Acute Effects of Exogenous Ketone Ester Administration in Heart Failure

Protocol

Clinicaltrials.gov identifier: NCT04633460

Version date: 1/15/2023

1. CLINICAL SIGNIFICANCE

Despite advances in the treatment of patients with heart failure (**HF**), there remains substantial residual morbidity and mortality. Even with insights into diverse mechanisms of myocardial dysfunction, virtually all current medical treatment is focused on modulating the "neurohormonal axis". Our project extends the HF treatment principles to an emerging frontier, the ketone "metabolic axis", as a means of ameliorating symptoms through exogenous delivery of a ketone ester. We seek to understand whether supplementation with a ketone ester can improve functional capacity in patients with HF and preserved ejection fraction (**HFpEF**), while simultaneously performing deep phenotyping to fully characterize metabolic, hemodynamic, and echocardiographic pathways altered with such therapy. If beneficial, our results would provide critical proof of concept for targeting the "metabolic axis" as a treatment strategy to help improve exercise capacity and quality of life in heart failure and would inspire larger, randomized trials of ketone therapy.

2. SPECIFIC AIMS

HF is a major public health problem: it affects 6.2 million people in the U.S.[1] The lifetime risk for developing HF after the age of 40 years approaches 20%,[2] and five-year survival after HF hospitalization is a dismal 35%, regardless of the underlying ejection fraction (EF).[3] These statistics highlight the urgent need to identify novel therapies to reduce the substantial morbidity and mortality of the disease process.

At its most basic level, however, HF is a metabolic imbalance - an inability of the heart to generate sufficient energy to adequately supply blood to the body, or only to do so at elevated filling pressures. The heart is a very metabolically demanding organ, which requires nearly 12 times its own body weight in ATP daily.[4] Abnormal myocardial energetics have been described in HFpEF.[5] Targeting the metabolic axis in HF therefore may hold great therapeutic promise.

Under physiologic conditions, fatty acids are the predominant energetic substrate for the heart, providing 50-70% of the ATP need. Glucose serves as a secondary source, necessary for flexibility and adaptive fuel utilization shifts during embryologic development and during a diverse array of nutritional and physiologic conditions.[6, 7] However, two important studies lead by our team have demonstrated that free fatty acid oxidation is impaired in advanced HF due to mitochondrial substrate reprogramming, and the hypertrophied and failing heart utilizes ketone bodies as a metabolic substrate, in both animals and humans.[4, 8] It was previously unknown whether this shift was adaptive or maladaptive, but recent small and large animal data demonstrates that ketone body oxidation is an adaptive feature of the failing heart. Importantly, delivery of exogenous ketones improves pathologic cardiac remodeling and dysfunction while enhancing mitochondrial bioenergetics.[7] Concordant with this animal data, medications that also increase systemic levels of ketone bodies, such as SGLT2 inhibitors, significantly decrease HF hospitalizations among patients with HFpEF raising the question as to whether therapeutic ketosis can provide benefit in HFpEF.

We now have a unique opportunity to study a nutraceutical ketone ester (**KE**), (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (commercially known as "DeltaG"), that provides systemic ketosis in a deep phenotyping study of HFpEF patients.[9] Initial studies of DeltaG demonstrated significant improvement in exercise capacity among endurance athletes.[10] Because exercise limitation is a cardinal feature of HF, KE therapy has the potential to likewise improve exercise capacity in HFpEF.

Our **Specific Aims** and **Hypotheses** are as follows:

Aim 1. To determine the acute effects of single dose DeltaG administration on maximal (peak VO_2) and submaximal exercise capacity (exercise time) assessed by cardiopulmonary exercise testing (**CPET**) among patients with HFpEF.

Hypothesis: DeltaG will improve peak VO_2 and submaximal exercise time compared to a matching placebo.

Subaim 1. To determine the impact of DeltaG on metabolic and biomarker profiles during exercise.

Hypothesis: DeltaG will reduce lactate production during exercise.

