CLINICAL RESEARCH PROTOCOL

INVESTIGATIONAL PRODUCT(S):		Empagliflozin Potassium Nitrate Potassium Chloride
STUDY NUMBER(S):	**IRB Number**	*849401*
	Other Protocol Identifiers	CHPS: pending

PROTOCOL(S) TITLE:	SGLT2i And KNO_3 in HFpEF – SAK HFpEF Trial
REGULATORY SPONSOR:	University of Pennsylvania
FUNDING SPONSOR(S):	National Institutes of Health
PRINCIPAL INVESTIGATOR	Payman Zamani, MD, MTR
ORIGINAL PROTOCOL DATE:	
VERSION NUMBER:	Version 3
VERSION DATE:	11 October 2021

PRINCIPAL INVESTIGATOR SIGNATURE

STUDY SPONSOR: National Institutes of Health

STUDY TITLE: SGLT2i and KNO_3 in HFpEF – SAK HFpEF Trial

STUDY ID

PROTOCOL VERSION: V3.0

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.

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
DKA – Diabetic ketoacidosis
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
EMPA - Empagliflozin
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
NO_m – Nitric oxide metabolites
OxPhos – Oxidative phosphorylation capacity
O_2 – Oxygen
PB – Placebo
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*
ΔAVO_2 – Arteriovenous O_2 content difference

^{31}P – 31-Phosphate
6MW – 6-minute walk distance

1 STUDY SUMMARY

1.1 Synopsis

Title:	**S**GLT2i **a**nd **K**NO_3 in **HFpEF** – The SAK HFpEF Trial
Short Title:	The SAK HFpEF Trial
Study Description:	This study will test whether Empagliflozin (Empa), with and without Potassium Nitrate (KNO_3), improves submaximal exercise endurance and skeletal muscle oxidative phosphorylation capacity (SkM OxPhos) in participants with Heart Failure with Preserved Ejection Fraction (HFpEF).
Objectives:	1. Compare the impact on submaximal exercise endurance of Empa, with and without KNO_3, as compared to active control (Potassium Chloride, KCl). 2. Assess the impact of Empa, with and without KNO_3, on SkM OxPhos using novel *in vivo* MRI-based imaging techniques that quantify SkM OxPhos and intramuscular perfusion with exercise 3. Assess the impact of Empa, with and without KNO_3, on SkM respiration, the metabolome, and the proteome.
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:	**Skeletal Muscle MRI:** 1. Change in SkM OxPhos following plantar flexion on MRI 2. Change in intramuscular perfusion following plantar flexion on MRI **Metabolic Efficiency:** 3. Change in the kinetics of O_2-consumption with submaximal exercise 4. Change in VO_2 efficiency with submaximal exercise 5. Change in the respiratory exchange ratio and venous concentration of substrates at standardized times during submaximal exercise 6. Change in tissue measures of substrate metabolism, muscle proteome, and the muscle metabolome.

	Hemodynamic: 7. Change in the exercise vasodilatory reserve with submaximal exercise **Quality of Life:** 8. Change in KCCQ overall summary score 9. Change in steps per day
Exploratory Endpoints:	Assess the impact of our interventions on: (1) Markers of intramyocardial filling pressure during exercise (2) Citrate synthase, a surrogate for mitochondrial content
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 Presbyterian Medical Center University of Pennsylvania
Enrolling Participants:	This will be a single center study
Description of Study Intervention:	Investigational Medications: • Empagliflozin (Empa) 10 mg daily • Potassium Nitrate (KNO_3) 6 mmol three times daily • Potassium Chloride (KCl) 6 mmol three times daily The order in which subjects will receive the following 3 interventions will be randomized and administered in a double-blind fashion:

1. Empa + KCl
2. Empa + KNO_3
3. KCl + Placebo for Empa

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:

- Empagliflozin + Potassium Chloride (KCl)
- Empagliflozin + Potassium Nitrate (KNO_3)
- Potassium Chloride (KCl) + Placebo for Empa

Study Overview:

Visit	Procedures	Primary Objective
Visit 1: Baseline Testing	a) Informed Consent and Randomization b) Max Effort CPET c) Submax verification study d) KCCQ	- Determine Peak VO_2 - Determine peak workload (PW) and 75%PW to be used in subsequent submaximal tests - 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 b) Submax (75%PW) Exercise test c) KCCQ	- Determine Phase A submax exercise time - Determine Phase A submax VO_2 on/off-kinetics - 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 - Intramuscular perfusion – vPIVOT
2-Week Washout, followed by 6 weeks of Period 2		
Visit 4: Period 2 Endpoint Assessment 1	a) Muscle Biopsy b) Submax (75%PW) Exercise test c) KCCQ	- Determine Phase B submax exercise time - Determine Phase B submax VO_2 on/off-kinetics - 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 - Intramuscular perfusion – vPIVOT
2-Week Washout, followed by 6 weeks of Period 3		
Visit 6: Period 3 Endpoint Assessment 1	a) Muscle Biopsy b) Submax (75%PW) Exercise test c) KCCQ	- Determine Phase C submax exercise time - Determine Phase C submax VO_2 on/off-kinetics - 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 - Intramuscular perfusion – vPIVOT

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,(*18-21*) suggesting that new treatment approaches are needed.**

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 differ 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.(*22*) 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 (**$VO_{2,peak}$**).(*22*) 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. **Moreover, because multiple abnormalities are present, improving any one abnormality has limited potential in improving exercise capacity.(*22*) On the other hand, therapies that target multiple pathways simultaneously stand a better chance to meaningful improve exertional intolerance in HFpEF patients.**

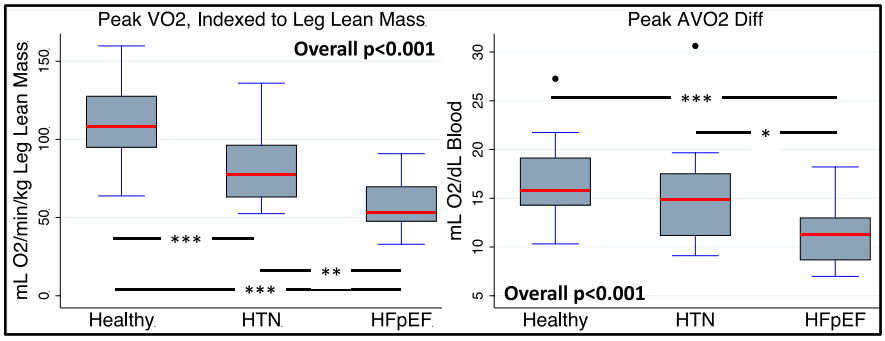


Figure 1 – *Peak VO_2 and the arteriovenous O_2 content difference are reduced in HFpEF.* Healthy (n=20), HTN (n=19), and HFpEF (n=20) individuals performed a maximal effort cardiopulmonary exercise test on a supine cycle ergometer. Echocardiography was used to determine cardiac output, and the ΔAVO2, 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 systemic 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.(*22-24*) 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.(*25, 26*) HFpEF subjects had markedly lower peak VO_2, compared to either control group. **Importantly, HFpEF subjects had a diminished ΔAVO_2** (**Fig. 1**), while peak cardiac output was no different,(*25, 26*) suggesting to us that the SkM may be an important site of exercise limitation in HFpEF.

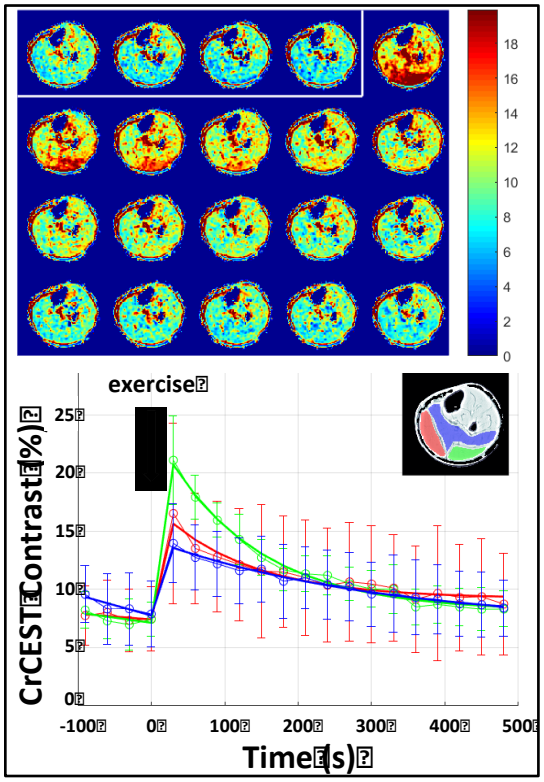


Figure 2 – Example of exercise-induced changes in Free Cr, measured by CrCEST. One healthy 36-year-old male performed plantar flexion exercise for 2 minutes (0.75 Hz). Baseline Cr maps are shown in the upper left (white box). Serial Cr maps following exercise are displayed at 30s intervals, with relative 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**).(*26-31*) 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.(*29, 32*) 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.(*27, 29-31*)

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**).(*26*)

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.

