laboratories.

3. *Focused efforts in high yield environments*: We will conduct directed recruitment in settings where HFpEF patients are likely to present in the course of routine clinical care, including outpatient cardiology practices, the clinical echocardiography laboratory, the clinical stress testing laboratory, and inpatient cardiovascular services.

4. *PennSeek and other Electronic Medical Record query tools:* PennSeek is a tool to search unstructured or semi-structured medical documents currently residing in Penn Medicine's EPIC electronic medical record and diagnostic applications (Radiology, Pathology, Cardiology, Ambulatory, etc.) to analyze and mine this data for patient care and research. PennSeek is designed to allow both refinement of search criteria and immediate review of identified cohort data to achieve the desired degree of specificity. PennSeek is available at CCRU workstations and will be used to identify lists of patients that meet eligibility for the proposed trials. Other similar search tools are available at UPenn, and new search tools are likely to be developed; these too may also be employed to identify potential participants.

5. *Real-time subject identification through EPIC:* The CCRU has worked with our health system electronic health records in the past to identify eligible patients and to enable 'pop-ups' when a patient who meets selection criteria is in clinic or is admitted to hospital. These efforts may again be employed. In addition, these events can generate notifications to the study team, providing real-time identification of potential candidates. Once identified, research coordinators may approach potential candidates and/or their physicians.

### *6.5.2 Retention Plans*

Our participant retention strategies begin with the introduction to the study and relies on frequent communication with our participants about the study protocol and the value they bring by participating in research. We include frequent phone calls to the prescreened and enrolled subjects at specified time points, including phone calls regarding the status of visits, check-ins for enrolled participants, and reminders for upcoming visits. Moreover, the frequent study visits in the describe protocol (8 visits within ~6 months) will allow many contact points between the study team and the participant, improving communication and the relationship between the participant and the study team. We will provide adequate participant compensation and will use the existing facilities in the CHPS unit, which allow flexibility of scheduling according to participants' needs, greatly facilitating study visits, retention, and satisfaction.

Based on recent experience, our retention rate is high (91% to date in the $KNO_3$CK OUT-HFpEF trial). We plan to exclude participants who are unlikely to complete study visits due to non-compliance, psychiatric illness or major comorbidities that would impede successful completion of the protocol, substance abuse, or other social or logistic circumstances that will be judged on a case-by-case basis.

In the event that recruitment is slow and jeopardizing the goals laid out in the milestones, we will consider several strategies to boost enrollment. If enrollment falls significantly below accrual benchmarks, we will expand recruitment efforts to include Penn Medicine hospitals that are farther from downtown Philadelphia, such as Chester County Hospital and Lancaster General Hospital. If enrollment is still below the benchmark, we will seek partnership with other local institutions such as Temple University and Lankenau Medical Center.

If the muscle biopsy and/or the MRI studies represent barriers to enrollment and subject participation, these too may be made optional.

We also provide subject compensation for patient time and transportation to mitigate the burden of participation according to the following schedule:

(1) Baseline Visit: Cycle exercise: $50
(2) Visit 2: Muscle Biopsy and cycle exercise: $100
(3) Visit 3: MRI study: $50
(4) Visit 4: Muscle Biopsy and cycle exercise: $100
(5) Visit 5: MRI study: $50
(6) Visit 6: Muscle Biopsy and cycle exercise: $100
(7) Visit 7: MRI study: $50
(8) Visit 8: 2 MRI studies (room air and 100% oxygen): $100
**Total: $600**

Participants will be paid via by Greenphire clincard after completing each visit. The study coordinator will keep track of patients using an electronic database to build-in schedule reminders. We also maintain close communication via phone/email with previous and active research subjects to mitigate patients becoming lost to follow-up.

### 6.6 End of Study Definition

A participant is considered to have completed the study if he or she has completed all phases of the study including the last visit or the last scheduled procedure shown in the Schedule of Activities (SoA), Appendix Section 12.1. This will include the baseline study visit, the 3 endpoint assessments (submaximal exercise and CrCEST MRI studies) following each interventional period, as well as the final MRI visit (CrCEST with and without supplemental oxygen), unless the subject chooses to curtail participation earlier for any reason. Subjects may also be removed from the study by the PI due to non-compliance, inability/unwillingness to comply with study instructions, or other unforeseen circumstances.

## STUDY INTERVENTION

### 6.7 Study Intervention(s) Administration

#### *6.7.1 Study Intervention Description*

The 3 arms to be tested during the supplementation portion of the study, and the target doses, are:

(1) Potassium Nitrate

a. $KNO_3$: 6 mmol three times daily

b. Placebo A: Two times daily (Placebo for Propionyl-L-Carnitine)

c. Placebo B: Three times daily (Placebo for Nicotinamide Riboside)

(2) Potassium Nitrate + Nicotinamide Riboside + Propionyl-L-Carnitine

a. $KNO_3$: 6 mmol three times daily

b. Propionyl-L-Carnitine: 1000 mg po two times daily

c. Nicotinamide Riboside: 300 mg po three times daily

(3) Potassium Chloride

a. KCl: 6 mmol three times daily

b. Placebo A: Two times daily (Placebo for Propionyl-L-Carnitine)

c. Placebo B: Three times daily (Placebo for Nicotinamide Riboside)

#### *6.7.2 Dosing and Administration*

Study drugs will be escalated to their targets after approximately one week, barring limiting side-effects. An initial two-week supply of the following regimens will be sent to subjects:

Initial starting regimens:

(1) Potassium Nitrate

a. $KNO_3$: 6 mmol two times daily

d. Placebo A: Once daily (Placebo for Propionyl-L-Carnitine)

b. Placebo B: Two times daily (Placebo for Nicotinamide Riboside)

(2) Potassium Nitrate + Nicotinamide Riboside + Propionyl-L-Carnitine

a. $KNO_3$: 6 mmol two times daily

b. Propionyl-L-Carnitine: 1000 mg po once daily

c. Nicotinamide Riboside: 300 mg po twice daily

(3) Potassium Chloride

a. KCl: 6 mmol two times daily

e. Placebo A: Once daily (Placebo for Propionyl-L-Carnitine)

b. Placebo B: Two times daily (Placebo for Nicotinamide Riboside)

A two-week supply will be given to account for potential delays in the shipment of the 2nd batch of study medications. Study medications are to be taken with meals to minimize the risk of GI upset.

***One Week Tolerability Assessment:***

After approximately one week (range 5-9 days to account for weekends/holidays), subjects will be contacted using his/her preferred method (for example, telephone, televideo, phone, text or email) to assess tolerability. Dietary restrictions will be reinforced during this interaction.

Subjects will be asked about side effects, including headache, dizziness, GI upset, nausea and lightheadedness. The presence of symptoms suggestive of orthostatic hypotension (i.e. sustained lightheadedness upon standing that does not dissipate quickly) will prompt an assessment for orthostatic vital signs (a drop in the systolic blood pressure of >20 mmHg 2-3 minutes after moving from the supine to standing position), and/or hypotension (systolic Bp<90 mmHg). If difficulties/barriers/subject preference preclude coming in for an in-person assessment (such as concerns regarding COVID-19), a subject may use a home Bp cuff to perform the orthostatic assessment, with guidance from the study staff:

1. Lie supine for approximately 5 minutes and then check the blood pressure.

2. Gradually move to a sitting position, rest there for a moment (~10-30s), and then stand upright. Subjects may hold on to something for balance.

3. Check blood pressure 2-3 minutes after standing.

4. Note the presence of any symptoms at the 2-3-minute mark after standing

*Note – transient lightheadedness that occurs soon after standing, but that resolves soon thereafter does not constitute a positive response.*

The presence of an orthostatic response (drop in systolic blood pressure of > 20 mmHg approximately 2-3 minutes after the transition from supine to standing) or hypotension (systolic blood pressure < 90 mmHg) will lead to exclusion from the study if confirmed on a repeat assessment. In cases in which home monitoring suggests resting or orthostatic hypotension, the study subject will be encouraged to come to UPenn for a formal assessment.

On the other hand, if study medications are well-tolerated, or tolerated with mild/minimal side effects, the doses will be increased to target:

*Weeks 2-6:*

(1) Potassium Nitrate

a. $KNO_3$: 6 mmol three times daily

f. Placebo A: Two times daily (Placebo for Propionyl-L-Carnitine)

b. Placebo B: Three times daily (Placebo for Nicotinamide Riboside)

(2) Potassium Nitrate + Nicotinamide Riboside + Propionyl-L-Carnitine

a. $KNO_3$: 6 mmol three times daily

b. Propionyl-L-Carnitine: 1000 mg po two times daily

c. Nicotinamide Riboside: 300 mg po three times daily

(3) Potassium Chloride

a. KCl: 6 mmol three times daily

g. Placebo A: Two times daily (Placebo for Propionyl-L-Carnitine)

b. Placebo B: Three times daily (Placebo for Nicotinamide Riboside)

If subjects experience symptoms that do not prompt exclusion from the study (such as mild dizziness or GI upset), the dose may be either kept at the Week 1 dose, or down-titrated to the Week 1 dose from the target dose if up titration was attempted. The default approach will be to up-titrate all participants to target doses unless a compelling reason, which may include the subject's strong preference, exists.

| | **Initial Dose** | **Target Dose** |
|---|---|---|
| **$KNO_3$** | 6 mmol BID | 6 mmol TID |
| **NR** | 300 mg BID | 300 mg TID |
| **PLC** | 1000 mg daily | 1000 mg BID |
| **KCl** | 6 mmol BID | 6 mmol TID |

**Serum Potassium Monitoring:**

Our study interventions all include potassium ($KNO_3$ or KCl). The amount of potassium provided in our study combinations is relatively small (<20 mEq/day). Moreover, subjects with a blood potassium greater than

5.0 mEq/L, or an eGFR < 30 mL/min/1.73 $m^2$, at baseline will be excluded from the study, minimizing the risk that clinically significant hyperkalemia (K ≥ 5.5 mEq/L) will develop as a result of our interventions. As an extra safety precaution, we will perform an additional assessment of the serum potassium following drug uptitration during the first phase of the study in participants who may be at higher risk for hyperkalemia, defined as:

1. Subjects who have a baseline serum K between 4.8 and 5.0 mEq/L, AND
2. Either:
   (a) Potassium-sparing diuretic use (e.g. amiloride, spironolactone, triameterene), OR
   (b) Estimated glomerular filtration rate of 30-39 mL/min/1.73m2

In these individuals, we will check a serum potassium ~1 week after the implementation of the 18 mmol/d dose. A serum potassium >5.5 mEq/L will prompt exclusion from the study. Noting that the potassium content of all 3 interventional regimens is the same (18 mEq/day), this additional safety laboratory assessment will only be done following the first study drug intervention. This laboratory assessment may be performed locally if more convenient for the participant and does not need to be fasting.

Given the small amount of potassium in our supplementation strategy, we will not plan to routinely alter a participant's pre-existing potassium supplementation, unless a high potassium (>5.0 mEq/L) is identified on baseline laboratories, prior to receiving any study medications. Changes to a participant's background medications will be done in conjunction with the participant's provider, and the potassium level will be rechecked following the change but prior to study drug administration. Participants will only be given study medications if the potassium upon recheck following withdrawal/reduction of potassium supplementation is < 5.0 mEq/L.

### *6.7.3 Preparation/Handling/Storage/Accountability*

All study medications will be dispensed by the UPenn IDS in a randomized double-blind manner.

(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. 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 $NO_3^-$/day, given as one capsule (6 mmoles) three times a day. A certificate of analysis will be obtained by the manufacturer.

(B) **Potassium Chloride:** Potassium chloride, granular, USP (450mg) plus lactose monohydrate, spray dried, NF (300mg) will be combined and packed into an identical capsule shell. The dose for this trial will be 18 mmoles of KCl per day, given as one capsule (6 mmoles) three times a day. A certificate of analysis will be obtained by the manufacturer.