Aim 2. To assess the effects of DeltaG on cardiovascular hemodynamics during exercise.

Hypothesis: DeltaG will reduce systemic vascular resistance as well as demonstrate improvements in cardiac output at peak exercise compared to placebo.

Aim 3. To profile the effects of ketone ester versus placebo on systemic and plasma carbohydrate metabolism during exercise using indirect calorimetry and stable isotopes (^{2}H-glucose) techniques

Hypothesis: Delta G will reduce systemic carbohydrate utilization at steady-state during submaximal exercise, with concordant reductions in plasma glucose oxidation

Overall impact: Our objective is to determine whether nutritional ketosis provides a therapeutic benefit in HFpEF). Strengths include the use a readily available KE drink with a favorable safety profile as well as detailed phenotyping to understand the mechanisms of potential benefit among patients with HFpEF. We leverage the expertise of collaborators with rich experience and expertise in ketone metabolism in HF, metabolomic profiling, deep phenotyping during early stage studies of HF therapeutics, and assessments of ventriculoarterial interactions. We anticipate that our results will enhance the understanding of "metabolic axis" of HF and identify a therapeutic intervention of substantial importance. Further, our methodology provides a robust framework to understand the impact of altered metabolism and exogenous ketone delivery in HFpEF that can easily be scaled and applied to other cardiovascular diseases, such as left ventricular hypertrophy, inherited cardiomyopathies, and/or cardiogenic shock, as well as test the ergogenic benefits of other pharmacologic therapies.

3. BACKGROUND AND SIGNIFICANCE

HF affects 6.2 million Americans and is associated with substantial morbidity and mortality in the contemporary era.[1] By 2030, the burden of HF is anticipated to surpass 8 million with an associated economic toll of a staggering $69.8 billion.[1] The cardinal clinical feature of patients with HF is reduced exercise capacity, which is associated with a substantial reduction in quality of life.[11] Despite significant advancements in therapy for patients with HF, particularly among those with reduced ejection fraction (EF), many still suffer from limitations in exercise capacity and a poor prognosis. As such, exploring other avenues to reduce morbidity from HF is of vital interest from societal, economic, clinical, and patient-centered perspectives.

Ketone bodies (acetoacetate, β-hydroxybutyrate [**BHB**], and acetone) are water-soluble molecules produced from acetyl-CoA following breakdown of fatty acids (and amino acids) mainly in hepatic cells. Ketone bodies are typically absent from the circulation during the normal fed state, but are produced continuously during metabolic stress conditions and/or periods of carbohydrate deprivation such as acute medical stress, prolonged exercise, or starvation, and become a key source of energy to vital organs including the brain and heart under these circumstances.[12] Ketone production has long been teleologically considered as a means to provide the brain with an efficient, alternative fuel source during starvation, sparing carbohydrate and protein stores, since the brain cannot effectively metabolize free fatty acids (which represent 99% of the body's energy stores).[12, 13] When completely oxidized in the citric acid cycle, ketones have a respiratory quotient similar to glucose (acetoacetate = 1.0, BHB = 0.89) and produce greater amounts of ATP, releasing approximately 30% more energy per molecule than pyruvate.[14, 15] The liver produces ketone bodies from mobilized fat stores that then can be utilized by neural tissue to produce ATP. It is therefore not surprising that several neurologic

diseases have been treated, or are under study, with ketone therapy, such as epilepsy, Parkinson's disease, and Alzheimer's disease.[16]