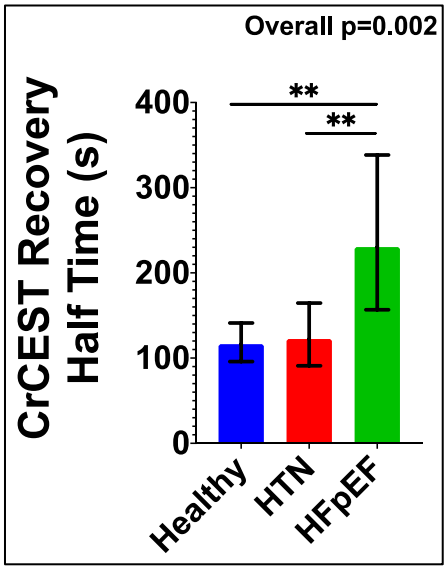
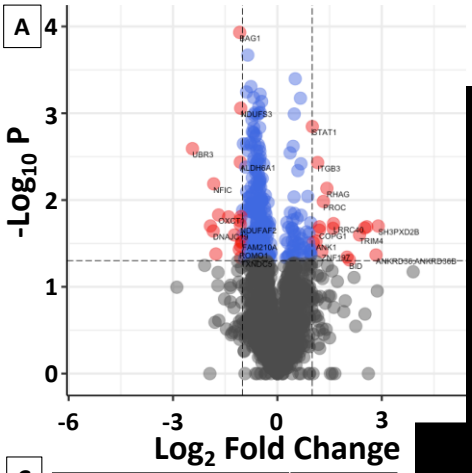


Figure 3 – SkM OxPhos is prolonged in HFpEF. The linear half-time of Cr recovery was calculated following a standardized plantar flexion exercise protocol. Data are displayed as the geometric mean with the 95% CI. ** Bonferroni-adjusted p<0.01

HFpEF skeletal muscle demonstrates reductions in the relative levels of mitochondrial complexes and enzymes in the OxPhos pathways. We then initiated a SkM biopsy program to investigate changes within the SkM of our HFpEF patients. We performed comprehensive LC-MS/MS proteomic analyses on muscle tissue from 12 HFpEF subjects, 9 HTN, and 13 healthy controls.(*26*) Proteins were extracted and digested into peptides, and then analyzed using LC-MS/MS, as reported previously by our team.(*26, 33*) In our comparison of HFpEF versus similarly-aged HTN SkM, a total of 2832 proteins were quantified, of which the relative abundance of 327 proteins was different between groups (p<0.05, **Fig. 4**). Using the Gene Ontology (GO) database,(*34, 35*) we found that the top biologic processes represented by these differentially abundant proteins were predominantly related to energy fuel metabolism and oxidative phosphorylation (false discovery rate [FDR]<1e-48).(*26*) **The proteomic data corroborate the functional findings, giving additional support for the existence of mitochondrial and SkM OxPhos abnormalities in HFpEF.**

Little research has focused specifically on the mitochondria in HFpEF. A recent phase 2b study evaluated the impact of neladenoson bialanate, a partial adenosine A_1 receptor agonist with potential mitochondrial effects, in HFpEF.(*36*) Unfortunately, the drug did not demonstrate any improvements in the six-minute walk distance, a marker of submaximal exercise capacity. Supporting data for this compound were largely drawn from animal studies in ischemic HFrEF and adults with angina,(*37-39*) two models with unclear relevance to HFpEF. As pointed out by the authors, the neutral findings suggest that either neladenoson bialanate: (a) did not effectively improve the mitochondrial abnormalities present in HFpEF participants, or (b) that any mitochondrial improvements caused by the drug did not translate into changes in six-minute walk distances.(*36*) Unfortunately, the design of the study precluded an ability to discern which conclusion should be drawn. **The current proposal will test pharmacologic agents that specifically target abnormalities demonstrated in our HFpEF patients. Within the context of a rigorous clinical trial, we will have the phenotypic depth to explore and understand the mechanistic basis of our interventions.**

A



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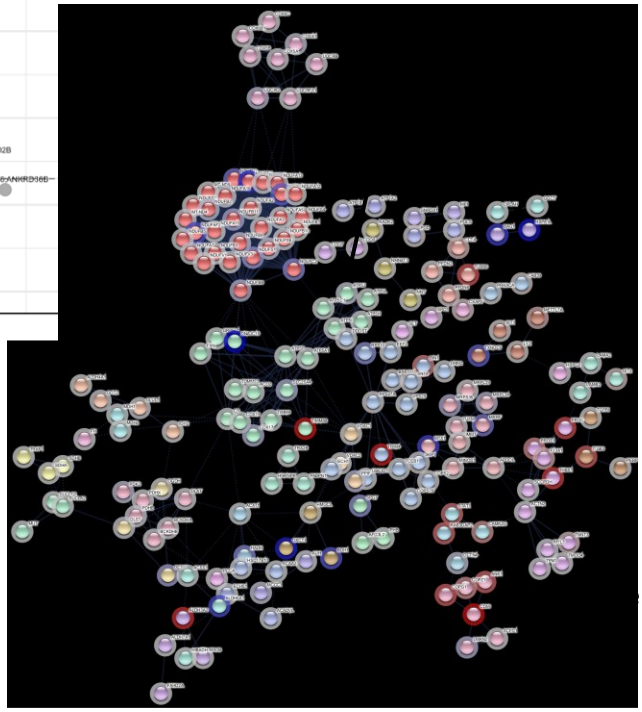

C

Biologic Process (GO)	False Discovery Rate
Generation of precursor metabolites and energy	7.76e-53
Oxidation-reduction process	4.32e-49
Small molecule metabolic process	7.62e-47
Cellular respiration	3.34e-44
Mitochondrial organization	1.85e-41

Figure 4 – Volcano plot, network map, and biologic enrichment of proteins significantly different between HFpEF and HTN participants. (A)Volcano plots were constructed with blue dots representing proteins with significantly different relative levels between HFpEF and HTN participants ($p<0.05$), and red dots and the gene names listed for proteins that had significantly different levels with an absolute log_2 fold-change (FC)>1. (B) All proteins with significantly different relative levels between HFpEF and HTN participants ($p<0.05$), and their log_2 fold-change (FC), were entered into the String Database (string-db.org). Interrelated proteins are displayed along with the connection between proteins and groups. Related proteins are shaded a similar color. The halo around each protein represents the log_2 FC, with blue indicating a relative decrease in protein level in HFpEF participants as compared to HTN participants, and red representing an increase. Though not inclusive, specific clusters of proteins related to energy fuel metabolism are enumerated. (C) Enrichment of the top 5 biologic (GO) processes, along with the false-discovery rate are listed. AKGDH= alpha-ketoglutarate dehydrogenase complex; BCKDH=branched-chain alpha-keto dehydrogenase complex; PDH=pyruvate dehydrogenase; TCA=tricarboxylic acid cycle.

In summary, HFpEF is a multifaceted disease that includes impediments to SkM OxPhos, impacting exercise tolerance. We reason that combinations of pharmacologic agents, which specifically target deficits identified in our HFpEF patients, will be needed to have a meaningful impact on exercise capacity.