(C) **Nicotinamide Riboside Chloride:** NR will be purchased from Chromadex as 300 mg capsules. The dose in this trial will be 300 mg three times a day. Capsules will be over-encapsulated to maintain blinding. A placebo pill of similar weight and color will be constructed by UPenn IDS.

(D) **Propionyl-L-Carnitine:** PLC will be purchased from Biovy as 1000 mg capsules and will be over-encapsulated by UPenn IDS to maintain blinding. A placebo pill of similar weight/color will be constructed by UPenn IDS.

### *6.7.4 Acquisition and accountability*

Both $KNO_3$ and KCl will be prepared by UPenn IDS, as we have done previously.[82] The $KNO_3$ and KCl powders will be obtained from a commercial supplier via purchase through IDS. The commercial supplier will provide a certificate of analysis. IDS will also furnish the placebo capsules for PLC and NR that are of similar color and weight to the study medications. Nicotinamide riboside (NR) will be obtained commercially (TruNiagen). Propionyl-L-Carnitine will be obtained commercially (Biovy).

The study team will randomly test and verify the composition of a random set of NR and PLC pills using mass-spectrometry and quantitative methods. Sealed bottles will be delivered to UPenn IDS. One NR/PLC pill from each lot will be given to the study team by IDS prior to dispensation of study medication from that lot to verify the authenticity of the compound using mass spectrometry/quantitative methods. The purity of the PLC/NR pills will be compared against PLC and NR obtained from a commercial supplier (e.g. SigmaAldrich) who provides a certificate of analysis. These results will be furnished to IDS and the PI prior to dispensation of study medications from that lot. We note that Niagen has been given “Generally Recognized As Safe” status. During this determination, data on the chemical purity of the compound (>99% purity) was presented (https://www.fda.gov/files/food/published/GRAS-Notice-000635--Nicotinamide-riboside-chloride.pdf; accessed 1/13/2020).

### *6.7.5 Formulation, Appearance, Packaging, and Labeling*

Potassium nitrate and potassium chloride will be obtained commercially (e.g. from Spectrum Pharmaceuticals) and encapsulated by the UPenn IDS. Nicotinamide riboside chloride will be obtained commercially from Chromadex. Propionyl-L-Carnitine will be obtained commercially from Biovy. The NR and PLC pills will be over-encapsulated to maintain blinding. UPenn IDS will construct a placebo pill for NR and PLC that will be similar in weight and appearance to the study medications.

## 6.8 Measures to Minimize Bias: Randomization and Blinding

Subjects will be randomized to one of six pre-specified sequences of interventions as shown in the Table below.

| Sequences use in the balanced 3-period 3-treatment crossover design | |
|---|---|
| Sequence | Order of Intervention[1] |
| 1 | ABC |
| 2 | ACB |
| 3 | BAC |
| 4 | BCA |
| 5 | CAB |

| | |
|---|---|
| 6 | CBA |
| [1]Each letter corresponds to one of the three interventions | |

The 3x3 crossover design involves 2 interventions plus an active control and 3 periods, leading to a total of 6 unique sequences (e.g. KCl in period [P] 1; $KNO_3$ in P2; $KNO_3$+NR+PLC in P3). Each sequence thus includes all three possible interventions e.g. in Sequence 1, coded ABC, one possible coding is A=KCl alone, followed by B=$KNO_3$ alone, followed by C= $KNO_3$+ PLC + NR. UPenn IDS will assign the codes linked to the intervention and will provide study medication based on the sequence code to which the subject is randomized. For purposes of blinding, none of the study team will have access to the IDS-assigned codes except in an emergency or at unblinding. If emergency unblinding is necessary, the investigators will access the treatment code by contacting UPenn IDS. This is unlikely given the safety profile of the study medications, and if time allows will be done in consultation with the medical monitor. Regardless, in the event of emergency unblinding, both the medical monitor and the Data Safety Monitoring Board overseeing this study will be informed.

***RANDOMIZATION PROCEDURE:***

The study statistician, Dr. Putt, will prepare a randomization table for inclusion in Redcap. Each subject who enrolls and consents will be randomized to the next available sequence based on consecutive rows of the table. In the event that a subject consents and enrolls but does not obtain medication, their sequence will be assigned to the next available subject. To ensure balance over the study duration, the randomization table will be prepared in random blocks of 6 or 12.

### 6.9 Study Intervention Adherence

Subjects will bring all study medications to their final endpoint assessment for each phase (i.e. Visit 3 for Period 1, Visit 5 for Period 2). Study coordinators will manually count the remaining pills in each bottle and compare against the number of pills expected to be taken to determine compliance. Non-compliance will be defined as taking < 80% of study medications. Remaining study medications will be returned to UPenn IDS for disposal.

### 6.10 Concomitant Therapy

For this protocol, a prescription medication is defined as a medication that can be prescribed only by a properly authorized/licensed clinician. Medications to be reported in the Case Report Form (CRF) are concomitant prescription medications, over-the-counter medications, and supplements.

Subjects will continue on all of their previously prescribed medications during this trial. Except in cases of clinical necessity, alterations to background medications will be discouraged. Due to concerns regarding excessive nitric oxide bioavailability and over-vasodilation, the following medication classes will not be permitted during this study:

- Phosphodiesterase-5 inhibitors (e.g. sildenafil or tadalafil)
- Organic nitrates (e.g. isosorbide mononitrate, sublingual nitroglycerin)

Changes to the medication regimen will be assessed during subject encounters.

# 7 STUDY INTERVENTION DISCONTINUATION AND PARTICIPANT DISCONTINUATION/WITHDRAWAL

## 7.1 Discontinuation of Study Intervention

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
- Significant non-compliance with study medications (<80%)
- Incorrect enrollment or randomization
- Any other reasons related to participant safety
- Termination of the study by the DSMB or regulatory authorities

The reason for study discontinuation will be recorded on the source documents. All such subjects will be asked to complete an early termination visit if possible. During this visit, we will document:

(1) vital signs
(2) compliance with the medications, including pill counts
(3) adverse effects
(4) specific reason for withdrawal

We note circumstances may make the performance of an in-person early termination visit challenging; therefore, this visit may also be performed virtually (e.g. over video conferencing).

## 7.2 Participant Discontinuation/Withdrawal from the Study

Participants are free to withdraw from participation in the study at any time upon request.
An investigator may discontinue or withdraw a participant from the study for the following reasons:

- Pregnancy
- Significant non-compliance with study medications (<80%)
- If any clinical adverse event (AE), laboratory abnormality, or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the participant
- If the participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation

The reason for participant discontinuation or withdrawal from the study will be recorded on a specific Case Report Form (CRF). Subjects who sign the informed consent form and who are randomized, but receive no study intervention, will be replaced.

## 7.3 Loss To Follow-Up

A participant will be considered lost to follow-up if he or she fails to return for an endpoint assessment and is unable to be contacted by the study site staff. Given the cross-over nature of this study design, the study-team will make significant efforts to retain all randomized subjects.

The following actions will be taken if a participant fails to return for a required study visit:

- The study team will attempt to contact the participant and reschedule the missed visit as soon as possible. Study medications may need to be extended, provided no limiting side-effects are present.

- The study team will counsel the participant on the value and importance of maintaining the assigned visit schedule and ascertain if the participant wishes to and/or should continue in the study.

- Participants who are unable to complete a particular study visit will be encouraged to complete subsequent visits, and will not be considered lost to follow-up

- Before a participant is deemed lost to follow-up, the study team will make every effort to regain contact with the participant (where possible, 3 telephone calls and, if necessary, a certified letter to the participant's last known mailing address or local equivalent methods). These contact attempts should be documented in the participant's study file.

- Should the participant continue to be unreachable, he or she will be considered to have withdrawn from the study with a primary reason of lost to follow-up.

# 8 STUDY ASSESSMENT AND PROCEDURES

## 8.1 Efficacy Assessments

***Pre-Visit Assessment:***

Prior to the initial study visit, subjects may be contacted by the study team to discuss the study. Subjects may be sent a copy of the informed consent for review. Data may be abstracted from the electronic medical record, and/or in conversation with the subject, to determine potential eligibility. Specific data to be reviewed and collected may include:

- Name, MRN, date of birth, gender
- Medical history and review of prior office visits, admissions/discharges, procedures, and surgeries
- Results of prior testing (e.g. echocardiograms, heart catheterizations, stress tests)
- Current medications and allergies
- Tobacco, alcohol, and drug use

Subjects will be asked to come to the Baseline visit in the fasted state, after avoiding caffeine for 24hrs, and strenuous activity for 48hrs prior.

***Baseline Visit:***

The main objectives of the baseline visit are to obtain written informed consent from the subject, ensure subject eligibility, and determine the submaximal workload to be used in future assessments (primary endpoint). Study procedures will not commence until after written informed consent has been obtained. The following will be performed during the baseline visit:

- **Anthropomorphic measurements**, including height and weight
- **Physical examination**, including seated blood pressure measurement and orthostatic blood pressure assessment
- **Urinary pregnancy test** for women of childbearing potential
- **Blood analysis and storage**:
    - Comprehensive metabolic profile
    - NT-proBNP
    - Complete Blood Count (hemoglobin, white blood cell count, and platelet)
    - Coagulation profile (PT/PTT/INR)
    - Methemoglobin

- Additional blood will be obtained for storage (plasma, serum, and whole blood)

- **Urine and saliva collection for storage**

- **Kansas City Cardiomyopathy Questionnaire:** Quality of life is significantly impaired in HFpEF patients and correlates with functional capacity.[12, 19] The Kansas City Cardiomyopathy Questionnaire (**KCCQ**) will be administered, and the KCCQ Overall Summary Score will be calculated and compared.[12, 19, 181, 182]

- **Low-nitrate diet:** Educational information and hand-out regarding foods that are high in inorganic nitrate, which are to be avoided during this study.

- **Comprehensive echocardiography**: Echocardiography will 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. 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, approximately 1-cm apical to the mitral valve plane. Additional images may 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. Additional echocardiographic images may also be taken, as new echocardiographic techniques become available, noting that there are no known risks associated with obtaining echocardiographic images.

- **Arterial tonometry**: Arterial tonometry will be performed using a high-fidelity applanation tonometer. Assessments may be obtained at the radial, carotid, brachial, and femoral arteries. Waveforms will be stored for offline analysis. Waveforms will be calibrated to the brachial blood pressure using a validated blood pressure device. Body surface measurements may be made to determine distances between anatomic landmarks and measurement sites, such as the suprasternal notch to the carotid, radial, femoral, and brachial arteries.

- **Dual-Energy X-ray Absorptiometry**: A DEXA scan will be performed. This data will be used to normalize exercise parameters to lean muscle mass and to assess body composition.

- **Maximal effort cardiopulmonary exercise test**: Participants will perform a maximal effort supine bicycle exercise test with expired gas analysis using a Parvo Medics TrueOne 2400 device. The device will be calibrated prior to each study. We will use a supine cycle ergometer designed for stress echocardiography (e.g. Stress Echo Ergometer 1505, Medical Positioning, Inc, Kansas City, MO) and a graded-exercise protocol, with resistance beginning at 15 W for 3 minutes, increasing to 25 W for 3 minutes, and then increasing by 25 W every 3 minutes thereafter until exhaustion.[64, 82] Electrocardiographic and vital sign monitoring will be performed during this test. Verbal encouragement will be given to encourage maximal effort.

    - Echocardiographic images will be obtained during the exercise tests. Lung ultrasound may be performed at rest and during exercise to identify B-lines, a marker of congestion.[183]
    - Peak workload (PW) will be defined as the maximal workload that could be sustained for at least 30 seconds.
    - Peak oxygen consumption will be defined as the average rate of oxygen consumption over the last 30 seconds of exercise.