Given the beneficial effects on glucose control and weight loss, ketone therapy has also been considered to treat diabetes mellitus.[17] Indeed, ketone therapy has been shown to reduce insulin, ghrelin, and GLP-1.[18] Macronutrient resources (low glucose, high free fatty acids) and hormonal signaling (low insulin, high glucagon, high cortisol) are the primary methods of regulating ketone production, and thus dietary modification has been studied extensively to treat these conditions (the "ketogenic diet"). Adherence to this diet, however, can be challenging, and may also be accompanied by dyslipidemia (due to increased flux of free fatty acids), impaired cardiac metabolism, or gastro-intestinal upset, leading to interest in alternative exogenous modes of delivery.[19, 20] Exogenous delivery of ketones holds several advantages over dietary modification such as increased compliance, lack of alteration on other fuel stores (such as intramuscular glycogen), and its unique effect of lowering both glucose and free fatty acids due to negative feedback on lipolysis.[21]

Different formulations of oral ketone therapy have been studied, including ketone salts and KE. Oral ketone salts, unfortunately, result in a very high amount of salt intake (which would be problematic for patients with HF) and results in half the plasma concentration of D-betahydroxybutyrate (D-BHB).[19] A novel KE compound, (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (DeltaG), has been extensively studied for the purposes of therapeutic exogenous ketosis and is approved as a nutraceutical by the Food and Drug Administration (FDA).[9, 10, 18, 19, 22-24] Advantages of DeltaG consumption include a rapid rise of blood levels of BHB in humans to ~3 mmol/L within 60 minutes of consumption of 282 mg/kg of KE, equivalent to a week of fasting, which is markedly increased from the levels of detectable ketones in normal populations (<0.5 mmol/L).[9, 19, 23] By way of comparison, a carbohydrate-restricted (high-fat) diet typically results in mild hyperketonemia, with BHB levels of $\geq$ 0.5 mmol/L, which is currently considered as the accepted definition of ketosis.[25]

Benefits of KE therapy among athletes have already been demonstrated. In a blinded, crossover study of endurance athletes, DeltaG increased exercise distance by an average of 411 meters over 30 minutes cycling duration, demonstrating that ketone therapy improves exercise capacity among highly trained athletes.[10] Importantly, it has been suggested that pathologic conditions that cause metabolic dysregulation, and where incremental improvements in energy transduction may translate to significant increases in exercise capacity, may afford the greatest clinical benefit, such as in HF.[10]

Recently, further data has emerged regarding potential benefits of intravenous infusions of sodium-3-hydroxybutyrate.[26] In a crossover study of 16 patients with HF and reduced EF (HFrEF) randomized to 6 hours of ketone infusion vs. isotonic saline (placebo arm), infusion of the ketone increased cardiac output by 2 L/min with an absolute improvement in left ventricular EF of 8%. Importantly, systemic vascular resistance decreased by 30% with the ketone infusion.[26] This may be uniquely important in HFpEF patients, who demonstrate an impaired ability to reduce systemic vascular resistance with exercise.[27] Furthermore, to contextualize the magnitude of increase, this improvement in cardiac output is comparable to that achieved with an intra-aortic balloon pump (typically increases cardiac output typically by 0.5-1.0 L/min) or an Impella 2.5 (typically increases cardiac output by 2.0-2.5L/min).[28] Notably, the hemodynamic effects of ketones were dose-dependent and detectable even in the physiological concentration range. While important, this trial was limited by use of an intravenous infusion (impractical for the outpatient management), enrollment only of patients with reduced EF, and concerns related to salt infusion, such as pH disturbances and sodium loading. In addition, this trial did not assess patient-centered metrics, such as exercise capacity, which is an important determinant of quality of life in HF.[29] However, the fact that intravenous ketone therapy increased cardiac output to this extent supports the hypothesis that there is a fundamental limitation of fuel utilization in HF that can be therapeutically targeted.