SGLT2i have changed the paradigm of how "antihyperglycemic" agents are viewed. The medical community witnessed a paradigm-shifting discovery with sodium-glucose cotransporter-2 inhibitors (**SGLT2i**). Following concerns for an increased risk of myocardial infarction with rosiglitazone,(*40*) the FDA mandated

cardiovascular outcomes trials during the development of antihyperglycemics.(*41*) Consequently, the EMPA-REG OUTCOME trial was conducted in ~7000 Type II diabetics,(*42*) finding a marked reduction in outcomes, driven primarily by reduced heart failure hospitalizations. Several subsequent studies of other SGLT2i (canagliflozin and dapagliflozin) recapitulated these remarkable findings.(*43-49*) Importantly, the antihyperglycemic effects of these agents are modest (generally 0.5-1% reduction in HbA_{1C}) and unlikely to explain the observed benefit.(*47, 50*)

Even more provocatively, the DAPA-HF trial randomized subjects with HFrEF, *with and without diabetes*, to dapagliflozin (DAPA) and found that DAPA reduced HF exacerbations and cardiovascular death by ~25% over a median of 18 months,(*51, 52*) alongside improvements in quality of life.(*53, 54*) The benefit of DAPA was demonstrated regardless of diabetes status(*55*),age,(*56*) gender,(*57*) or the use of other guideline-directed medical therapy.(*58*) Similar findings in HFrEF have subsequently been demonstrated for empagliflozin,(*59-61*) another SGLT2i that is more selective for the SGLT2i receptor.(*62*)

The SOLOIST-WHF Investigators examined the effect of sotagliflozin, a combined SGLT2i and gut SGLT1-inhibitor, in Type 2 DM participants who were hospitalized for worsening heart failure. While the trial was ended early due to loss of funding, an analysis of 1222 randomized patients who were followed for 9.0 months showed that the rate of primary endpoint (total cardiovascular deaths and hospitalizations/urgent visits for heart failure) was reduced by sotagliflozin. Two-hundred and fifty-six of these participants (~20% of the study population) had an LVEF >=50%, and there was no heterogeneity of the treatment effect by ejection fraction, suggesting a potential role in HFpEF.

Given these studies, SGLT2i are approved for the treatment of HFrEF patients; however, the utility of SGLT2i in HFpEF remains unknown. Two ongoing studies are examining the impact of SGLT2i on cardiovascular outcomes in HFpEF (DELIVER; NCT03619213 and EMPEROR-Preserved; NCT03057951). While both studies will also look at quality of life measures, neither is testing the impact of SGLT2i on exercise capacity despite its important relationship to quality of life and independence.(*10, 63*)

SGLT2i have the potential to improve exercise by improving mitochondrial function and energy fuel metabolism through several mechanism, many of which target abnormalities specifically described in human HFpEF:

1. **Increased mitochondrial biogenesis**: In a human HFpEF SkM biopsy study, citrate synthase activity and porin expression, two markers of mitochondrial volume density,(*64*) were both reduced in HFpEF subjects as compared to age-matched controls, suggesting impaired mitochondrial biogenesis in HFpEF.(*65*) EMPA has been shown to increase the AMP/ATP ratio, leading to activation of AMP-kinase (AMPK),(*66-68*) a master regulator of mitochondrial energy homeostasis.(*69, 70*) Amongst its many targets, AMPK activation leads to an increase in PGC-1α activity,(*69, 71, 72*) potentially leading to an SGLT2i-induced increase in SkM mitochondrial biogenesis.(*73-75*) In both murine and porcine models of ischemic HFrEF, EMPA increased PGC-1α and AMPK activation,(*67, 68, 76*) leading to increased mitochondrial biogenesis and volume.(*76, 77*) Through this mechanism, EMPA has the potential to target the impaired SkM OxPhos seen in our HFpEF participants.
2. **Increased fatty acid oxidation**: AMPK also phosphorylates a host of enzymes including acetyl-CoA carboxylase (ACC),(*67, 69, 70, 78*) leading to a reduction in malonyl-CoA and CPT-1 inhibition.(*79, 80*) Enzymes involved in fatty acid import are also increased,(*70*) collectively leading to increased fatty acid oxidation (**FAO**).(*69-71*) SGLT2i may also attenuate a HIF-1α mediated decrease in FAO in diabetes.(*81, 82*) In murine models of heart failure due to coronary artery ligation, EMPA attenuated the post-infarction decrease in exercise capacity,(*83*) increased the levels of transporters involved in fatty acid import,(*76*) and increased mitochondrial FAO.(*83*) In a porcine model of ischemic HFrEF, EMPA increased myocardial fatty acid uptake.(*68*) In several human studies of diabetic patients, SGLT2i have been shown to increase resting FAO.(*84-86*) Pertinent to this proposal, one of the main adaptations to aerobic conditioning is an

increase in fatty acid oxidation,(*87-92*) and animal studies in heart failure,(*93, 94*) including HFpEF,(*95*) demonstrate impaired FAO in this condition. This suggests that perhaps by increasing rates of FAO, SGLT2i could improve the markedly reduced exercise tolerance seen in HFpEF participants.

3. **A shift away from carbohydrate**. As glucose levels fall, insulin declines, leading to an increase in the glucagon:insulin ratio,(*84-86, 96-98*) and an increase in lipolysis.(*85, 96*) In turn, acetyl-CoA production at the liver increases, leading to ketone body production and export,(*96*) which can then be oxidized at the SkM.(*99-101*) Several studies have shown an increase in plasma ketones with SGLT2i in both animal(*67, 68, 76, 83, 102*) and human studies.(*84, 96, 98, 103-105*) EMPA has also been shown to increase utilization of ketone bodies,(*68, 76*) providing an alternative carbon source for energy production.(*106*) Glycogen stores are intrinsically linked to exercise endurance.(*107-110*) An SGLT2i-induced shift away from carbohydrate utilization could have a glycogen-sparing effect,(*99, 111, 112*) allowing its depletion to be delayed, and prolonging exercise capacity.(*99, 109, 113-115*) In addition to proteomics, we also assessed the phosphoproteome using LC+MS/MS, as done by our Co-Investigators previously,(*33*) and compared differences in protein phosphorylation between HFpEF and HTN subjects. While the main differences in phosphorylation were on contractile proteins, we also found reductions in phosphorylation on serine-293 (log2 fold-change: -2.18 for HFpEF versus HTN, $p<0.05$) and serine-232 (log2 FC: -1.55, $p<0.05$) on pyruvate dehydrogenase (PDHA1). Phosphorylation at these sites inhibits PDH activity;(*116, 117*) therefore, the reduced phosphorylation suggests greater carbohydrate oxidation in our resting HFpEF SkM samples, which were obtained in the fasted state, as compared to HTN. Interestingly, in a porcine model of HFrEF, EMPA decreased PDH activity,(*68*) suggesting that EMPA might target the increased PDH activity implied by our phosphorylation studies. Additionally, EMPA has been shown to decrease myocardial glucose uptake in humans with Type II DM.(*105*) Collective, these data suggest that SGLT2i have the potential to diminish reliance on glucose for energy production.
4. **Increased hemoglobin concentration**. Several studies, including our own, have documented a lower hemoglobin concentration (**[Hgb]**) in HFpEF subjects.(*25, 118, 119*) SGLT2i increase [Hgb],(*105, 120-125*) potentially through increased erythropoietin levels.(*104, 121, 123, 125, 126*) Increased [Hgb] would increase oxygen delivery to the SkM for any given blood flow, supporting increased rates of SkM OxPhos.
5. **Replenish metabolite deficiencies identified in HFpEF** – In our muscle biopsy program, we identified decreased levels of propionyl-CoA and acetylcarnitine (C2-carnitine) in the SkM of HFpEF participants (**Fig 5**). Propionyl-CoA is formed via the breakdown of branched chain amino acids and odd chain fatty acids and can provide additional carbon backbones to the tricarboxylic acid cycle (anapleurosis).(*127, 128*) Acetylcarnitine interacts with carnitine acetyltransferase (CrAT) to determine the acetyl-CoA/CoA ratio, which regulates glycolytic flux at pyruvate dehydrogenase, provides a readily available source of CoA in times of need, and assists in the efflux of fatty acid moieties from the mitochondria.(*129-132*) In an in-depth phenotyping study of diabetics with cardiovascular disease, 25 participants were given Empa 10 mg daily, with serum drawn at baseline and after 1 month.(*98*) Empa increased circulating levels of Propionyl-CoA and acetylcarnitine, along with significant increases in many of the TCA cycle intermediates, suggesting that Empa might replenish some of the deficits we identified in HFpEF SkM.