- *Note:* Subjects will be excluded if the arterial saturation falls to < 90% during this maximal effort study or if significant exercise-induced segmental wall motion abnormalities are noted on exercise echocardiography that are consistent with inducible ischemia. Given the high prevalence of 'false-positive' ECG stress tests in HFpEF,[184] isolated EKG abnormalities, in the presence of preserved echocardiographic wall motion, will not be exclusionary.

- **Submaximal workload verification study:** After a rest interval of at least 1 hour and a standardized low-nitrate lunch, subjects will undergo a submaximal exercise test at 75% of the peak workload (75%PW) achieved during the preceding maximal effort test. Subjects will begin with 1 minute of unloaded (0W) exercise, followed by a step increase to 75%PW, performed until exhaustion.

  - A pedal cadence of 60 RPMs will be maintained, and exhaustion will be determined as the time at which the subject indicates that he or she cannot continue, or as an inability to maintain the pedal cadence >50 RPM for >10s, despite verbal encouragement.[185]
  - The cycle ergometer allows for increments of 5W. If the 75%PW does not fall exactly on a possible value that can be input into the cycle ergometer (e.g. a number that is not divisible by 5), the nearest integer divisible by 5 will be selected (e.g. 92.5 W→ 95W; and 91W → 90W.)

- **Workload titration for subsequent submaximal studies:** The primary exercise endpoint of this study is the time to exhaustion during submaximal exercise. Following the submaximal verification study during the baseline visit, we will titrate the workload to be used in subsequent assessments in order to target a time to exhaustion of 3-6 minutes, as longer tests may be limited by boredom or discomfort as opposed to a physiologic limitation.[185] If the subject exercises for >6 minutes during the submaximal workload verification study, the workload to be used in subsequent assessments will be increased by 10W or 15%, whichever is a greater change (but not greater than the peak workload during the maximal effort test); if the exercise time is <3 minutes, the workload will be reduced by 10W or 15%, whichever is a lesser change. A similar schema previously used in HFpEF patients led to exercise times within the desired range.[74]

***Submaximal Workload Titration Table Based on Results of Verification Test:***

| Peak Workload during Maximal Effort Study | 75% PW from Maximal Effort Study | >6 min to Exhaustion on SubMax Verification Study *(+10W or 15%, whichever Δ is greater)* | <3 min to Exhaustion on SubMax Verification Study *(-10W or 15%, whichever Δ is less)* |
|---|---|---|---|
| 15 | 10 | 15* | 5** |
| 25 | 20 | 25* | 15 |
| 50 | 40 | 50 | 35 |
| 75 | 55 | 65 | 45 |
| 100 | 75 | 85 | 65 |
| 125 | 95 | 110 | 85 |
| 150 | 115 | 130 | 105 |
| 175 | 130 | 150 | 120 |

** Targeted workload cannot be greater than peak workload achieved.*
*** 5W is the minimum loaded workload allowable by the cycle ergometer*

  - *The workload determined after this titration procedure will be used during all subsequent submaximal exercise assessments for the subject.*
  - We note that some individuals may not be able to maintain a pedal cadence of 60 RPMs. In these instances, the self-selected cadence that can be maintained during the submaximal verification study will be recorded. During subsequent visits, the subject will be coached to maintain this same

cadence such that the exercise conditions (workload and cadence) will be consistent across all submaximal exercise endpoint assessments at the end of each period (i.e. Visits 2/4/6).

*Baseline Visit – List of Procedures*

- Eligibility assessment
- Informed consent
- Medical history and ascertainment of concomitant medications
- Dietary counseling re: low nitrate diet
- KCCQ administration
- Physical exam (including orthostatic vital signs, anthropometric measurements)
- Laboratory tests, blood, urine and saliva collection
- Urine pregnancy test, as applicable
- Echocardiography and arterial tonometry
- Maximal effort cardiopulmonary exercise test
- Standardized Low-Nitrate Meal
- Submaximal workload verification study

We note that several studies have demonstrated that inorganic nitrate supplementation does not lead to clinically significant increases in methemoglobin;[82, 186-188] therefore, this will not be serially measured during our study. However, subjects with a significantly increased methemoglobin level at baseline ($\geq$ 5%, where ~1% is considered normal),[189] will not be enrolled, as these individuals may harbor defects in their endogenous capacity to reduce methemoglobin and manage oxidant stress.

Following the baseline study visit, the data will be verified to ensure inclusion/exclusion criteria are met. Subjects will then be randomized, and the study intervention will be delivered to the subject.

***One-week Assessment:***

As described in **Section 7.1.2**, subjects will start on the initial doses of study medications. Subject will be contacted approximately one week later to assess tolerability of the study medications and for adverse effects. The following may then result:

(a) The subject describes mild/no side effects at which point study medications will be up-titrated, as detailed in **Section 7.1.2**;

(b) The subject describes moderate side effects that are tolerable, and the subject and the investigators which to continue – in this case, study medications will be maintained at the initial doses;

(c) The subject describes limiting side effects at which point study medications will be discontinued, an early termination visit will be attempted to be arranged, and the subject will be terminated from the study.

**Visit 2 – Assessment #1: Muscle Biopsy and Submaximal Exercise Endurance**

After taking study medications for 6 weeks (range 5-7 weeks), subjects will return to the exercise unit. Prior to this visit, subjects will receive an actigraphy monitor to be worn for the week prior to the visit. This device will collect activity information, including steps taken per day. The average steps per day during the monitoring period will be calculated. The subject will return this device at the study visit.

Subjects will come to the exercise unit in the morning and in the fasted state, though routinely prescribed medications should be taken according to their schedule. Subjects will be asked to refrain from caffeine for 24hrs prior to this visit, and to avoid strenuous activity for 48 hours prior. A standardized low-nitrate meal will be sent to subjects for the evening meal prior to the study visit, and the last doses of study medications will be taken in the evening prior to this visit. We note that muscle markers of $NAD^+$ metabolism,[117] and blood and muscle nitrate[82, 190] remain elevated well after the last dose. This approach will allow for trough sampling on the morning of the study visit, reflective of our chronic supplementation strategy, and minimize differences in muscle metabolite concentration levels consequent to differences in the timing of the last dose of study medications and the performance of the biopsy and exercise test.

The following will be performed:

- **Anthropometric measurements**
- **Physical exam**: including seated and orthostatic blood pressure assessments
- **Kansas City Cardiomyopathy Questionnaire**
- **Intravenous catheter placement:** 2 venous catheters will be placed: (a) antecubital and (b) a retrograde intravenous catheter on the dorsum of the hand
- **Blood draw**:
    - Comprehensive metabolic panel
    - Complete blood count
    - Storage of blood for later analysis, including whole blood, plasma, and serum
- **Saliva and urine collection for storage**
- **Urine pregnancy test:** for female of childbearing potential
- **Skeletal muscle biopsy of the vastus lateralis:** Prior to exercise, the lateral aspect of the thigh will be sterilized and anesthetized using local anesthetic (e.g. lidocaine with sodium bicarbonate). A small incision will be made overlying the muscle and extended down through the fascia. A modified Bergstrom needle, with suction, will be used to obtain at least 200 mg of muscle tissue.[191] Multiple passes through the same incision may be required to obtain sufficient muscle.
    - **The following studies may be performed on muscle tissue:**
        - **Mitochondrial respiration** – both isolated mitochondrial and permeabilized fiber preparations will be made. Respiration may be assessed following the provision of carbohydrate and fatty acid substrate. Citrate synthase activity may be assessed as a marker of mitochondrial content and used to normalize the respirometry data.
        - **Muscle metabolomics** – including measurement of the $NAD^+$ metabolome, acylcarnitine profile, Acyl-CoA species (including propionyl-CoA), and nitrate/nitrite concentrations

- **Muscle proteomics**
- **Tissue processing for H&E and OCT blocks** for subsequent histologic/immunofluorescent assessments
- We note that additional studies, such as muscle homogenate $^3$H-palmitate flux, may be performed if additional tissue remains and the budget allows.
- Remaining tissue will be stored for later analysis.

- **Submaximal exercise test until exhaustion – Primary Endpoint**: Following the muscle biopsy, and after hemostasis has been achieved, subjects will perform a submaximal exercise test at the workload determined during the baseline visit (75% PW). Strong verbal encouragement will be given to encourage maximal effort until the time of exhaustion. While time to exhaustion is the primary endpoint, several other pieces of data will be obtained. We will mainly compare data obtained at 4 minutes, when steady state hemodynamic conditions are likely to be present:

  - **Arteriovenous Metabolite Concentrations:** The hand with the retrograde IV in its dorsum will be placed in a heated hand box prior to, and during, cycle ergometry. Heated hand boxes have been used to 'arterialize' the blood within the vein, allowing us to obtain blood that approximates arterial content without the use, and risk, of an arterial catheter. The hand will be placed in the hand box prior to exercise (at least 10 min prior to exercise) and remain in the chamber throughout exercise for sampling. The hand box air temperature will be set at $\leq$ 55°C (for comparison, typical sauna temperatures are >65 °C). When used appropriately, and for periods of up to 4 hours for serial sampling, no skin burns have been reported (http://www.uvm.edu/~dmatthew/dem_res/?Page=heated_hand.html, accessed on 9/29/20). Arterialized and venous blood will be obtained immediately prior to exercise, at 4 minutes of exercise when steady-state conditions likely are present, and at the time of exhaustion. Substrates to be measured may include:
    - Glucose
    - Lactate
    - Non-esterified fatty acids
    - Triglycerides

    *Other metabolites/hormones/proteins, such as insulin and acylcarnitines, may also be measured.*

  - **Aortic input impedance:** Radial arterial tonometry and echocardiographic cardiac flow from the left ventricular outflow tract Doppler signal will be obtained at rest, at 4 minutes, and at the time of exhaustion. We will primarily compare differences in arterial hemodynamics at the 4-minute time point across submaximal exercise transients (V2/V4/V6). The vasodilatory reserve (the percent change in systemic vascular resistance relative to baseline) will be the main endpoint.

  - **Respiratory exchange ratio**: the ratio of the rate of carbon dioxide production ($VCO_2$) and the rate of oxygen consumption ($VO_2$) will be assessed ($VCO_2/VO_2$) as a marker of systemic substrate metabolism.[192] Lower values (RER of 0.7) correspond to greater fatty acid oxidation, as compared to carbohydrate (RER of 1.0).[192-195] The RER will be compared at 4 minutes of exercise and will

be computed as the average value over a 30 second time period.[196]

- **$VO_2$ kinetics** – the rate of increase or decrease in oxygen consumption following the onset or cessation of exercise is related to SkM OxPhos.[48, 197-200] In HFpEF, $VO_2$ kinetics are slowed,[49, 50] and SkM OxPhos is insufficient to meet energetic demands, leading to greater biochemical perturbations (e.g. accelerated lactate production) and ultimately the early cessation of exercise. Our interventions may impact $VO_2$ kinetics, as they affect SkM OxPhos. Both "on" and "off" $VO_2$ kinetics may be modeled from the submaximal studies using state-of-the-art single- and double-exponential models, as appropriate.[201]

- **Myocardial diastolic parameters and surrogate markers of congestion** – Given that our interventions may impact arterial properties, which in turn, can impact myocardial performance,[202-208] we will also measure the mitral inflow velocity (E) and tissue doppler signals to arrive at the E/e' ratio, a non-invasive surrogate for left ventricular filling pressures.[209, 210] We may also interrogate echocardiographic estimates of RV function including the tricuspid regurgitant jet velocity[211] and the tricuspid annular plane systolic excursion.[212] Lung ultrasound may also be performed using echocardiography.[183, 212]

  *Note: if the subject is willing, an additional muscle biopsy may be performed at the time of exhaustion. This will be done through the same incision as the resting biopsy performed earlier in the day, using a fresh sterile needle biopsy set-up, though the needle may be angled in a different orientation to sample undisturbed tissue. We note that many studies have employed serial muscle biopsies either during one study visit or on a different days following an intervention,[117, 163, 190, 213-236] demonstrating the acceptability of this practice. We also note that the Molecular Transducers of Physical Activity Consortium (MoTrPAC), an NIH sponsored collaborative effort to understand the impact of exercise on the human body, employs serial muscle biopsies before and at several time points following exercise (pre-exercise, 0.5 hours after exercise, 4 hours after exercise, and 24 hours following exercise).[236]*

**Visit 3 – Assessment #2: SkM MRI for SkM OxPhos and Intramuscular Perfusion**

The subject will continue on study medications for an additional 3-10 days prior to returning to UPenn for the CrCEST/vPIVOT MRI assessment. Subjects will be given a standardized low-nitrate meal for the evening prior to the scan. The last dose of study medications will be taken with this standardized meal in the evening prior to the MRI assessment. Subjects will come to the MRI scanner in the morning and in the fasted state, though routinely prescribed medications should be taken according to their schedule. Subjects will be asked to refrain from caffeine for 24hrs prior to this visit, and to avoid strenuous activity for 48hrs prior. Subjects will be asked to arrive at the MRI scanner at least 1 hour prior to their scan time, to avoid the confounding influence caused by travel. A standardized low-nitrate meal will be given during this rest period prior to the MRI scan.