There is rationale to suspect that such benefits extend to the HFpEF population, which accounts for at least half of all cases of HF in the United States.[30] Among patients with preserved EF and pressure overload induced by aortic stenosis (conditions that simulate non-valvular HFpEF with resultant left ventricular hypertrophy), there is a significantly greater myocardial uptake of ketones compared to controls.[31] Because "late" left ventricular systolic loading from reflected waves in the periphery induce left ventricular hypertrophy and diastolic dysfunction, it stands to reason that the vasodilatory effect of KE therapy may be beneficial.[32, 33] In addition, among diabetic patients (a population at significant risk for HFpEF), SGLT2 inhibitors reduce HF hospitalization and also increase systemic ketosis, an intriguing pair of findings that may be causally linked.[34]

We therefore seek to determine whether exogenous ketosis can improve exercise capacity among patients with HF. We will assess whether exogenous ketosis can acutely affect systolic function (myocardial strain), diastolic function, autonomic tone, and ventriculo-vascular interactions. We further seek to understand the changes in metabolism induced by ketone therapy during exercise.

4. Preliminary Results

DeltaG is a ketone ester (KE) that undergoes complete enzymatic hydrolysis to form BHB (and R-1,3-butanediol) by carboxylesterases expressed widely, including within the gastrointestinal tract, liver, and blood. R-1,3-butanediol, itself, is further metabolized in the liver by first-pass metabolism to produce BHB by alcohol dehydrogenase and aldehydye dehydrogenase. BHB undergoes further metabolism to acetoacetate and acetone, though only BHB and acetoacetate are taken up by extra hepatic tissues as energy sources.

KE is eliminated through at least first order kinetics and capacity limited elimination. Elimination is accomplished via 3 predominant means, including (1) conversion to acetone and lung exhalation, (2) renal elimination (of BHB and acetoacetate, though this contribution is negligible particularly during exercise), and (3) uptake by tissues using monocarboxylate transporters for conversion to ATP.[10, 23]

Concentrations of the KE compound of roughly 3-5 mmol/L are reached within 60 minutes at doses studied here.[9, 10] The half-life of the compound is 0.8-3.1 hours.[9] The KE compound has a very favorable safety profile with adverse effects limited to dose-dependent, gastrointestinal distress that typically occur only at the highest levels of administration (714 mg/kg).[9, 35]

5. Research Design and Methods

Overview: The overall study design will be a randomized, double-blind crossover comparison of DeltaG therapy vs. KE-free vehicle in 20 patients with HFpEF. The main outcomes will be measures of exercise performance (peak and submaximal exercise) and associated metabolic and cardiovascular metrics. The CHPS will provide many study services requested through a Doris Duke Charitable Foundation award (to SS).

The overall structure include 3 visits:

- 3 Visits during which cardiopulmonary exercise testing will be performed (and related testing at these visits), with 1 baseline visit and 2 exercise endpoint assessment visits.

Study population (Inclusion criteria)

Participants aged 18-85 years of age, stable medical therapy for 2 weeks, and New York Heart Association (NYHA) class II or III will be included for this study. We will recruit 20 HFpEF participants. Participants will be recruited from UPHS enterprise. Given the challenges of the diagnosis of HFpEF, and to include a more homogenous cohort, we have the following inclusion criteria:

1. Left ventricular ejection fraction ≥ 50%
2. Evidence for elevated filling pressures as follows (at least one of the following between a-d):
    a. Mitral early (E)/mitral septal tissue annular (e') velocity ratio > 8 in addition to one of the following:
        i. Large left atrium (LA>4.0 cm width or LA volume index >34 mL/m^2)[36, 37]
        ii. Chronic loop diuretic use for control of symptoms
        iii. Elevated natriuretic peptides within the past year (NT-proBNP>125 pg/ml or BNP>35 pg/ml)
    b. Mitral E/e' ratio > 14[37] at rest or with exercise
    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)[38]
    d. Prior episode of acute heart failure requiring IV diuretics with evidence of volume overload on exam/radiology or elevated natriuretic peptides.