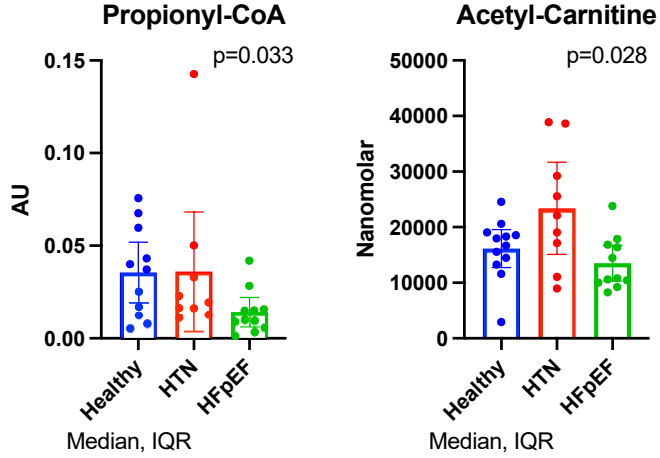

Figure 5 – Intramuscular metabolites in SkM biopsy samples. Biopsy samples were taken from HFpEF (n=11), HTN (n=9), and Healthy (n=11) SkM and subjected to MS-based analysis. Propionyl-CoA and acetyl-carnitine were lower in HFpEF SkM.

Despite these potential benefits, EMPA did not improve 6-minute walk distance in EMPERIAL-PRESERVED. As illustrated above, SGLT2i have several metabolic effects that could translate into an ergogenic benefit, specifically in HFpEF patients. Despite this, results from the EMPERIAL-PRESERVED study showed that 12 weeks of EMPA did not improve six-minute walk (**6MW**) distance in 315 symptomatic HFpEF patients.(*133*) It is certainly possible that the choice of endpoint may have played a role in masking an ergogenic benefit, especially considering that SGLT2i have been shown to improve peak VO_2 in HFrEF,(*134*) yet also did not increase 6MW in this patient population.(*133*) While the 6MW test is convenient, its administration must be rigorously standardized,(*135*) as different degrees of encouragement influence the test.(*136*) Additionally, the 6MW is self-paced and impacted by patient motivation, yet provides no objective metric of effort.(*135*) *Moreover, and key to this proposal, there are other physiologic reasons, centered on nitric oxide bioavailability, that potentially could explain why EMPA did not yield an ergogenic benefit.*

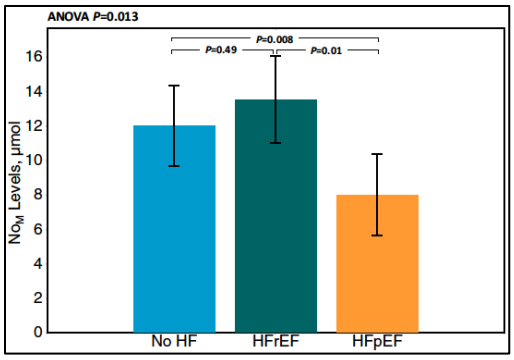

Figure 6 – Serum nitric oxide metabolites are lower in HFpEF. Comparison of NO_m levels between subject without heart failure (HF) and subjects with HFpEF and HFrEF. From Chirinos…Zamani, JAHA 2016.

Nitric oxide bioavailability may be needed to unlock the benefits of SGLT2i in HFpEF. We,(*137, 138*) as well as others,(*139, 140*) have demonstrated impaired nitric oxide (**NO**) bioavailability in HFpEF. In two studies, we demonstrated a reduction in serum nitric oxide metabolites (**NO_m**) in HFpEF patients, compared to those with HFrEF and individuals without heart failure (**Fig 6**).(*137, 138*) Additionally, in a human myocardial biopsy study, HFpEF hearts had lower tissue NO_m levels.(*139, 140*) We demonstrated that supplementation with inorganic nitrate, in the form of potassium nitrate (**KNO_3**), significantly increased serum concentrations of NO_m;(*141, 142*) other work showed that inorganic nitrate supplementation also increases muscle NO_m.(*143*) While NO has many effects, this proposal will focus on 2 beneficial effects of NO that could specifically bolster the effects of SGLT2i: (1) the impact of NO on FAO; and (2) the impact of NO on SkM blood flow.

Nitric oxide is required for efficient fatty acid oxidation. A central mechanism for the postulated ergogenic benefit of SGLT2i is an increase in FAO. However, efficient FAO requires nitric oxide. Previously, our Co-Investigators demonstrated that S-nitrosylation, a NO-mediated post-translational modification, of proteins participating in FAO, markedly increased the rate of palmitate oxidation.(*144*) Moreover, mice deficient in endothelial nitric oxide synthase ($eNOS^{-/-}$) lacked S-nitrosylation of FAO proteins, which coincided with reduced liver, heart, and SkM FAO.(*145, 146*) Treatment of $eNOS^{-/-}$ mice with NO-donors restored both FAO protein S-nitrosylation and FAO efficiency.(*146*) With restoration of FAO, the physical activity of the $eNOS^{-/-}$ mice improved to approximate that of wild-type mice, suggesting an important link between FAO and physical activity.(*146*) In rats, nitrate supplementation has been shown to increase soleus SkM FAO by increasing the activity of key enzymes involved in fatty acid metabolism, such as 3-hydroxylacyl-CoA dehydrogenase, PGC-1α, and PPARα, and by decreasing malonyl-CoA content.(*80, 147*) **These data demonstrate a central role for NO in efficient FAO, suggesting that without sufficient NO-signaling, SGLT2i cannot improve FAO to its full potential, hence limiting its ergogenic benefit.**

Increased nitric oxide bioavailability increases SkM blood flow during exercise, which is necessary to support greater FAO. Increased reliance on FAO would, of necessity, require greater oxygen delivery to the SkM, as more O_2 is required to generate the same amount of ATP from fatty acids as compared to carbohydrates (e.g. lower P:O ratio for fatty acids).(*148*) This is all the more relevant as several studies have identified constrained blood flow to the SkM during both large(*25, 149, 150*) and small(*151*) muscle mass exercise in HFpEF patients. Because NO contributes to the exercise vasodilatory response,(*152-156*) we tested whether increasing NO bioavailability could improve SkM exercise blood flow. In a 2x2 cross-over trial of 17 HFpEF subjects, we found that a single dose of 12.9 mmol of inorganic nitrate, which can be reduced to NO in the hypoxic and acidic environment of exercising muscle,(*157-160*) led to significant improvements in the vasodilatory reserve during exercise, allowing for greater cardiac output, higher peak VO_2, and higher ventilatory thresholds (**Fig. 7**).(*141*) Using near infrared spectroscopy on the calf muscle during exercise, we found a tendency for oxyhemoglobin to fall to a lesser degree following inorganic nitrate than following placebo (-11.3% vs. -15.8%, p=0.07), suggesting an improvement in exercise intramuscular perfusion with inorganic nitrate.

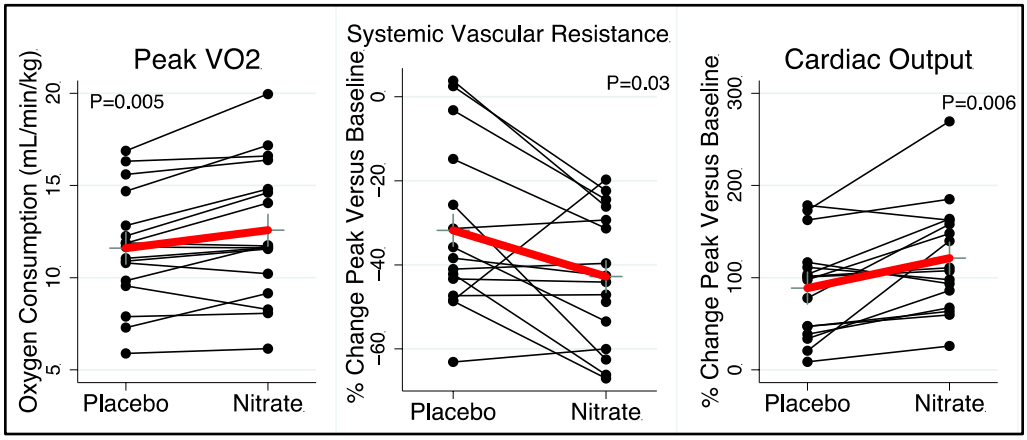

Figure 7 – Inorganic nitrate enhances the vasodilatory reserve in HFpEF subjects (n=17) allowing for higher cardiac output and peak VO_2. Red line indicates mean difference.