Subjects will be screened for metal objects on their clothes and in their pockets and asked to remove such items. Then the subject will be placed on the MRI patient bed and the radiofrequency coil placed around the part of the body under investigation. The subject will be given earplugs to dampen the sound made by the scanner during the imaging process. The subject will then be placed inside the magnet until the part of the body under investigation is in the center. The study will then proceed. The MR operator will be in constant two-way voice communication with the subject. The subject will be given periodic updates throughout the examination. Subjects will not be asked to remain in the magnet for more than 1.5 hours.

During the scan, the subject will be asked to perform 2 bouts of plantar flexion exercise against a pneumatically controlled foot pedal. Immediately following exercise, SkM OxPhos (CrCEST protocol, following the first bout of exercise) and intramuscular perfusion (vPIVOT protocol, following the second bout of

exercise) will be assessed during the post-exercise recovery period (**Figure 8)**. When the study is complete, the subject will be brought out of the magnet, the coil will be removed, and the subject will be allowed to get up slowly. Subjects are encouraged to take their time in getting up from the patient bed in order not to induce lightheadedness after the prolonged supine period. Data may be reviewed during the scan session to assess for quality. If one portion of the scan is deemed to be of insufficient quality for accurate data interpretation (CrCEST or vPIVOT portion), a third exercise bout may be performed at the end of the study to repeat this portion, if time allows and the subject is willing.

For each MRI scan, the following represent the main measures:

- **CrCEST-based metrics of SkM OxPhos**:
  - Half-time of CrCEST recovery ($t_{1/2,Cr}$) – primary outcome measure for this assessment
  - The linear slope of CrCEST recovery over the early phase (first 2 minutes) of recovery
  - Baseline and increase in CrCEST asymmetry with exercise in the posterior compartment muscle groups, including the lateral gastrocnemius
- **vPIVOT measures**:
  - Baseline and peak intramuscular perfusion of the muscles involved in calf plantar flexion exercise, as well as the recovery characteristics of intramuscular perfusion, particularly of the lateral gastrocnemius muscle.
  - Baseline and peak conduit artery (e.g. popliteal artery) blood flow
  - Relative measure of capillary oxygenation (T2*) before and serially after exercise

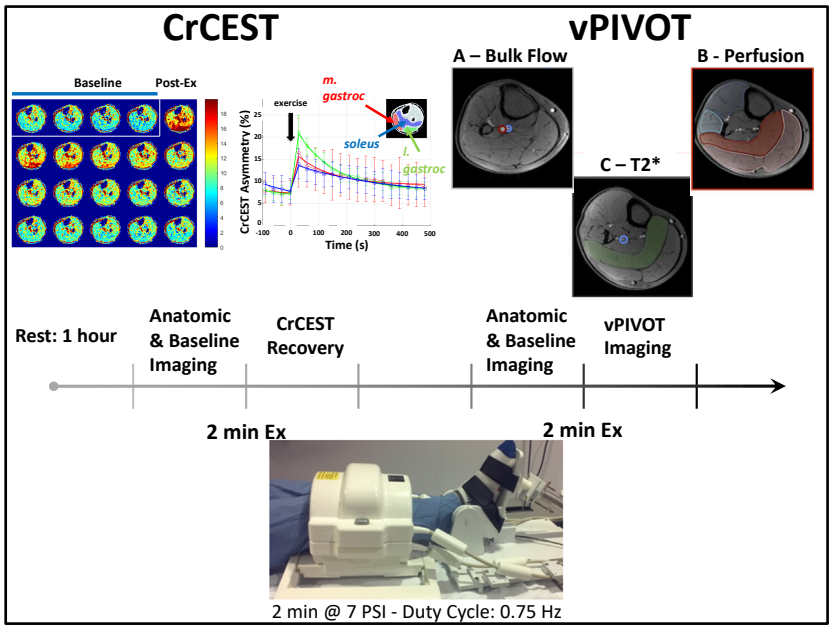


**Figure 8. CrCEST and vPIVOT imaging protocol.** Following rest, the scanning protocol begins with anatomic and baseline CrCEST imaging. A standardized plantar flexion exercise protocol is then started for 2 minutes. Additional CrCEST images are obtained every 30s during recovery for 8 minutes. After a rest period, anatomic and baseline vPIVOT images are obtained, followed by an identical exercise transient and vPIVOT data collection during recovery. In vPIVOT, bulk conduit artery blood flow (A – bulk conduit artery flow), intramuscular perfusion (B – Perfusion), and relative T2* (C – T2*) are measured serially. Adapted from Englund, 2018.

The primary endpoint of the MRI assessment will be the half-time of CrCEST recovery of the lateral gastrocnemius muscle, used as an *in vivo* metric of SkM OxPhos. Additional endpoints include the slope of early (first 2 minutes) Cr recovery, peak lateral gastrocnemius perfusion and its timing and recovery characteristics, relative capillary oxygenation (T2*) and its timing and recovery characteristics, and peak conduit (popliteal) artery blood flow and its timing and recovery characteristics.

Scans will be performed on a 3T MRI scanners. The sequences used in this protocol are not FDA approved. Appropriate language stating as such has been added to the informed consent, as suggested by CAMRIS.

Following both the MRI and exercise assessment, the subject will have completed Period 1. All study medications will be collected and counted by the study team to determine medication compliance. Study medications will then be returned to the UPenn IDS for disposal.

*We note that due to scheduling concerns and/or subject availability, the MRI assessment may be performed prior to the exercise assessment. In this instance, a delay of at least 2 days will be arranged to minimize the small potential for the calf exercise performed during the MRI assessment to impact the cycle ergometry exercise study. In all cases, the subject will remain on study medications until both the exercise and MRI assessments for the period are completed.*

**Wash-Out Period and Subsequent Periods: *(Visits 4-7)***

Subjects will then enter a 2-week washout period, before beginning Period 2. After the washout, the next study intervention in the subject's sequence will be given to the subject by UPenn IDS. The exact same study procedures and protocols enumerated for Period 1 will be repeated, including **Assessment #1: Muscle Biopsy and Submaximal Exercise Endurance** (Visit 4) and **Assessment #2: SkM MRI for SkM OxPhos and Intramuscular Perfusion** (Visit 5)**.** Following Period 2, the subjects will again have a 2-week washout period, before entering Period 3, which will again be identical in its performance (Visit 6 – Muscle biopsy and submaximal exercise and Visit 7 – SkM MRI), as the subject receives the final treatment in his/her sequence.

**Visit 8 – Assessment #3: The Response of SkM OxPhos to Supplemental Oxygen:**

A central goal of this proposal is to understand the relationship between SkM OxPhos and SkM $O_2$ delivery with exercise. If oxygen delivery to, and within, the skeletal muscle is impaired and insufficient to meet mitochondrial needs, SkM OxPhos will be reduced. Increasing $O_2$ delivery will increase SkM OxPhos only if mitochondrial reserve capacity is present and mitochondrial rates of oxidative energy production are less than the rate of SkM energy utilization. In healthy individuals for large muscle mass exercise, maximal rates of isolated mitochondrial $O_2$ consumption exceed maximal systemic and skeletal muscle $VO_2$ achieved during exercise, demonstrating an excess mitochondrial reserve capacity.[237-239] Accordingly, in athletes, SkM OxPhos and PCr recovery kinetics are speeded with supplemental oxygen, demonstrating functional mitochondrial reserve.[240] On the other hand, in sedentary individuals, decreased mitochondrial reserve may limit SkM OxPhos, such that supplemental oxygen is of lesser benefit.[241-243]

Disease state may also impact the SkM response to oxygen. A recent elegant study demonstrated that supplemental $O_2$ did not speed SkM OxPhos in sedentary controls, but it did in sedentary diabetics.[244] These findings were all the more intriguing as diabetic patients were found to have reduced mitochondrial content on muscle biopsy,[244] *demonstrating that even in the context of impaired mitochondrial content, a mitochondrial reserve may be present such that SkM OxPhos rates could be increased with supplemental* $O_2$.

At least 30 days from the last dose of study medications, subjects will again come to the MRI scanner for a final visit. This will be a single-day, 2x2 cross-over study to assess the impact of supplemental $O_2$ on SkM OxPhos in HFpEF. Equal numbers of subjects will be randomized to receive either low ($FIO_2$=0.21, room air) followed by high ($FIO_2$=1.0) $FIO_2$ or high followed by low $FIO_2$. Inhaled gas randomization sequence will be prespecified and stored in RedCap in order to ensure balance (50% with $FIO_2$=0.21 first and 50% with $FIO_2$=1.0 first).

Subjects may be sent a standardized meal for the evening prior to the MRI scan. Subjects will arrive at least one hour prior to their scan time in the fasted state and will then rest, to avoid any confounding influence caused by travel. Subjects will be asked to avoid caffeine for 24 hrs prior to the MRI scan,[174, 175] and to avoid strenuous activity for 48hrs prior. Water intake will not be restricted, and the subjects will take their usually prescribed medications according to their schedule. Upon arrival and during rest, a standardized meal will be given to the subject. Aside from the inspired gases ($FIO_2$=0.21 or $FIO_2$=1.0), the MRI scanning procedure will be identical. The subject and the MRI technician who will interpret the images will both be blinded to the $FIO_2$

order. Both scans will be performed on the same day to minimize biologic variability,[175, 245-248] with at least an hour separating each session to allow for adequate rest and oxygen washout. The same standardized meal will be given to the subject an hour before the second scan. We note that previous studies in sedentary individuals used between 5-30 minutes of rest between repeat studies.[241, 242, 244] Tanks with room air and 100% oxygen will be kept in the control room and attached to a non-rebreather facemask with a reservoir to ensure constant delivery throughout the scanning protocol. Inhaled gases will be started ~15 minutes prior to each scanning sequence for adequate equilibration.[249]

*(Note: Regulators providing 25L/min of flow are available for 100% oxygen; however, regulators for room air are only available that provide a maximum of 15L/min. As the subjects will be breathing the inhaled gases through a reservoir, and the contents of the room air gas should be identical to the content of the air in the MRI scanner, additional intake of ambient room air should not lead to any significant difference than if regulators were able to provide 25L/min of room air. Should a room air regulator that provides 25 L/min of flow become available, this will be used).*

**Primary Endpoint.** The primary endpoint will be the change in SkM OxPhos on CrCEST in response to supplemental oxygen (**ΔSkM OxPhos**). We hypothesize that two basic HFpEF phenotypes of impaired SkM OxPhos will emerge in response to 100% $O_2$:

1. $O_2$-Responsive **(↑ΔSkM OxPhos)**: Those with moderate or major SkM OxPhos improvements with $O_2$, suggesting available mitochondrial reserve, but a defect in $O_2$ delivery.
2. $O_2$-non-responsive **(↔ΔSkM OxPhos)**: Those with no or little improvement in SkM OxPhos with $O_2$, suggesting a lack of mitochondrial reserve.