Exclusion Criteria

1. Intentional ketogenic (high fat, low carbohydrate) diet in the last week or use of ketogenic medications (SGLT2 inhibitors)
2. Significant liver disease (liver function tests > 3x upper limit of normal, cirrhosis) or alcohol abuse disorder (>14 drinks/week).
3. Contraindications to stress testing, conditions that limit exercise, and other clinically-significant causes of exertional limitation (claudication with peripheral artery disease, atrial fibrillation and heart rate >110 at rest, systolic blood pressure>180 mmHg or diastolic blood pressure>110 mmHg, infiltrative/hypertrophic/inflammatory cardiomyopathy, clinically significant pericardial disease, joint or neuromuscular disease that precludes exercise, acute coronary syndrome within the last 2 months, estimated glomerular filtration rate<30 mL/min/1.73 m^2, and hemoglobin < 9 mg/dL).
4. Clinically significant lung disease. This would be defined by severe obstructive lung disease (Gold stage 3), a requirement for supplemental oxygen, or chronic obstructive pulmonary disease with an exacerbation requiring steroids or antibiotics within the last 2 months.
5. ≥ Moderate aortic stenosis, >mild mitral stenosis, ≥ moderate aortic or mitral regurgitation on screening echocardiogram
6. Type 1 diabetes mellitus
7. Pregnant women. Due to unknown affects of nutritional ketosis in pregnant women, pregnancy will be an exclusion. Accordingly, women of childbearing age with a menstrual cycle within the past year will be asked to submit a urine specimen for pregnancy testing.
8. Angina due to epicardial coronary disease or known presence of clinically-significant, unrevascularized epicardial coronary disease, in the investigator's opinion.
9. Prior reduced LVEF to < 45%

Participant clinical data collection

We will assess the following clinical data parameters on study intake, which may include but are not limited to:

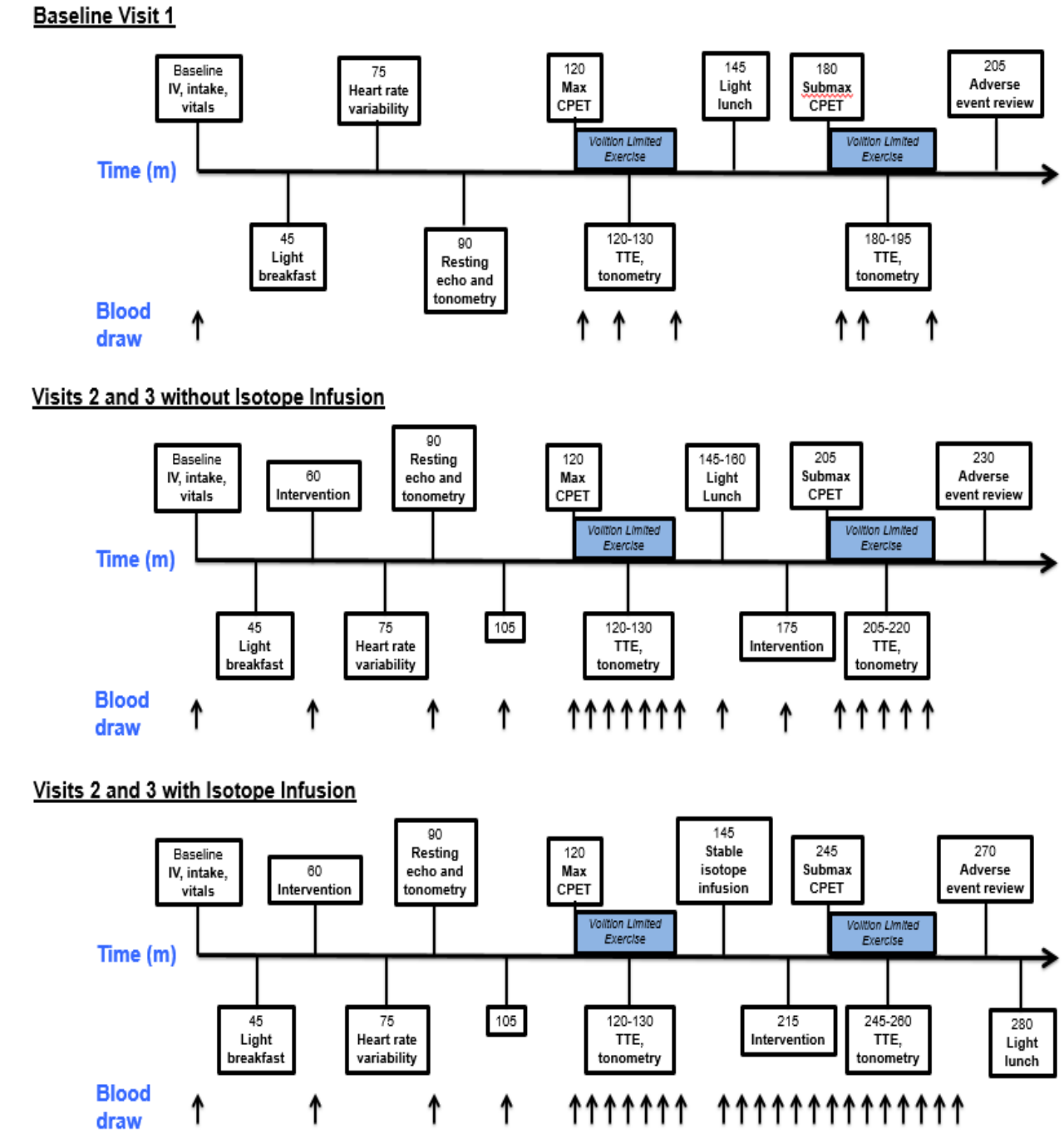
1. Age, date of birth, gender, race (white, black, Hispanic, other)
2. New York Heart Association class
3. Smoking status, alcohol status.
4. Comorbidities (supplemented by chart view of the problem list): previous HF hospitalization in last 6 months, myocardial infarction, stroke, coronary artery bypass graft surgery, percutaneous coronary intervention, chronic obstructive pulmonary disorder, asthma,