These data suggest that <u>combining</u> inorganic nitrate with SGLT2i may uncover a metabolic and ergogenic benefit in HFpEF patients by increasing SkM blood flow and supporting increased rates of fatty acid oxidation during exercise.

HFpEF patients remain extremely limited.(*161*) Even small improvements in exercise tolerance could allow patients to perform more activities of daily living, increasing quality of life and independence, and enhancing the likelihood that a patient engages in prescribed exercise training.(*162*) All of these factors can reduce the burden on the healthcare system. Thus, our study has the potential to impact the lives of millions of patients who currently suffer from this debilitating condition, without any effective pharmacologic treatment options.

2.2.1 Known Potential Risks with SGLT2i

a. Empagliflozin –

i. **Overall considerations:** In the EMPERIAL program, participants with HFpEF and HFrEF were randomized to Empa 10 mg po qd for 12 weeks versus PB.(*133*) The rates of premature medication discontinuation were no different between Empa and PB: 8.3% in PB, 9.7% with Empa in HFrEF, and 7% with Empa in HFpEF. There was no difference in the frequency of AEs, and serious adverse events (SAE) were less frequent with Empa. Similarly, “any adverse event” and “serious adverse events” were no different in the EMPEROR-Reduced study of HFrEF participants randomized to Empa versus PB.(*59*) Adverse events were also not more frequent in the dapagliflozin arm of the DAPA-HF trial in HFrEF participants.(*51*)

ii. **Genital infections:** Empagliflozin has been studied in many clinical trials of diabetics and patients with heart failure (with and without diabetes). In the EMPA-REG OUTCOME trial,

7020 diabetic participants were randomized to 10 mg or 25 mg of Empa versus placebo. As described above, Empa reduced rates of cardiovascular outcomes including HF hospitalization, without significance dose heterogeneity.(*42*) Genital infections were more frequent in the Empa groups (6.4% vs. 1.8%). In EMPEROR-Reduced, a study in heart failure patients, the incidence of urinary tract infections was no different between Empa and PB (Empa 4.9% versus PB 4.5%); the incidence of genital infections was also no different (Empa 1.7% versus PB 0.6%).

iii. **Risk of Hypoglycemia and Diabetic Ketoacidosis (DKA):**
   1. The EMPA-REG OUTCOME trial enrolled 7020 Type II DM to Empa versus PB. Hypoglycemic episodes requiring assistance (Empa 1.3% versus PB 1.5%) and diabetic ketoacidosis (DKA, Empa 0.1% versus <0.1% in PB) were no different between Empa and PB in the EMPA-REG OUTCOME Trial.(*42*)
   2. The DAPA-HF Trial enrolled 4744 participants with HFrEF to dapagliflozin 10 mg daily versus placebo; approximately 40% of participants had diabetes mellitus, and about half of study participants were on biguanide medications (e.g. metformin).(*51*) Hypoglycemia requiring the assistance of another person was extremely rare (0.2% in either group) and did not differ significantly between groups. Similarly, DKA was extremely infrequent, occurring in 0.1% of those randomized to Dapa and 0 of those randomized to PB.(*51*)
   3. In EMPEROR-Reduced, 3730 HFrEF participants were randomized to Empa 10 mg daily versus placebo. Approximately 50% of participants had diabetes. In the Empa group, HbA1c was reduced by 0.28±0.03%, as compared to -0.12±0.03 in PB (Absolute difference of -0.16% in favor of greater reduction with Empa). Hypoglycemic events were defined as plasma glucose <=70 mg/dL or that required assistance. The overall incidence of hypoglycemic events was 1.4% in the Empa group as compared to 1.5% in the PB group. Looking at diabetic participants only, the incidence of hypoglycemic events was 2.2% in Empa versus 2.4% in PB; lower rates were seen in non-diabetics (0.7% in Empa versus 0.6% in PB). Overall, the incidence of hypoglycemic events was no different between groups. There were 0 episodes of DKA throughout the trial.(*59*)
   4. Large meta-analyses of SGLT2i use in diabetics shows that while rare, there is an increased risk of DKA with SGLT2i use (~1 per 1000 person years for SGLT2i).(*163*)

iv. **Amputations:** Earlier studies with canagliflozin, another SGLT2i, suggested an increased risk of amputations in diabetics.(*44*) Subsequent analyses showed that this was specific to Cana and not present for Empa or Dapa.(*164, 165*) A more recent study using Cana in diabetics with nephropathy, which instituted regular foot exams, did not identify an increased risk of amputation, though Cana did significantly reduce the risk of worsening renal function.(*166*)

v. **Dose considerations:** Because the 10 mg dose of Empa has been shown to lower adverse events in both diabetics and HFrEF participants,(*42*) and lead to metabolic changes,(*98*) this dose will be used throughout this project.

b. **Potassium Nitrate –** Inorganic nitrate has been tested in several human studies in HFpEF to date and is very well tolerated.(*141, 142, 167*) In our Dose-Finding study of 12 individuals with HFpEF, 9 were treated with KNO_3 as we propose to do in this study.(*142*) KNO_3 did not lead to clinically significant hypotension or methemoglobinemia.(*142*)

   i. **Bp effects:** There was a systolic blood pressure lowering effect (approximately 12 mmHg),(*142, 168*) though we note that HFpEF patients generally have elevated blood pressures.(*2*)

ii. **Other symptoms:** 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,(*169*) and may be related to the potassium content, as potassium is known to cause GI side effects in clinical practice.(*170*)

c. **Potassium Chloride:**
   i. **GI side effects:** The main side effect of potassium chloride in clinical practice are gastrointestinal symptoms.(*169, 170*) We will ask subjects to take study medications with food to minimize this.
   ii. **Bp effects:** Potassium itself also has an effect on the vasculature, lowering blood pressure (average of ~3/2 mmHg).(*170, 171*) 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 the combination of Empa + KNO_3 has not been studies before and so data regarding additional adverse effects with the combination are not available. 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 with inorganic nitrate, and the data in the field regarding SGLT2i, support the conclusion that the supplementation strategies to be tested in HFpEF are rationale and target HFpEF-specific deficits. 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.

3 STUDY OBJECTIVES AND ENDPOINTS

Aim 1: Compare the impact of Empa, without and without KNO_3, on submaximal exercise endurance

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Primary		
Compare the impact on submaximal exercise endurance for Empa, with and without pharmacologic therapy aimed at increasing nitric oxide signaling (KNO_3), 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 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 SkM mitochondrial properties, O_2 delivery, and exercise endurance. Through their impact on these mechanisms, our interventions may affect VO_2 kinetics.
Assess the impact of our intervention on VO_2 efficiency	**VO_2 Efficiency**: Ratio of total work performed to total oxygen consumed above basal metabolic rate during the exercise study.(*26, 172*)	VO_2 efficiency reflects the muscle's capacity to convert O_2 into mechanical work.
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 compared to the SVR at 4 minutes of exercise. Aortic input impedance will also be determined to further characterize changes in pulsatile load.	Both KNO_3 and Empa may have vascular effects that could influence exercise, cardiac performance, and SkM blood flow and O_2 delivery.
Assess the impact of our interventions on venous substrate concentrations during exercise	We will measure blood concentrations of substrates (e.g. glucose, lactate, non-esterified fatty acids, and triglycerides) at baseline, 4 minutes of exercise, and at the time of exhaustion. Comparisons at 4 minutes of exercise will be the focus.	Empa and KNO_3 may alter SkM substrate metabolism during exercise.

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Assess the impact of our interventions on the respiratory exchange ratio	We will also assess the respiratory exchange ratio, a metric of whole-body substrate metabolism, obtained from expired gas analysis. Comparisons at 4 minutes of exercise will be the focus.	Empa and KNO_3 may alter SkM substrate metabolism 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 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.
Tertiary		
Assess the impact of our interventions on intramyocardial filling pressure during submaximal exercise	**E/septal e':** Mitral early inflow velocity (E) to septal tissue Doppler velocity (e') is non-invasive marker of intramyocardial filling pressures that correlates with invasively-derived metrics.(*173, 174*)	Through multiple mechanisms, SLGT2i may reduce intramyocardial filling pressures at rest and with exercise, which could serve as another mechanism for improved exercise tolerance.