In addition to defining differences between the $O_2$-responsiveness/non-responsive subjects, we will also compare ΔSkM OxPhos to the contrasts in exercise duration from the interventional portion of this study. The difference in submaximal exercise duration between the $KNO_3$ vs $KNO_3$+NR+PLC interventions may be attributable to the addition of agents selected to improve mitochondrial reserve (NR+PLC). Therefore, we predict that individuals with large improvements in submaximal exercise duration following combination therapy versus $KNO_3$ will be $O_2$-non-responsive, harboring a lack of mitochondrial reserve (↔ΔSkM OxPhos) at baseline. In contrast, individuals with small differences in submaximal exercise duration following combination therapy versus $KNO_3$ alone will be $O_2$-responsive (↑ΔSkM OxPhos), demonstrating the presence of mitochondrial reserve at baseline.

It is possible that in the future, the response to supplemental $O_2$ could be used to phenotype HFpEF patients, identifying those HFpEF individuals who are more likely to benefit from a mitochondrial intervention and enriching clinical trials that otherwise have been hampered by the heterogenous nature of the disease.[250]

## 8.2 Subject Safety and Risk Assessments

***Potential Risks***

1. **Cardiopulmonary exercise test:** Exercise testing 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.[251] These procedures will be performed by qualified personnel according to established American Heart Association guidelines.[252, 253] Non-revascularized myocardial ischemia, which may increase the risk of cardiovascular complications with exercise,[254] is an exclusion criterion for the study, as is current angina due to epicardial coronary artery disease.

   Subjects may feel uncomfortable as a result of pushing themselves during an exercise test. Subjects will likely feel short of breath and tired 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 exercise. Although exercise testing may result in exhaustion, rarely do people develop abnormal heart rhythms or significant cardiovascular complications, such as an acute myocardial infarction,[254] during exercise tests. The risk of this happening is the same as if the participant were to exert him/herself during stressful situations or during exercise elsewhere, though subjects are monitored in our exercise lab setting.

We will perform EKG, heart rate, and blood pressure 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 non-invasively using a pulse oximeter. Of note, oxygen levels can decrease with exercise, even in individuals without significant cardiopulmonary disease.[255, 256] If the arterial saturation falls to below 88% ("severe exercise induced hypoxemia"[256]), we will alert the subject and his/her care provider, if possible, as this may prompt consideration for additional/alternative causes for arterial hypoxemia and shortness of breath.

2. **Echocardiography:** Our exercise protocols routinely incorporate echocardiography during exercise for determination of cardiac output.[64, 82] This is a non-invasive procedure and does not have any known significant risks. It is possible that subjects may experience mild, temporary discomfort or skin irritation from the echo probe, or from wearing the electrodes needed to monitor heart rate during the examination. We may also use the ultrasound machine to obtain images of the lungs for signs of congestion.[183]

3. **Skeletal muscle vastus lateralis biopsy:** Muscle needle biopsy with suction will be performed,[191] following local anesthesia (for example, with 1% lidocaine with sodium bicarbonate). In a large series of 13,500 muscle needle biopsies performed at one center, the overall complication rate was 0.16% in adult patients:

**Table 1.** Biopsy complication rates.

| | Adult (>18 y), $n$ = 13,626 | | Pediatric (<18 y), $n$ = 288 | |
|---|---|---|---|---|
| Complication description | Male | Female | Male | Female |
| Arterial bleed | 1 | 0 | 1 | 0 |
| Ecchymosis/hematoma | 0 | 2 | 0 | 0 |
| Local skin infections ($n$ = 8) | 0 | 0 | 0 | 0 |
| Occlusive dressing | 3 | 0 | 0 | 0 |
| Stitch left in >10 days | 2 | 0 | 0 | 0 |
| Unexplained | 1 | 1 | 0 | 1 |
| Localized numbness | 3 | 2 | 0 | 0 |
| Localized pain >3 days | 3 | 2 | 0 | 0 |

*From Tarnopolsky et al. Muscle Nerve 2011; 43: 717.*

The most significant risks occurring in adults included arterial bleeding (1/13,626 = 0.007%) and local skin infection (8/13,626 = 0.06%). To date, we have performed vastus lateralis biopsies in over 50 subjects without significant complications. We note that our protocol of pre-exercise muscle biopsies has been extensively employed in many prior physiologic studies.[217, 218, 220-228, 230, 257-263] Moreover, several studies have employed serial muscle biopsies either during one study visit or on a different days following an intervention,[117, 163, 190, 213-232, 235, 236] demonstrating that this is an acceptable study procedure. We note that in many of these studies, muscle biopsies were performed before and after exercise, or multiple times during a single exercise transient. In fact, the Molecular Transducers of Physical Activity Consortium (MoTrPAC), an NIH sponsored collaborative effort to understand the impact of exercise on the human body, employs serial muscle biopsies before and at several time

points following exercise (pre-exercise, 0.5 hours after exercise, 4 hours after exercise, and 24 hours following exercise).[236]

Other considerations specific to the muscle biopsy:

- **Post-exercise muscle biopsy:** In individuals who elect to also allow us to obtain a post-exercise muscle biopsy, we do not anticipate a significant increase in risk, noting that a new sterile needle will be used *through the same skin incision* as the biopsy performed prior to exercise, though we may attempt to angle the needle into a different segment of muscle.
- **Thrombocytopenia:** Biopsies will not be performed on individuals with platelet count < 100k on baseline laboratories (**Visit 1**), unless a subsequent check shows that the platelet count has recovered or was erroneous.
- **Treatment with anticoagulation** (e.g. warfarin, dabigatran, apixiban, rivaroxaban, or a different anticoagulant): Given the significant prevalence of atrial fibrillation in HFpEF (~35% in recent large clinical trials such as PARAGON and TOPCAT[264, 265]), we expect that a substantial proportion of enrolled subjects will be on anticoagulation. In these instances, risk stratification regarding the temporary cessation of anticoagulation will be performed (see **Table**):

**Table 1. Suggested risk stratification for perioperative thromboembolism[7]**

| Risk category | MHV | Atrial fibrillation | VTE |
|---|---|---|---|
| High (> 10%/y risk of ATE or > 10%/mo risk of VTE) | Any mechanical mitral valve | $CHADS_2$ score of 5 or 6 | Recent (< 3 mo) VTE |
| | Caged-ball or tilting disc valve in mitral/aortic position | Recent (< 3 mo) stroke or TIA | Severe thrombophilia |
| | | | Deficiency of protein C, protein S or antithrombin |
| | Recent (< 6 mo) stroke or TIA | Rheumatic valvular heart disease | Antiphospholipid antibodies |
| | | | Multiple thrombophilias |
| Intermediate (4%-10%/y risk of ATE or 4%-10%/mo risk of VTE) | Bileaflet AVR with major risk factors for stroke | $CHADS_2$ score of 3 or 4 | VTE within past 3-12 mo |
| | | | Recurrent VTE |
| | | | Nonsevere thrombophilia |
| | | | Active cancer |
| Low (< 4%/y risk of ATE or < 2%/mo risk of VTE) | Bileaflet AVR without major risk factors for stroke | $CHADS_2$ score of 0-2 (and no prior stroke or TIA) | VTE > 12 mo ago |

TIA indicates transient ischemic attack; AVR, aortic valve replacement; ATE, arterial thromboembolism; VTE, venous thromboembolism; and MHV, mechanical heart valve.

*Table from Spyropoulos and Douketis, 2012*[266]

- In subjects at high risk for thromboembolic events, anticoagulation will not be interrupted, and the muscle biopsy will not be performed.
- In individuals at intermediate (expected to be the majority of subjects) or low (expected to be infrequent, given the age and comorbidities in HFpEF subjects) risk for thromboembolic events, we will have an informed conversation with the subject about his/her risk with withholding anticoagulation for the performance of the muscle biopsy. Anticoagulation will only be held if the subject consents to do so. We may also discuss this with the subject's provider.
- For illustrative purposes, an average of 5 days of non-therapeutic anticoagulation in a subject with intermediate risk of thromboembolic events would translate to:
    - 4-10%/year risk → 7% average annual risk → 0.02% daily risk → 0.1% 5-day risk → 1:1000 risk of a thromboembolic event during the peri-biopsy period.

- With the subject's consent, and generally after a discussion with the subject's provider, anticoagulation will be discontinued prior to the muscle biopsy (~5 days prior for warfarin, and 2 days prior for direct oral anticoagulants). This is similar to what is done for clinical procedures, such as a routine colonoscopy.
- In the case of warfarin, the INR will be checked prior to the biopsy, and the biopsy will be performed if the INR is <1.5[266] If the INR is >1.5, we will attempt to reschedule the study visit, if possible, or forego the biopsy.
- Warfarin may be restarted on the evening of the biopsy, and oral anticoagulants may be restarted 2 days following the biopsy.

- **Antiplatelets:** Biopsies can be performed while on aspirin.[191] Additional antiplatelet agents (e.g. clopidogrel, ticagrelor, prasugrel) will be treated similarly to anticoagulation and will not be interrupted without a discussion with the subject and his/her provider. Biopsies will not be performed in subjects on dual antiplatelet therapy (aspirin, in addition to another antiplatelet).
- **Allergy to lidocaine or related anesthetic medication.** In these cases, the nature of the allergy will be discussed with the subject, and the use of an alternative agent may be pursued. For example, in subjects with a lidocaine allergy, which is an amide local anesthetic, alternatives could include the use of an esther local anesthetic (such as tetracaine) or diphenhydramine.[267] These decisions will be made in discussion with the Investigational Drug Pharmacy and the subject, and in light of the agents that the subject may have received previously for local anesthesia.