hypertension, peripheral artery disease, atrial fibrillation or flutter, dyslipidemia, implanted cardioverter-defibrillator, pacemaker, hypothyroidism, diabetes mellitus, and others.

5. Vital signs and anthropomorphic measures, including: height, weight, waist circumference, heart rate, systolic blood pressure, diastolic blood pressure, oxygen saturation, and others
6. Quality of life, assessed using a validated tool (Kansas City Cardiomyopathy Questionairre)[39]
7. Medications will be obtained from the medical chart and confirmed with the participant.

Overall structure of Exercise Visits (visits 1-3)

Figure 1. Flow Diagram of CPET Study Visit Days



Times and number of blood draws are approximate and only for illustrative purposes

Partipants first come for a baseline visit (**Figure 1**) during which they will undergo cardiopulmonary stress testing in order to determine ~75% of peak work rate. This will allow us to determine the workload for submaximal testing (**Table 1**). We will also perform baseline echocardiography at this visit.

Rationale for selection of exercise tolerance 75% peak work rate and titration procedure:
We aim to target a workload that resembles the workload experienced by subjects during routine daily activities, increasing clinical applicability. Selecting too low of a workload, however, could lead to subjects stopping exercise prematurely due to mental fatigue, as opposed to the physiologic processes we seek to modify. Given this, we will perform a familiarization exercise test at ~75% peak work rate during the baseline visit, following the maximal effort study, and modify our submaximal exercise workload based on the subject's performance. We will titrate the workload 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. Subjects will begin with a 1 minute period of exercise at 0W, followed by a step increase to 75% peak workload performed until exhaustion. If the subject exercises for >6 minutes, the workload to be used in subsequent studies will be increased by 10W or 15%, whichever is greater; if the exercise time is <3 minutes, the workload will be reduced by 10W or 15%, whichever is less.

While the preceding maximal effort study may confound submaximal exercise time, the submaximal protocol will always follow a maximal effort study during the assessments. Moreover, adding another study visit to perform this familiarization study would increase subject burden.