AIM 2: Compare the impact of Empa, with and without KNO_3, on SkM OxPhos and Intramuscular Perfusion using MRI

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Primary		
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 (half-time of CrCEST recovery), 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 altering

		SkM energy fuel metabolism
Secondary		
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 capacity 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.

AIM 3: Assess the impact of Empa, with and without KNO_3, on SkM respiration, the metabolome, and the proteome

Assess the impact of our interventions on muscle tissue respirometry, specifically FAO	***Ex vivo* tissue measures of substrate metabolism.** We will measure tissue rates of substrate (fat and carbohydrate) metabolism. Citrate synthase, a marker of mitochondrial content, will also be assessed.	In addition to our CrCEST-based measures of SkM OxPhos, we will 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 fatty acid and ketone oxidation will be measured, as well as proteins related to mitochondrial biogenesis.	We postulate that Empa+KNO_3 will lead to greater improvements in FAO than either Empa alone or active control, as reflected by changes in the muscle proteome. As Empa may alter mitochondrial biogenesis, we will also measure proteins key to the process of mitochondrial expansion.
Assess the impact of our interventions on the muscle metabolome	**Muscle Metabolome:** Targeted quantitative metabolomics will be performed, including the acylcarnitine profile (specifically C4-OH carnitine, a marker of ketone metabolism, and acetylcarnitine [C2]), malonyl-CoA, and glycolytic intermediates	Changes in substrate metabolism may be reflected in changes in substrate concentrations within the muscle.

4 STUDY PLAN

4.1 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) Empa; (C) Empa + KNO_3. 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; Empa will be purchased by IDS and over-encapsulated to maintain blinding.

While changes to the mitochondrial proteome occur early after the initiation of an exercise program (1-2 weeks),(*175, 176*) the timing of mitochondrial biogenesis in humans is less well known, suggesting that the longer duration of therapy chosen in this study is warranted.(*177*) 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.(*178, 179*) Similar findings of decreased mitochondrial oxidative capacity were noted during a 12-day unilateral leg suspension protocol.(*180*) 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.

4.2 Scientific Rationale for Study Design

HFpEF subjects suffer from multiple comorbidities that may impact exercise capacity.(*181-183*) 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.

4.3 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) Empagliflozin alone (4 pills daily)

   a. Empa 10 mg daily

   b. Potassium Chloride 6 mmol three times daily

(2) Empagliflozin + Potassium Nitrate (4 pills daily)

a. Empa 10 mg daily

b. KNO_3: 6 mmol three times daily

(3) Potassium Chloride alone (4 pills daily)

a. KCl: 6 mmol three times daily

b. Placebo: Placebo for Empa to be given once daily

Both the KNO_3 and the KCl will be made by the UPenn IDS, as we have done previously,(*142*) with pharmaceutical grade product obtained from a commercial supplier (e.g. Spectrum Laboratory Products (Gardenia, CA)). Empagliflozin will be purchased commercially by UPenn IDS and over encapsulated to maintain blinding. A similarly appearing placebo will also be made for Empagliflozin by UPenn IDS.

5 STUDY POPULATION

5.1 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, defined as: no addition/removal/changes in antihypertensive medications or beta-blockers in the preceding 30 days and continuation of a stable diuretic regimen, if applicable
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)(*184, 185*)
        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)(*186*)
    b. Mitral E/e' ratio > 14(*185*) at rest or during exercise(*186*)
    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)(*186, 187*)
    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.(*186*)

5.2 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 be 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 < 45 mL/min/1.73m^2. We note that while the FDA packing insert suggests a lower limit of 45 mL/min/1.73 m^2 for Empa, the EMPERIOR Reduced trial enrolled HFrEF participants with an eGFR >= 20 mL/min/1.73m^2.(*59*)
18. Methemoglobin > 5%
19. Serum potassium > 5.0 mEq/L on baseline testing
20. Type I Diabetes
21. History of ketoacidosis
22. Current use of, or prior intolerance to, an SGLT2i
23. Ongoing maintenance of a 'Ketogenic Diet' (low carbohydrate, high fat)
24. Allergy to beets
25. Severe right ventricular dysfunction
26. Baseline resting seated systolic blood pressure > 180 mmHg or < 100 mmHg
27. Orthostatic blood pressure response to the transition from supine to standing (>20 mmHg reduction in systolic blood pressure 2-3 minutes after standing, or a fall in SBP to < 90 mmHg)
28. Active participation in another study that utilizes an investigational agent (observational studies/registries allowed)
29. 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 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.

5.3 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.(*188, 189*)
- 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.(*190-193*)
- Subjects will be asked to avoid a ketogenic diet (high fat, low carb) or excessive alcohol.(*194, 195*)
- Subjects will be asked to withhold study medications during periods of fasting or illness that inhibit adequate oral intake, as these situations may increase the risk for DKA.(*194, 195*)

5.4 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 that 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.

5.5 Strategies for Recruitment and Retention

5.5.1 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.(*25, 26, 141, 142, 196*) 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 Division of Cardiology and 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_3CKOUT-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 may conduct directed recruitment in settings where HFpEF patients are likely to present during 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.

5.5.2 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 (7 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. 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

Total: $500

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

5.6 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, and the 3 endpoint assessments (submaximal exercise and CrCEST MRI studies) following each interventional period, 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

5.7 Study Intervention(s) Administration

5.7.1 Study Intervention Description

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

(1) Empagliflozin alone (4 pills daily)

a. Empagliflozin 10 mg daily

b. Potassium Chloride: 6 mmol three times daily

(2) Empagliflozin + Potassium Nitrate (4 pills daily)

a. Empagliflozin 10 mg daily

b. KNO_3: 6 mmol three times daily

(3) Potassium Chloride (4 pills daily)

a. KCl: 6 mmol three times daily

b. Placebo: A placebo for Empagliflozin will be taken once daily

5.7.2 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) Empagliflozin alone

a. Empagliflozin 10 mg daily

b. Potassium Chloride: 6 mmol twice times daily

(2) Empagliflozin + Potassium Nitrate

a. Empagliflozin 10 mg daily

b. KNO_3: 6 mmol twice times daily

(3) Potassium Chloride

a. KCl: 6 mmol two times daily

b. Placebo: A placebo for Empagliflozin will be taken once daily

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 in the supine position.

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 and without another identifiable cause (for instance, if a concurrent GI illness is ongoing, study medications can be paused until the participant recovers, before restarting with close contact/instruction from the study team). In cases in which home monitoring suggests resting or orthostatic hypotension, the study subject may 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) Empagliflozin alone

a. Empagliflozin 10 mg daily

b. Potassium Chloride (KCl): 6 mmol three times daily

(2) Empagliflozin + Potassium Nitrate

a. Empagliflozin 10 mg daily

b. Potassium Nitrate (KNO_3): 6 mmol three times daily

(3) Potassium Chloride

a. Potassium Chloride: 6 mmol three times daily

b. Placebo: A placebo for Empagliflozin will be taken once daily

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**
Empagliflozin	10 mg daily	10 mg daily
KNO_3	6 mmol twice daily	6 mmol three times daily
KCl	6 mmol twice daily	6 mmol three times daily

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 < 45 mL/min/1.73 m^2, at baseline will be excluded from the study, minimizing the risk that clinically significant hyperkalemia (K $\geq$ 5.5 mEq/L) will develop because 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. Chronic use of a potassium-sparing diuretic (e.g. amiloride, spironolactone, triameterene)

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/medication alteration is < 5.0 mEq/L.

5.7.3 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) **Empagliflozin:** Empagliflozin will be purchased by UPenn IDS from a commercial pharmaceutical supplier (e.g. Boehringer Ingelheim). Pills will be over-encapsulated to maintain blinding. A placebo of similar appearance and weight will be constructed by IDS.

5.7.4 Acquisition and accountability

Both KNO_3 and KCl will be prepared by UPenn IDS, as we have done previously.(*142*) 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. UPenn IDS will also purchase IDS from a commercial supplier. IDS will furnish the placebo capsule for Empagliflozin, which will be similar in color and weight the over-encapsulated Empagliflozin pill.