4. **Phlebotomy and blood draws:** The CHPS unit in which the studies will be performed employs a critical-care trained nurse practitioner and a research nurse who have extensive experience in blood draws and maintaining venous catheters. Risks from the peripheral (e.g. antecubital fossa) venous catheterization include minor discomfort, minor bruising, bleeding, hematoma and/or fainting associated with the drawing of blood. There is also a very small chance (less than 1%) of infection at the blood draw site. We anticipate that completion of the entire protocol (all visits) will lead to less blood drawn than that which is removed during a standard red-blood cell donation (approximately 1 pint or 473 mL):

| | **Baseline Visit** | **Period 1: Assessment #1** | **Period 2: Assessment #1** | **Period 3: Assessment #1** |
|---|---|---|---|---|
| **Resting** | CMP – 5 mL | CMP – 5 mL | CMP – 5 mL | CMP – 5 mL |
| | NTproBNP – 5 mL | CBC – 3 mL | CBC – 3 mL | CBC – 3 mL |
| | Met-hgb – 6 mL | (Coag – 3 mL)* | (Coag – 3 mL)* | (Coag – 3 mL)* |
| | CBC – 3 mL | | | |
| | Coag – 3 mL | | | |
| | **Storage**: | | | |
| | WB: 5 mL | | | |
| | Plasma: 15 mL | | | |
| | Serum: 10 mL | | | |
| **Submaximal Exercise** | | | | |
| | ***"Arterialized" & Venous Blood*** | **Rest (0-min):** | **Rest (0-min):** | **Rest (0-min):** |
| | | WB: 5 mL | WB: 5 mL | WB: 5 mL |
| | | Plasma: 20 mL | Plasma: 20 mL | Plasma: 20 mL |
| | | Serum: 15 mL | Serum: 15 mL | Serum: 15 mL |

| | | | | |
|---|---|---|---|---|
| | | **4-min:** | **4-min:** | **4-min:** |
| | | WB: 5 mL | WB: 5 mL | WB: 5 mL |
| | | Plasma: 20 mL | Plasma: 20 mL | Plasma: 20 mL |
| | | Serum: 15 mL | Serum: 15 mL | Serum: 15 mL |
| | | **Exhaustion:** | **Exhaustion:** | **Exhaustion:** |
| | | WB: 5 mL | WB: 5 mL | WB: 5 mL |
| | | Plasma: 20 mL | Plasma: 20 mL | Plasma: 20 mL |
| | | Serum: 15 mL | Serum: 15 mL | Serum: 15 mL |
| | | | | |
| **Approx. Total:** | **52 mL** | **131 mL** | **131 mL** | **131 mL** |

CMP = comprehensive metabolic panel, CBC = complete blood count, Coag = coagulation profile, Met-hgb = methemoglobin %, WB = whole blood. * = if subject is on warfarin and muscle biopsy is to be performed

5. **Dual Energy X-Ray Absorptiometry (DEXA):** DEXA scanning will occur during Visit 1 in the dedicated and certified lab space of the CHPS unit according to the practice guidelines of the American College of Radiology.[268] Participants will be instructed to never look directly into laser of the scanning arm that passes over the body. In most instances, scans will occur one time. However, in the event that the images and data collection from the first scan are not clear, a second scan may occur. However, in any event, no more than 2 scans will occur for a participant. The length of a single scan will take approximately 15 minutes. The radiation dose received during a DEXA scan is less than that of a chest X-ray.[269] This data will be used to normalize exercise parameters to lean muscle mass.

6. **Kansas City Cardiomyopathy Questionnaire:** Participants may become uncomfortable with questions or feel sadness as a result of completing questionnaires.

7. **Lower Extremity MRI:** The non-invasive nature, lack of ionizing radiation, and lack of gadolinium contrast make the risks associated with our MRI studies small. MRI scans requires the subject to be in a partially enclosed space inside the scanner, which some people may find uncomfortable. The MRI scanner produces different noises during a scan. Patients are given special earplugs to reduce the noise. The MRI scanner has a strong magnet that attracts certain metals. If anyone has these types of metal in their body, the MRI's strong magnetic field can cause the metal to move, which may cause injury. Screening of MRI-related exclusion criteria will be done during the baseline visit and with an on-site questionnaire at the time of, or prior to, the first scan. Subjects with be thoroughly screened for the presence of body metal and excluded from the MRI portion of the study if they are unable to safely undergo an MRI. We will not perform MRI scans in subjects who are not suitable candidates for an MRI. The presence of the following will be evaluated:
    - **a.** Central nervous system aneurysm clips
    - **b.** Implanted neural stimulators
    - **c.** Implanted cardiac pacemaker or defibrillator
    - **d.** Cochlear implant
    - **e.** Ocular foreign body (e.g. metal shavings)
    - **f.** Other implanted medical devices: (e.g. drug infusion ports)
    - **g.** Insulin pump
    - **h.** Metal shrapnel or bullet
    - **i.** Severe claustrophobia
    - **j.** Extreme obesity rendering the patient unable to fit into narrow-bore scanners
    - **k.** Unwillingness of the patient to undergo an MRI scan.

**I.** Pregnancy: Women with childbearing potential will have a urine pregnancy test as part of each period assessment during the interventional medication portion of this study. Attestation of non-pregnant status will be obtained prior to the MRI scans and during **Assessment #3 (**MRI with and without oxygen). We note that the subject's assent of non-pregnancy status is an acceptable practice put forth by CAMRIS.

All patients with metallic implants will be individually evaluated prior to the first MRI. In cases where there is uncertainty, we will consult with our radiologists/MRI technicians. In these cases, the MRI will only be performed if deemed to be safe by our radiologists/technicians. Standard safety practices, as per our institutional standards, will be followed. Voice contact with patients will be maintained throughout the scan. Some individuals may experience leg stiffness/soreness as a result of lying on the table in the MRI scanner with his/her leg relatively still in between bouts of calf exercise. This is anticipated to be minor and should abate as the participant gets off the table and begins to walk around.

***We note that many contemporary ICD/pacemakers are MRI-compatible. In participants with an implantable device (defibrillator or pacemaker), the specific details of the device will be reviewed and discussed with our radiologists in order to ensure the safety of the participant with that device during scanning at 3T. The participant will only undergo the MRI scan if it is deemed to be safe.*

8. **Confidentiality:** There is a potential for a breach of confidentiality. We are committed to protecting confidentiality as described in detail below.

9. **Active Pharmacologic Therapy:** This proposal includes 3 different pharmacologic interventions/combinations: (a) potassium nitrate; (b) potassium nitrate + nicotinamide riboside (NR) + propionyl-L-carnitine (PLC); (c) potassium chloride. Risks are described in **Section 3.1.1**.

10. **Acute Oxygen Supplementation:** There are little data to guide the risk/benefit discussion of acute oxygen supplementation in heart failure overall, and no data in HFpEF specifically, in the absence of hypoxia.[270] As in non-HF patients, hyperoxia can have negative hemodynamic effects in HFrEF patients, such as an increase in systemic vascular resistance and reductions in cardiac output.[271-273] Despite this, oxygen supplementation has been shown to increase exercise time in HFrEF subjects.[274] The impact of oxygen in HFpEF is unknown. Over longer periods of time than that in this study (not anticipated to exceed 1.5-2 hours), supplemental oxygen can have a detrimental effect on lung function. In studies of monkeys, more than 5 days of exposure to 90% oxygen were needed before symptoms developed.[275] Exposure to $FIO_2$=0.5 in healthy subjects for a mean of 44 hours led to an increase in inflammatory mediators found on bronchoalveolar lavage.[276] Pre-existing pathology may also exacerbate oxygen toxicity. In a study of human subjects who were intubated (therefore presumably with some degree of lung injury present), excessive $FIO_2$ above that needed to maintain saturations to >92% have been associated with worse oxygenation at 48 hrs.[277] Indeed, pre-existing lung injury can exacerbate/predispose to oxygen toxicity, worsening lung injury.[275] Even in the setting of an acute myocardial infarction, where less lung injury is reasonably expected to be present than in acute respiratory distress syndromes, supplemental oxygen can have detrimental effects in the absence of hypoxia.[278, 279] Hyperoxia can be associated with absorption atelectasis due to the higher solubility of oxygen in blood than nitrogen.[280] In patients with severe COPD and baseline hypoxia, supplemental oxygen can lead to a decrease in respiratory drive.[281] We note, however, that our study will be performed in stable outpatient in whom known significant pulmonary disease (as defined in the exclusion criteria) will be exclusionary. Moreover, subjects with clinically significant coronary artery disease (as defined above, and per investigator judgement) will be excluded from the study, minimizing

the risk of supplemental oxygen leading to myocardial ischemia. Additionally, the increase in arterial oxygen content with supplemental oxygen may help mitigate the impact of decreased coronary flow on oxygen delivery. During the scan, we will maintain verbal contact with the subjects, checking in on them frequently. The study will be stopped if the subject experiences significant untoward or concerning side effects such as chest pain.

11. **Actigraphy and Step Counts:** The actigraphy monitor is a watch-like device. There are no risks associated with actigraphy and acquisition of the step count data. It is possible that a subject might get mild irritation from the "watch strap" if the monitor is worn on the wrist. In that case, the device may be worn on the hip.

### 8.3 Adverse Events and Serious Adverse Events

**Adverse events**

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 protocol following applicable regulations. 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 IRB and the Data Safety Monitoring Board in a timely fashion (i.e., at continuing review).

**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 Medical Monitor 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 in 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 Medical Monitor (i.e., if any one of these believes it is serious, it must be considered serious).

**Unanticipated Problem**: An Unanticipated Problem (UP) is a medical adverse event OR a non-medical event that is:
(1) unforeseen (unexpected), AND
(2) suggests that research places subjects at greater risk than was previously known or recognized, AND

(3) is related or possibly related to a subject's participation in research

**Classification of AE**

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. This also includes "unlikely related" events (No temporal association or the cause of the event has been identified, or the drug or device is unlikely to be implicated, or there is a low likelihood that a causal relationship exists) |
| Related | There is evidence to suggest a causal relationship, and the influence of other factors is unlikely. This includes the category of "possibly related (reasonable evidence to suggest a causal relationship between the drug and adverse event) |

| 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 SAE shall be determined according to the specified reference document containing safety information (e.g., most recent protocol or informed consent). Any AE/ADE that is not identified in nature, severity, or specificity in the current study reference document(s) is considered unexpected. Events that are mentioned in the reference documents 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. Lightheadedness
7. Worsening renal function
8. Edema
9. Shortness of breath at rest or during/after exertion
10. Fatigue at rest or during/after exertion

The following are potential expected side effects of Potassium Nitrate ($KNO_3$):

1. Slight headache
2. Dizziness
3. New onset or worsening lightheadedness
4. Low blood pressure
5. GI Upset: Stomach discomfort, stomachache, diarrhea, nausea, or vomiting
6. Worsening shortness of breath
7. Worsening fatigue
8. Flushing
9. Rash
10. Orthostatic hypotension

As above, while methemoglobinemia is a *theoretical* side effect of $KNO_3$, we did not observe it in our prior work in HFpEF participants using the same dosing regimen; therefore, we will consider any occurrence of clinically significant methemoglobinemia (>5%) to be unexpected.[82]

The following are potential expected side effects of Potassium Chloride (KCl):

1. GI upset
2. Stomach discomfort
3. Nausea
4. Vomiting

The following are potential expected side effects of Nicotinamide Riboside:

1. GI Upset: nausea, vomiting, diarrhea, transient changes in stool, bloating
2. Muscle soreness/cramping
3. Rash/flushing
4. Headache

The following are potential expected side effects of Propionyl-L-Carnitine:

1. Nausea
2. GI upset/gastric discomfort

**Recording and Reporting of Adverse Events & Unanticipated Problems (UP)**
The PI will continuously supervise all aspects of the trial and review the records of the study subjects following each visit and at the end of their participation. The PI will be responsible for ensuring that all adverse events are noted, followed, and reported to the IRB. UPs will be reported in an expedited manner per applicable regulations.

- **AEs occurring from the time of *signed informed consent* to one week after the administration of the last study drug, or one day after the final MRI visit if participating in MRI studies, will be collected.**
- AEs will be classified according to the guidelines/definitions specified above.
- We note that given the pharmacology of our study medications, events occurring > 2 days after the last dose are unlikely to be related to their administration.[82, 123, 282] As an additional safety margin, we will consider the possibility of an AE being related to study medications up to 5 days after the last dose.
- Any AE rated >=3 in severity and all SAEs must be reported within 1 working day of first becoming aware of the event to the medical monitor. The IRB should also be notified per their reporting guidelines.
- The medical monitor will make a determination about the necessity to modify the protocol, include additional information in the consent form, inform previous participants, temporarily hold enrollment of subjects, or terminate the study. In addition, we will report AEs/SAEs to NIH, FDA, and the DSMB per their regulations, as applicable.
- 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. The DSMB will review adverse events at regularly scheduled meetings. Meetings will be convened based on landmarks in participant completion, for example:
    - First 5 participants complete
    - 10 participants complete
    - 30 participants complete
    - Enrollment complete (53 participants)
        - *Additional meetings may be convened at the request of the DSMB chair*
        - *The above schedule may be modified by the DSMB*
- Events significant enough to necessitate modification of study drug dosing will be captured on an appropriate eCRF module.