Table 1. Visits and Related Rationale for Testing

Visit Number	Purpose for CPET Testing	Procedures performed	Intervention
1	Perform incremental CPET testing to determine 75% of peak workload (PW) for submaximal testing for Visits 2 and 3. Familiarization study at 75% PW after maximal intensity protocol.	Blood draws, CPET testing, rest and stress echocardiography, arterial tonometry test, HRV test KCCQ	None
2	Perform CPET testing using a maximal incremental effort protocol to determine peak VO_2, then after 1-2 hours, perform CPET testing using a submaximal protocol (75% PW)	Same as visit 1 and also includes possible additional IV with stable isotope infusion	KE drink or placebo
	WASHOUT PERIOD (1-4 week)		
3	Perform CPET testing using a maximal incremental effort protocol to determine peak VO_2, then after 1-2 hours, perform CPET testing using a submaximal protocol (75% PW).	Same as visit 1 and also includes possible additional IV with stable isotope infusion	KE drink or placebo (the opposite arm from Visit 2)

Approximately within 2 weeks of Visit 1 (range of 1-4 weeks), participants will undergo cardiopulmonary (VO_2) exercise testing during Visit 2 following a dose of KE versus PB. Visit 2 includes a maximal intensity CPET, followed by a submaximal intensity CPET at 75% PW. After a >5 day washout period (1-4 weeks), participants return for Visit 3. Visit 3 is identical to Visit 2, except that the study participant will receive the other intervention not received during the previous visits (cross-over). These visits will allow us to determine the difference in maximal and submaximal exercise

capacity between KE drink and placebo. We note that in our previous studies of HFpEF subjects, our patients were able to perform a maximal effort study, followed by a submaximal testing, without difficulty.[40]

Some participants will also be enrolled in a companion study performed by our team looking at the impact of exogenous ketones on lactate production on MRI. In these participants, the MRI scanning sessions may be interleaved with the visits delineated in this protocol. For example, following CPET Visit 2, participants may undergo skeletal muscle MRI with plantar flexion exercise ~3-7 days later, receiving the same interventional compound as Visit 2 (Placebo or Ketone). Following CPET Visit 3, participants may then undergo a skeletal muscle MRI with plantar flexion exercise, receiving the same interventional compound as Visit 3 (Ketone or Placebo). If scheduling does not allow for this, the MRI studies will be performed following a waiting period of at least a week after the last CPET study.

CPET Study Design

Patients will be asked (though not required) to avoid alcohol and caffeine consumption in the 24 hours prior to testing and to refrain from strenuous activity for 48hrs prior to study visits. Subjects will be asked to fast beginning at midnight prior to study initiation,[19] though water consumption will be freely permitted. Treatment order will be randomized prior to commencement using a random number generator by the University of Pennsylvanina Investigational Drug Pharmacy. Participants will be randomized in a crossover, double-blind fashion to: 1) 500 mg/kg (prior to max incremental CPET) and 250 mg/kg (prior to submax CPET) KE versus a placebo drink. By way of comparison, the liver produces 30-60 grams of ketones after overnight fasting, accounting for 2-6% of energy needs.[23, 41] Weight-based dosing of ketone therapy has been shown to be crucial to replicating ketone levels between participants.[19] The control drink will be furnished by the UPenn Nutrition Core and made of foodstuffs to roughly match taste.[42] Compliance will be defined as consumption of >85% of the drink volume. Light breakfast and lunch will be provided through CHPS.

The study intervention will be administered approximately 1 hour prior to exercise for the maximal incremental protocol.[9, 10] Because of logistical issues, up to 105 minutes after drink consumption will be allowable without deviation. For the submax protocol, we will provide a half-dose of study drug ~30 minutes prior to exercise since it is expected that some of the compound will be in the body still from the max effort CPET. For similar logistical issues, up to 60 minutes will be allowable without deviation. For comparison, website marketing of the commercial drink recommends ingestion 30 minutes prior to athletic performance, with additional dosing every 1.5-2.0 hours (https://hvmn.com/ketone-ester, accessed 12/7/2019).