5.7.5 Formulation, Appearance, Packaging, and Labeling

Potassium nitrate and potassium chloride will be obtained commercially (e.g. from Spectrum Laboratory Products) and encapsulated by the UPenn IDS. UPenn IDS will purchase Empagliflozin from a commercial supplier (e.g. Boehringer Ingelheim) and over-encapsulated the pills to maintain blinding. UPenn IDS will construct a placebo pill for Empagliflozin that will be similar in weight and appearance to the over-encapsulated Empagliflozin pill.

5.8 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; Empa alone in P2; Empa + KNO_3 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=Empa alone, followed by C= Empa + KNO_3. The study statistician will provide the sequence orders. UPenn IDS will assign the codes linked to the intervention and will provide study medication based on the sequence order to which the subject is randomized. For purposes of blinding, none of the study team will have access to the IDS-assigned codes. Furthermore, the study statistician will not release the sequence orders to the Study Team. If emergency unblinding is necessary, the investigators will access the treatment identity by contacting UPenn IDS and receive information regarding the specific participant's active treatment at that time (and without other information as to the sequence). This is expected to be infrequent given the safety profile of the study medications. If time allows, any unblinding 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, that 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.

5.9 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, or UPenn IDS, 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.

5.10 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 all 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.

Background Diabetic Medications and Diuretics

Given the overall low incidence of hypoglycemic events (Reviewed in Section 2.2.1), the study protocol does not include plans to modify background diabetes therapy. Furthermore, given that SGLT2i did not increase the risk of volume depletion in large HFrEF studies (DAPA HF: Dapa 1.2% versus 1.7% in PB;(*51, 197*); EMPEROR-Reduced: Empa 10.6% versus PB 9.9%), the study protocol does not include plans to modify background diuretic therapy.

6 STUDY INTERVENTION DISCONTINUATION AND PARTICIPANT DISCONTINUATION/WITHDRAWAL

6.1 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 participant 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 or the telephone).

6.2 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.

- If a participant decides to withdraw from the study AFTER he/she has been randomized, this participant will be included in the intention to treat cohort (regardless of whether the participant took any study medication or not).
- If a randomized participant decides to withdraw from the study before taking any medication, as reported to a study coordinator and/or through return of their medication, the subject will be 'replaced' on the study by increasing the total number of subjects accrued to the study. The randomization sequence of the subject who withdraws will not be re-used, i.e., the next individual randomized will be assigned the sequence subsequent to that of the participant who withdrew.

6.3 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.

7 STUDY ASSESSMENT AND PROCEDURES

7.1 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, 198*) The Kansas City Cardiomyopathy Questionnaire (**KCCQ**) will be administered, and the KCCQ Overall Summary Score will be calculated and compared.(*12, 198-200*)

- **Low-nitrate diet:** We will provide educational information and a 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 may 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, may be assessed in the 4-chamber view. Tissue Doppler imaging may 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) may 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 may 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 may 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 metabolic cart (for example, 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.(*25, 26, 141, 142*) Electrocardiographic and vital sign monitoring will be performed during the test. Verbal encouragement will be given to encourage maximal effort.

    - Echocardiographic images may be obtained during the exercise tests. Lung ultrasound may be performed at rest and during exercise to identify B-lines, a marker of congestion.(*201*)
    - 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.
    - A pedal cadence of 60 RPMs will be targeted, 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.(*202*)

We note that some individuals may not be able to maintain a cadence of 60 RPMs. In these instances, we will coach the subject to maintain the highest possible sustainable cadence over the course of the exercise test.

- *Note:* Subjects will be excluded if the arterial saturation falls to < 90% on a reliable assessment during this maximal effort study or if significant exercise-induced segmental wall motion abnormalities develop on exercise echocardiography that are consistent with inducible ischemia. Given the high prevalence of 'false-positive' ECG stress tests in HFpEF,(*203*) 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 (0 Watts) 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.(*202*)
  - 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)

  - *Note: In participants who could not maintain a pedal cadence > 60 RPM during the maximal effort incremental study, the highest sustained cadence during the maximal effort study will be used during the submaximal exercise assessment.*

- **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.(*202*) 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 was previously used in HFpEF patients.(*167*)

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;(*142, 204-206*) 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),(*207*) 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 approximately 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 blood and muscle nitrate(*142, 208*) 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:** 1-2 venous catheters will be placed.
- **Blood draw**:
    - Comprehensive metabolic panel
    - Complete blood count
    - Storage of blood for later analysis, including 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.(*209*) 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 may 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 acylcarnitine profile, Acyl-CoA species (including malonyl-CoA), glycolytic intermediates, ketones, 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:
  - **Blood Metabolite Concentrations:** 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 (but not limited to):
    - Glucose
    - Lactate
    - Non-esterified fatty acids
    - Triglycerides
    - Acylcarnitines
    - Ketones

    Other metabolites/hormones/proteins, such as insulin and glucagon, may also be measured.
  - **Aortic input impedance:** Radial arterial tonometry and echocardiographic cardiac flow from the left ventricular outflow tract Doppler signal may 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.(*148*) Lower values (RER of 0.7) correspond to greater fatty acid oxidation, as compared to carbohydrate (RER of 1.0).(*148, 210-212*) The RER will be compared at 4 minutes of exercise and will be computed as the average value over a 30-second time period.(*172*)

- **VO_2 kinetics** – The rate of increase or decrease in oxygen consumption following the onset or cessation of exercise is related to SkM OxPhos.(*156, 213-216*) In HFpEF, VO_2 kinetics are slowed,(*26, 217, 218*) 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.(*219*)
- **VO_2 Efficiency:** Because 75%PW will likely be above where lactate levels can be maintained at a steady state (above 'critical power'), it is expected that subjects may evince an additional metabolic cost ('VO_2 slow component') that will drive VO_2 to maximal/peak rates of oxygen consumption during the submaximal execise studies.(*220-222*) By improving SkM blood flow, it is possible that the study interventions could *decrease* VO_2 at the same work rate by reducing the slow-component. On the other hand, increased fatty acid oxidation could lead to greater oxygen consumption for the same work-rate due to the lower ATP-generated-to-O_2-consumed ratio for fats as compared to carbohydrate. Net VO_2 efficiency, defined as the ratio of total work performed to total oxygen consumed (above baseline metabolic rate) during the exercise study will be computed,(*141, 142, 172*) reflecting the muscle's capacity to convert oxygen into mechanical work.(*172*)
- **Myocardial diastolic parameters and surrogate markers of congestion** – Given that our interventions may impact arterial properties, which in turn, can impact myocardial performance,(*223-229*) 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.(*230, 231*) We may also interrogate echocardiographic estimates of RV function including the tricuspid regurgitant jet velocity(*232*) and the tricuspid annular plane systolic excursion.(*233*) Lung ultrasound may also be performed using echocardiography.(*201, 233*) These markers will primarily be compared at 4 minutes of exercise between interventions.

  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 different days following an intervention,(78, 87, 89, 91, 108, 143, 176, 208, 234-253) 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).(252)

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 may 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 will be placed around the part of the body under investigation. The subject may 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 two-way voice communication with the subject. The subject may 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 may 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) may 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 to avoid 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. Additional ancillary sequences may also be performed to gather additional information, if time allows.