**Safety Event 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, improvement, or the event is otherwise explained.

The DSMB will review detailed safety data, as detailed above.

**Management of Suspected (Related) 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 to the regulatory authorities (e.g. DSMB, NIH, etc.). The Principal Investigator, along with the Medical Monitor, will assess all SAEs and evaluate for “unexpectedness” and relationship to study drug. The Principal Investigator is required to complete a report for any event identified as serious, study drug related, and unexpected, and submit it to the Medical Monitor within 1 working day of the PI becoming aware of it.

**Pregnancy**

Pregnancy is a contraindication to enrollment in the study. Pregnancy occurring during the study period, although not considered an SAE, must be reported 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. Study drugs will be discontinued immediately, and the 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.

# 9 STATISTICAL CONSIDERATIONS

## 9.1 Statistical Hypotheses

**Statistics for the Trial Evaluating the Impact of Study Medications on Submaximal Exercise Endurance:** The primary analyses will be intention-to-treat. The 3x3 crossover design involves 2 interventions plus an active control and 3 periods, leading to a total of 6 unique sequences (e.g. KCl in period [P] 1; $KNO_3$ alone in P2; $KNO_3$+NR+PLC in P3). For each block of 6 participants, the design is 'balanced' across periods, with each treatment appearing twice in each period. Each subject will have paired measurements of the primary outcome, submaximal exercise endurance, on each treatment pair ($KNO_3$ vs KCl), ($KNO_3$+NR+PLC vs KCl) and ($KNO_3$ vs $KNO_3$+NR+PLC). We will analyze each treatment pair contrast (e.g. $KNO_3$ vs. KCl) separately using least squares regression, to minimize assumptions on the covariance matrix of the repeated measurements.[283] The regression model will be adjusted for period effects and for baseline submaximal exercise endurance.[283, 284] The model-based estimates and hypothesis tests will be used to determine which, if any, of the individual interventions is/are effective.

The proposed sample size of study completers is 48 (8 subjects per each of 6 treatment sequences); we will enroll 53 subjects to account for dropouts. For the primary outcome, and Bonferroni adjusting for two multiple comparisons of each intervention ($KNO_3$ or $KNO_3$+NR+PLC) to control (KCl), the proposed sample size yields at least 80% power to detect mean differences in submaximal exercise time on the order of 80 seconds. The calculation uses a family-wise two-sided Type I error rate of 0.05 and conservatively assumes a standard deviation (SD) of submaximal exercise time of 170 seconds, around 20% larger than the roughly 140 seconds reported by Eggebeen et al.[74] This effect is hypothesized to be clinically important given the severity of impairment in this cohort (mean submaximal exercise duration of $363 \pm 125$ s seen in Eggebeen et al.).[74] The comparison of $KNO_3$+NR+PLC versus $KNO_3$ alone is expected to yield somewhat smaller effects; without adjustment for multiple comparisons, we have 80% power to detect a mean difference of 70 seconds. In addition to hypothesis testing, 95% confidence intervals will be created to evaluate potential effect sizes for possible future studies.

In general, for the secondary endpoints (e.g. CrCEST recovery time, $VO_2$ recovery kinetics, vasodilatory reserve, respiratory exchange ratio, steps per day, KCCQ overall summary score), we have 80% power to detect reasonable effect sizes (Mean difference/SD) of 0.41. Within each of the physiologic outcome groups (e.g. quality of life, muscle metabolomics, arteriovenous substrate gradients, mitochondrial respirometry and muscle proteome), we will follow the general strategy described above. In these exploratory analyses, in addition to hypothesis testing, we will focus attention on confidence intervals. Conclusions will be based on the coherence of the results for the individual endpoints within pre-defined outcome groups.

The statistical analysis will be blinded to intervention group; exploratory analyses will stratify by sex as a biological variable in order to assess whether males and females tend to respond differently to the interventions. The study is not powered to detect these differences between groups, but sex effects will be more rigorously examined in future studies should differences be apparent. While missing data are expected to

be minimal, we will explore the impact of any missing data using multiple imputation.[284, 285] Analyses will be carried out using the latest version of R or Stata.

*Proteomics Analysis:* The protein assignment and MS2 intensity values will be generated by Spectronaut (Biognosys AG; or similar software) and used to analyze the proteome data. Perseus (or similar software) will be used for proteomics data processing and statistical analysis.[286] The data will be $log_2$-transformed and normalized by subtracting the median for each sample after each intervention. Protein intensities will be compared between matched intervention pairs using paired Student's t-tests. Proteomic comparisons between $KNO_3$, $KNO_3$+NR+PLC, and KCl will identify differentially abundant proteins, and volcano plots will be generated using EnhancedVolcano package in R (or similar software).

Comparisons will be made between individual proteins that are key regulators of fatty acid oxidation (e.g. *CD36, FABP3, SLC27A6, FABP5, CPT1B, CPT2, CRAT, ACADVL, ACADM, ACADS, ACADSB, HADHA, HADHB, ECHS1, HADH, ACAA2, ACAD9, ECI1, ECI2, DECR1, ECH1, ACC)*, mitochondrial biogenesis *(*e.g. *PGC-1$\alpha$, HIF-1, NRF1/2, ERR$\alpha$, TFAM, MXI1, C-MYC, AMPK, ANT,* and *mTOR),* $NAD^+$ metabolism (SIRT1&SIRT3,[83] FOXO,[91] CD38,[287] PARPs,[108] NAMPT, NAMNT, NRK, and CX43[288]), and energy fuel metabolism, with adjustment for multiple comparisons. Additionally, lists of differentially abundant proteins will be used for downstream bioinformatic analysis using the STRING database (string-db.org) and MetaCore (Clarivate Analytics) to identify pathway enrichment, and its associated false discovery rate,[289-291] for each comparison (other similar software may be used).

*Metabolomics Analysis:* Period-adjusted levels of metabolites will be compared between matched intervention pairs using paired Student's t-tests. Candidate metabolites will be selected based on an FDR no greater than 0.05.

**Visit 7 – Assessment #3: The response of SkM OxPhos to Supplemental Oxygen:**

The design and analysis plan is similar to that of the main trial, except that the design is an AB:BA (two-treatment, two-period) crossover trial with each letter corresponding to two different oxygen levels ($FIO_2$=0.21 or $FIO_2$=1.0). Briefly, Dr. Putt will provide a coded randomization sequence in RedCap, which the study coordinator will access on the day of each subject's study.

**Statistics for the Impact of Supplemental Oxygen on SkM OxPhos.**

The primary endpoint will be the change in SkM OxPhos on CrCEST in response to supplemental oxygen (**Δ SkM OxPhos**) using paired statistics. We will then graph the distribution of SkM OxPhos for the individual $FIO_2$ levels as well as for the change in $FIO_2$ levels, as a function of $FIO_2$ order. We hypothesize that the HFpEF population represents a mixture of two 'classes' of response to $O_2$ i.e., 'responders' and 'non-responders'. Under this hypothesis, the variability in SkM OxPhos under 100% $FIO_2$ should far exceed that seen under room air, with some individuals showing a pronounced increase in SkM OxPhos with 100% $FIO_2$ compared to room air, and others showing little change. To formally test this hypothesis, we will fit the change in SkM OxPhos to a latent class model. We will fit two models, one with a single class, and one with two classes and compute the Akaike Information Criteria (AIC). We will then repeatedly permute the assignment of the randomization sequence (Room air followed by 100% $O_2$ or vice versa) in order to determine the distribution of the AIC under the null hypothesis of a single class of response to 100% $O_2$. The observed AIC will be compared to the permutation distribution in order to determine a p-value. Additionally, we will use the latent class model to estimate the proportion of $O_2$-responders by assigning individuals to a class based on the larger of their individual probabilities of class membership. A 95% confidence interval for this proportion will be

determined using the bootstrap method. Based on individual participants' class assignment, we will estimate the threshold in the change in SkM OxPhos corresponding to a 'responder' versus a 'non-responder'.

***Comparison with data from Submaximal Exercise Studies:***

We will then regress the outcome variable, the change in skeletal muscle oxidative phosphorylation capacity on CrCEST (ΔSkM OxPhos) with 100% oxygen supplementation, on the difference in submaximal exercise duration for combination therapy versus $KNO_3$, adjusting for baseline exercise duration. Submaximal exercise duration post-intervention will be pre-adjusted for period effects. We hypothesize a negative slope; individuals with large improvements in submaximal exercise duration between combination therapy and $KNO_3$ should be $O_2$ non-responsive (↔ΔSkM OxPhos); whereas, individuals with limited improvement in submaximal exercise duration between combination therapy and $KNO_3$ are likely $O_2$-responsive (↑ΔSkM OxPhos). With 48 subjects and a Type I error rate of 0.05, we have 80% power to detect moderate Pearson correlations on the order of 0.39 or larger ($r^2$ of at least 0.15).

Further analyses will allow us to probe our findings. First, we will regress submaximal exercise endurance on combination therapy as a function of submaximal exercise endurance on $KNO_3$. We anticipate a large positive intercept term and a moderate slope as combination therapy is expected to be most effective in rescuing the mitochondrial deficit in individuals with low response to $KNO_3$ alone. Alternatively, combination therapy may uncover mitochondrial reserve across the spectrum of $KNO_3$ response; in this case we anticipate a positive intercept term and a null slope. We will regress ΔSkM OxPhos on period-adjusted exercise duration separately for each of the three interventions. We hypothesize a null slope for KCl, a strong positive slope for $KNO_3$, and a positive slope to $KNO_3$ for the combination therapy (which also contains $KNO_3$). The strong positive slope for $KNO_3$ is anticipated based on previous findings; we cannot yet predict the results for the combination as this will depend on which subjects are most impacted by improvements in mitochondrial reserve. Linearity will be assessed by including spline terms in the model and comparing the spline-based models and the linear model using an information criteria (AIC or BIC). Analyses will be repeated, stratified by male versus females, and assessed for evidence of sex-specific differences in the response.

The secondary analysis will consider individual level ΔSkM OxPhos and include only those individuals in the lower and upper quartiles of ΔSkM OxPhos. The outcome variable will be submaximal exercise endurance under each of the treatment regimens. Using a two-group t-test, we will assess whether there are differences in the mean exercise endurance for each treatment group between the upper and lower quartiles of ΔSkM OxPhos. We predict that mean submaximal exercise endurance under the $KNO_3$ intervention will be higher in the upper versus lower quartiles of ΔSkM OxPhos response; mean submaximal exercise endurance under the combination intervention will be higher in the lower versus upper quartiles of ΔSkM OxPhos response.

Using only the 24 subjects with the highest and lowest quartiles of ΔSkM OxPhos, we have 80% power to detect differences in the mean submaximal exercise endurance of 203 seconds. The calculation assumes a familywise Type I error rate of 0.05 adjusted for the two different models and standard deviation of submaximal exercise endurance of 170 seconds.

# 10 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

## 10.1 Regulatory, Ethical, and Study Oversight Considerations

### *10.1.1 Informed Consent Process*

#### *10.1.1.1 Consent Procedures and Documentation*

Written informed consent will be obtained from participants by the investigators prior to entry into the research study. This will be performed in accordance with the guidelines and under the supervision of the University of Pennsylvania Institutional Review Board. The study procedures, interventions, and the associated risks will be explained to participants during the informed consent process. Only IRB-approved consent forms will be used. We expect that all privacy practices put in place by those entities should translate into sufficient privacy for all participants. All informed consent procedures will be carried out by personnel approved to work on the study. The consent process will be carried out in a private area. Each potential participant will be given the consent form to read for him/herself and given adequate time to read the entire consent form. Study personnel will go through the consent form with the potential participant, answering any questions. We will explain the HIPAA form to all potential participants. Potential participants will also be informed of the voluntary nature of their participation and the lack of direct benefits. If the participant agrees to participate, an approved study team member will witness the participant sign and date the consent form, and then verify the process by signing the form as well. At no point will any undue influence be applied to the potential participant by any means including the embellishment of monetary compensation or understatement of risks associated with this study. The language to be used in the consent form will be written such that all potential participants can understand what is being asked of them, should they voluntarily choose to participate in the study. Consent forms will be kept in a locked file cabinet within the Principal Investigator's research space.

Although not directly targeted, economically or educationally disadvantaged persons, and/or employees or students of the University of Pennsylvania will not be denied enrollment and any special protections and/or additional safeguards will be undertaken in order to protect the rights and welfare of these subjects from coercion or undue influence, as appropriate.

### *10.1.2 Study Discontinuation and Closure*

This study may be temporarily suspended or prematurely terminated by the Data Safety and Monitoring Board, the Medical Monitor, or the PI if there is sufficient reasonable cause. Written notification, documenting the reason for study suspension or termination, will be provided by the suspending or terminating party to study participants, investigators, and the funding agency. If the study is prematurely terminated or suspended, the Principal Investigator (PI) will promptly inform study participants, the Institutional Review Board (IRB), and the sponsor (NIH) and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule.

Circumstances that may warrant termination or suspension may include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Demonstration of efficacy that would warrant stopping
- Insufficient compliance to protocol requirements

- Data that are not sufficiently complete and/or evaluable
- Determination that the primary endpoint has been met
- Determination of futility

The study may resume once concerns about safety, protocol compliance, and data quality are addressed, and satisfy the sponsor, DSMB, and the IRB.

In terminating the study, the Principal Investigator will assure that adequate consideration is given to the protection of the subjects' interests.

### *10.1.3 Confidentiality and Privacy*

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, the sponsor, and the UPenn IRB. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence.

All research activities will be conducted in as private a setting as possible.

The study monitor, other authorized representatives of the sponsor, representatives of the Institutional Review Board (IRB), or regulatory agencies may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records.

The study participant's contact information will be securely stored for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IRB, institutional policies, or sponsor requirements.

Study participant research data, which is for purposes of statistical analysis and scientific reporting, will be maintained indefinitely in an electronic database. This will not include the participant's contact or identifying information. Rather, individual participants and their research data will be identified by a unique study identification number. The study data entry and study management systems used by clinical sites and by the research staff will be secured and password protected. At the end of the study, all study databases will be de-identified and archived.

If any data or samples are shared with collaborators at UPenn or elsewhere, only de-identified samples/data will be given, without any linking information.

### *10.1.4 Future Use of Stored Specimens and Data*

Data collected for this study will be analyzed and stored at UPenn. After the study is completed, the de-identified, archived data will be available at UPenn for use by other researchers including those outside of the study. Permission to transmit data and/or share de-identified samples (e.g. muscle, blood, images, exercise data, etc.) to collaborators will be included in the informed consent.

With the participant's approval and as approved by local Institutional Review Boards (IRBs), de-identified biological samples will be stored at UPenn. These samples could be used to research the causes of HFpEF, its complications, and other conditions for which individuals with HFpEF may have and that could improve with

treatment. We cannot anticipate all of the possible future uses of the data and samples. Collaborators and study investigators may be provided with information linking the biological specimens with the phenotypic data from each participant, while maintaining the blinding/confidentiality of the identity of the participant.

During the conduct of the study, an individual participant can choose to withdraw consent to have biological specimens stored for future research. In this case, no future specimens will be collected. However, biosamples and specimens already collected may be retained and used.

When the study is completed, access to study data and/or samples will be provided through the Principal Investigator.

**Commercial Products:** Samples/data may be shared with other entities, including for-profit companies, for research purposes. Any company/entity (at UPenn or elsewhere) receiving information/samples related to this study may ultimately discover products, drugs, tests, etc. as a result, which may lead to commercial products. Study participants will not be entitled to compensation for these discoveries. This information has been included in the informed consent.

### *10.1.5 Safety Oversight*

Safety oversight will be under the direction of a Medical Monitor and a Data and Safety Monitoring Board (DSMB) composed of individuals with the appropriate expertise, including a cardiologist and a statistician. Members of the DSMB should be independent from the study conduct and free of conflict of interest, or measures should be in place to minimize perceived conflict of interest. The DSMB will meet regularly to assess safety data on each arm of the study. The DSMB will operate under the rules of an approved charter that will be written and reviewed at the organizational meeting of the DSMB. At this time, each data element that the DSMB needs to assess will be clearly defined. The DSMB will provide its input to the Principal Investigator and the Medical Monitor.

### *10.1.6 Clinical Monitoring*

A Data and Safety Monitoring Plan has been assembled for this study and is enumerated within a separate document.

### *10.1.7 Quality Assurance and Quality Control*

All monitoring and audits are to be performed according to ICH GCP E6(R2).

We will perform internal quality management of study conduct, data and biological specimen collection, documentation and completion. As in the Data Safety and Monitoring Plan, an individualized quality management plan will be developed to describe a site's quality management.

Quality control (QC) procedures will be implemented beginning with the data entry system, and data QC checks will be run on the database. Any missing data or data anomalies will be communicated to the study team for their resolution.

Following written Standard Operating Procedures (SOPs), the monitors will verify that the clinical trial is conducted, data are generated, specimens are collected, documented (recorded), and reported in compliance with the protocol, International Conference on Harmonization Good Clinical Practice (ICH GCP), and

applicable regulatory requirements (e.g., Good Laboratory Practices (GLP), Good Manufacturing Practices (GMP)).

The investigational site will provide direct access to all trial related sites, source data/documents, and reports for the purpose of monitoring and auditing by the sponsor, Medical Monitor, and inspection by local and regulatory authorities.

### *10.1.8 Data Handling and Record Keeping*

#### *10.1.8.1 Data Collection and Management Responsibilities*

Data collection is the responsibility of the clinical trial staff under the supervision of the Principal Investigator. The investigator is responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported.

All source documents should be completed in a neat, legible manner to ensure accurate interpretation of data.

Hardcopies of the study visit worksheets will be provided for use as source document worksheets for recording data for each participant enrolled in the study. Data recorded in the electronic case report form (eCRF) derived from source documents should be consistent with the data recorded on the source documents.

Clinical data (including adverse events (AEs), concomitant medications, and expected adverse reactions data) and clinical laboratory data will be entered into REDCAP. The data system includes password protection and internal quality checks, such as automatic range checks, to identify data that appear inconsistent, incomplete, or inaccurate. Clinical data will be entered directly from the source documents.

#### *10.1.8.2 Study Records Retention*

Study documents should be retained for a minimum of 2 years after the completion of the study. These documents should be retained for a longer period, however, if required by local regulations.

### *10.1.9 Protocol Deviations*

The PI and the study team should document all scenarios where the protocol is not followed and provide details which may include:

- Who deviated from the protocol?
- What was the deviation?
- When did the deviation occur?
- How did the deviation happen?
- What is the impact of the deviation?
- A root cause analysis of why the deviation occurred

If the assessment results in a determination that any of the following are potentially affected, the deviation would be considered of significant impact:

- having the potential to adversely affect subject safety; OR
- increases risks to participants; OR

- adversely affects the integrity of the data; OR
- violates the rights and welfare of participants, OR
- affects the subject's willingness to participate in research, OR
- there is a potential for an overall impact on the research that should be shared with the IRB for consideration and development of next best steps to address it

### *10.1.10 Publication and Data Sharing Policy*

**1) Trial Registration:** The clinical trial proposed in this application will be registered in www.ClinicalTrials.gov prior to initiation. Registration will be updated to reflect significant protocol amendments during the course of the study. Trial results will be uploaded at the time of publication. Informed consent documents will include a specific statement relating to posting of clinical trial information at ClinicalTrials.gov. The University of Pennsylvania has an internal policy in place to ensure that clinical trials registration and results reporting occur in compliance with policy requirements.

**2) Publication:** Study results will be published in a peer-reviewed publication. The final, peer-reviewed journal manuscript will be submitted to PubMed Central upon acceptance for publication.

Data sharing may be considered upon reasonable request to the Principal Investigator, with appropriate agreements in place.

### *10.1.11 Conflict of Interest Policy*

The independence of this study from any actual or perceived influence, such as by the pharmaceutical industry, is critical. Therefore, any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed. Furthermore, persons who have a perceived conflict of interest will be required to have such conflicts managed in a way that is appropriate to their participation in the design and conduct of this trial.

## 10.2 Protocol Amendment History

| Version | Date | Description of Change | Brief Rationale |
|---|---|---|---|
| | | | |
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| Version | Date | Description of Change | Brief Rationale |
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# 11 APPENDIX

## 11.1 Schedule of Activities (SoA)

| **Procedures** | **Screening Day -7 to -1** | **Baseline Visit Day 1** | **Study Visit 2 Day 42 +/-7 d** | **Study Visit 3 Day 3 +/- 1 day** | **Study Visit 4 Day 21 +/-1 day** | **Study Visit 5 Day 28 +/-1 day** | **Study Visit 6 Day 35 +/-1 day** | **Study Visit 7 Day 42 +/-1 day** | **Study Visit 8 Day 49 +/-1 day** |
|---|---|---|---|---|---|---|---|---|---|
| Informed consent | | X | | | | | | | |
| Demographics | X | X | | | | | | | |
| Medical History | X | X | | | | | | | |
| Randomization | | X | | | | | | | |
| Dietary Counseling re: Low Nitrate Diet | X | X | X | | X | | X | | |
| Concomitant Medications | | X | X | | X | | X | | |
| Physical Exam (height, weight) | | X | X | | X | | X | | |
| Orthostatic Vital Signs | | X | X | | X | | X | | |
| Kansas City Cardiomyopathy Questionnaire | | X | X | | X | | X | | |
| Complete Blood Count | | X | X | | X | | X | | |
| Comprehensive Metabolic Profile | | X | X | | X | | X | | |
| Coagulation Profile | | X | *Only select cases* | | *Only select cases* | | *Only select cases* | | |
| Methemoglobin % | | X | | | | | | | |
| NTproBNP | | X | | | | | | | |
| Urine Collection/Storage | | X | X | | X | | X | | |
| Saliva Collection/Storage | | X | X | | X | | X | | |
| Blood Collection/Storage | | X | X | | X | | X | | |
| Urine Pregnancy Test *(if applicable)* | | X | X | | X | | X | | |

| Procedures | Screening Day -7 to -1 | Baseline Visit Day 1 | Study Visit 2 Day 42 +/-7 d | Study Visit 3 Day 3 +/- 1 day | Study Visit 4 Day 21 +/-1 day | Study Visit 5 Day 28 +/-1 day | Study Visit 6 Day 35 +/-1 day | Study Visit 7 Day 42 +/-1 day | Study Visit 8 Day 49 +/-1 day |
|---|---|---|---|---|---|---|---|---|---|
| Echocardiogram and Arterial Tonometry | | X | X | | X | | X | | |
| Maximal Effort Exercise Test | | X | | | | | | | |
| Submaximal Exercise Test | | X | X | | X | | X | | |
| Muscle Biopsy | | | X | | X | | X | | |
| CrCEST/vPIVOT MRI Assessment | | | | X | | X | | X | X |
| Administration of Supplemental Oxygen during MRI | | | | | | | | | X |
| Adverse Event Assessment | | X | X | X | X | X | X | X | X |
| Complete Case Report Forms (CRFs) | X | X | X | X | X | X | X | X | X |

**END OF DOCUMENT**

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