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(*26*)
  - 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

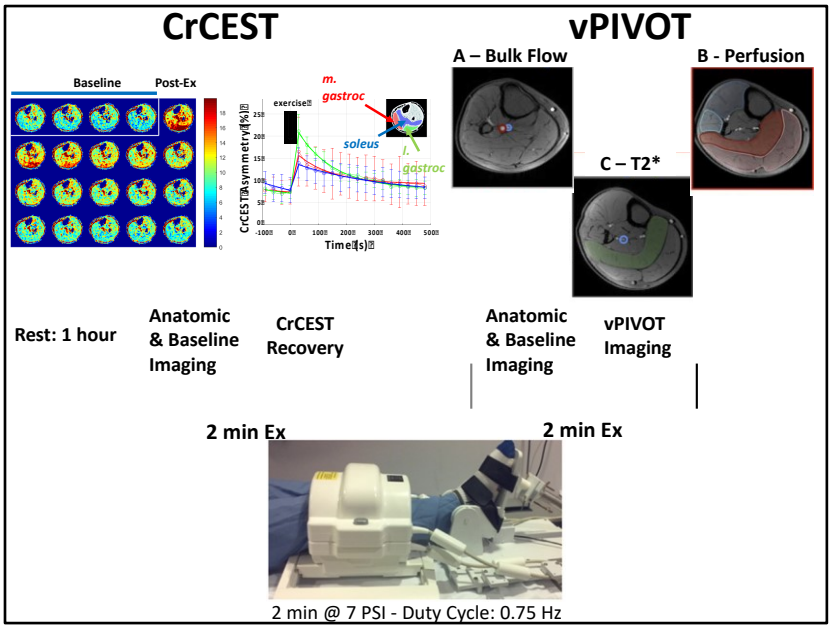

Figure 8. CrCEST and vPIVOT imaging protocol. Following rest, the scanning protocol begins with anatomic and baseline CrCEST imaging. A standardized plantar flexion exercise protocol is then started for 2 minutes. Additional CrCEST images are obtained every 30s during recovery for 8 minutes. After a 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.

- Relative measure of capillary oxygenation (T2*) before and serially after exercise

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 or UPenn IDS 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 an approximate 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 an ~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.

7.2 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.(*254*) These procedures will be performed by qualified personnel according to established American Heart Association guidelines.(*255, 256*) Non-revascularized significant epicardial coronary disease, which may increase the risk of cardiovascular complications with exercise,(*257*) 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 because of the exercise test. Various other complaints, such as nausea, lightheadedness, and other aches and pains are also possible because 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,(*257*) during exercise tests. The risk of this happening is the same as if the participant were to exert him/herself during stressful situations or 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.(*258, 259*) If the arterial saturation falls to below 88% on a reliable assessment ("severe exercise induced hypoxemia"(*259*)), 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,(*25, 26, 141, 142*) and other parameters. 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.(*201*)

3. **Skeletal muscle vastus lateralis biopsy:** Muscle needle biopsy with suction will be performed,(*209*) 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 serious complications. We note that our protocol of pre-exercise muscle biopsies has been extensively employed in many prior physiologic studies.(*108, 238, 240-249, 253, 260-266*) Moreover, several studies have employed serial muscle biopsies either during one study visit or on different days following an intervention,(*87, 89, 91, 108, 143, 176, 208, 234-250, 252, 253*) 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 visit. 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).(*252*)

Other considerations specific to the muscle biopsy:

- **Post-exercise muscle biopsy:** In individuals who 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(*267, 268*)), 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(269)

- 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 possibly 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.(*269*) 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.(*209*) 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 possibly 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 ester local anesthetic (such as tetracaine) or diphenhydramine.(*270*) These decisions will be made in discussion with Investigational Drug Services and the subject, and in light of the agents that the subject may have received previously for local anesthesia (e.g. during dental procedures or skin biopsies).

4. **Phlebotomy and blood draws:** The CHPS unit in which the studies will be performed employs a research staff with 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): *(values are approximate)*

	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:			
	Plasma: 15 mL			
	Serum: 10 mL			
Submaximal Exercise				
	Venous Blood	**Rest (0-min):**	**Rest (0-min):**	**Rest (0-min):**
		Plasma: 20 mL	Plasma: 20 mL	Plasma: 20 mL
		Serum: 15 mL	Serum: 15 mL	Serum: 15 mL
		4-min:	**4-min:**	**4-min:**
		Plasma: 20 mL	Plasma: 20 mL	Plasma: 20 mL
		Serum: 15 mL	Serum: 15 mL	Serum: 15 mL
		Exhaustion:	**Exhaustion:**	**Exhaustion:**
		Plasma: 20 mL	Plasma: 20 mL	Plasma: 20 mL
		Serum: 15 mL	Serum: 15 mL	Serum: 15 mL
Approx. Total:	**47 mL**	**113 mL**	**113 mL**	**113 mL**
			Grand Total:	**~386 mL**

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

5. **Dual Energy X-Ray Absorptiometry (DEXA):** DEXA scanning may 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.(*271*) Participants will be instructed to never look directly into the 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 during the visit 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.(*272*) 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 because of completing the 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 require 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.
    l. 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. 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 because 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. Some individuals may experience lightheadedness upon sitting up from the table. We may ask participants to get up slowly and sit for a bit so that this dissipates.

***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 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) Empagliflozin with potassium chloride; (b) Empagliflozin + potassium nitrate; (c) potassium chloride. Risks are described in **Section 2.2.1**.

10. **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.

7.3 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 the event is 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 the 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 affecting 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, data safety and monitoring plan, and/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.(*142*)

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 Empagliflozin:

1. Urinary tract infections
2. Genital mycotic infections
3. Diabetic ketoacidosis
4. Hypoglycemia
5. Slight worsening of renal function (though we note that long-term studies in diabetics and in HFrEF participants show that SLGT2i are beneficial for renal function over the long term (*59, 166, 273, 274*))
6. Volume depletion
7. Orthostatic hypotension
8. Increased urination

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 will be collected.**
- AEs will be classified according to the guidelines/definitions specified above.
- 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 decide 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
    - 15 participants complete
    - 30 participants complete
    - 40 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.

8 STATISTICAL CONSIDERATIONS

8.1 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; Empa + KCl in P2; Empa + KNO_3 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 (Empa + KCl vs. Empa + KNO_3), (Empa + KCl + KCl vs. KCl) separately using least squares regression, to minimize assumptions on the covariance matrix of the repeated measurements.(*275*) The regression model will be adjusted for period effects and for baseline submaximal exercise endurance.(*275, 276*) 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 (Empa + KCl or Empa + KNO_3) 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.(*167*) This effect is hypothesized to be clinically important given the severity of impairment in this cohort (mean submaximal exercise duration of 363 ± 125 s seen in Eggebeen et al.).(*167*) 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, venous substrate concentrations, 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 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.(*276, 277*) 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.(*26, 278*) 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 Empa vs. Empa + KNO_3, 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-1α, HIF-1, NRF1/2, ERRα, TFAM, MXI1, C-MYC, AMPK, ANT,* and *mTOR),* 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,(*26, 35, 279, 280*) 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.

9 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

9.1 Regulatory, Ethical, and Study Oversight Considerations

9.1.1 Informed Consent Process

9.1.1.1 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. The informed consent may be sent to potential participants ahead of time to allow participants more time to review the material. 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.

9.1.2 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.

9.1.3 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.

9.1.4 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 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.

9.1.5 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.

9.1.6 Clinical Monitoring

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

9.1.7 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.

9.1.8 Data Handling and Record Keeping

9.1.8.1 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.

9.1.8.2 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.

9.1.9 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

9.1.10 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.

9.1.11 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.

Of note, Dr. Chirinos, a Co-Investigator on the study team, is named as Inventor on a patent application that has been assigned to Penn regarding the use of potassium nitrate in the treatment of HFpEF. As a content expert in HFpEF and in arterial hemodynamics, areas of direct relevance to this proposal, Dr. Chirinos adds significant scientific value to the study team and will provide scientific input and advice on study matters ("Protocol Advisor"). Dr. Chirinos and his lab will assist in developing/refining software that he developed to model biologic signals (e.g. tonometry, echocardiography, and gas exchange data) and serve as a "Core Lab Director." Hemodynamic/biologic signal analyses will be performed in a blinded manner and prior to data unblinding, negating the potential to introduce bias. His group will not provide 'clinical' reads on study data. Dr. Chirinos will not participate directly in enrollment or consenting subjects for this study. His involvement in the study and the potential for its financial implications will be enumerated in the informed consent document and will also be reviewed by the Conflict of Interest Standing Committee at the University of Pennsylvania.

9.2 Protocol Amendment History

Version	Date	Description of Change	Brief Rationale
2	8/20/21	DSMB meeting plan updated to include additional visit	Request of DSMB
3	9/28/21	1)Exclusion criteria updated to exclude subjects on SGLT2i 2)Clarified replacement procedure	1) Request of DSMB 2) Clarify ambiguity

10 APPENDIX

10.1 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
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	
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
Adverse Event Assessment		X	X	X	X	X	X	X
Complete Case Report Forms (CRFs)	X	X	X	X	X	X	X	X

END OF DOCUMENT

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