At the end of Visit 3, the participants will be asked if they knew which arm they were randomized to and how confident they were using a validated blinding assessment tool.[43]

<u>Cardiopulmonary exercise testing</u>

We will use a supine cycle ergometer along will a metabolic cart to measure expired gases.

Maximal intensity protocol: Subjects will perform a maximal exertion-limited exercise test using a graded-exercise protocol. Resistance will begin at 15 Watts (W) for 3 minutes, increasing to 25W for 3 minutes, then increasing by 25W every 3 minutes thereafter. Vital signs will be monitored during the test.

We will define peak oxygen uptake (VO_2) as the average value during the final 30 seconds of exercise. The gas exchange/ventilatory threshold (VT) will be determined using both the V-slope and

ventilatory equivalent methods, with the results of the two measurements averaged when both are valid. VE/VCO_2 slope will be calculated from the beginning of exercise until exhaustion. The respiratory exchange ratio (RER) will be calculated as the ratio of VCO_2 to VO_2 during the final 30 seconds of exercise. We will model heart rate and VO_2 recovery following exercise.

The primary outcome of the maximal testing study will be difference in peak VO_2 following ketone therapy as compared to the placebo drink. Secondary cardiopulmonary exercise testing endpoints include exercise efficiency (ratio of work performed to oxygen consumed), total work performed, ventilatory threshold, VE/VCO_2 slope, VO_2 and heart rate recovery.

Submaximal intensity protocol: Resistance will begin with 1 minute of unloaded pedaling followed by a step-increase to 75% of peak workload (or the workload determined at the first visit). Workload will then be held at this intensity until volitional exhaustion or limiting symptoms prevail (e.g. subject cannot continue despite verbal encouragement, or subject is unable to maintain pre-specified pedal cadence despite verbal encouragement). Vital signs will be monitored during the test. We will assess exercise endurance time at 75% peak work rate. We may also assess VO_2 kinetics, including Tau, which is the time needed to reach 63% of the total response amplitude, and describe the rate/slope of VO_2 recovery. We will also assess VO_2 efficiency (total O_2 consumed to total work performed), and the respiratory exchange ratio, amongst other parameters gathered during the exercise tests.

The primary outcome for this submaximal part of the study is the difference in exercise endurance at 75% peak work rate following ketone therapy as compared to placebo.

<u>Electrocardiography</u>
A resting 12-lead ECG and prolonged rhythm strip will be obtained prior to exercise, and will be analyzed for rate, rhythm, intervals, and morphology. Heart rate variability metrics will be computed following KE and following the placebo intervention.

<u>Blood sampling and metabolomic profiling</u>
Standard blood testing is performed at Visit 1 for baseline analysis. This may include a CMP, CBC, NT-proBNP, lipid profile, hemoglobin A1c (approximately 20 mL of blood).

We will draw blood frequently before drug, after drug, and during exercise and recovery for measurements. The lab testing protocols are in **Table 2** with **approximate** times noted for planned lab draws (note: given the multitude of lab draws, missing laboratory draws will not be counted as deviations nor is exact timing required for laboratory draws). At Visit 2 and 3, a tertiary goal of blood sampling is to assess the pharmacokinetic (assess circulating ketone levels resulting from the DeltaG) and pharmacodynamic (assess biologic effects of the DeltaG) impact of DeltaG. BHB will be the primary means of determining therapeutic absorption. We will recreate pharmacokinetic curves including C_{max} (peak serum concentration achieved by drug), T_{max} (time at peak serum concentration), and area under the curve (AUC).

Blood samples will be stored at -80C for later analysis. We may perform comprehensive metabolomic profiling from this peripheral blood, in addition to other tests.

Table 2. Timing of Laboratory Draws during Study Protocol for Visit 2 and 3 WITHOUT Stable Isotope Infusion

Study Time	Plasma and/or Serum Tube Draw
0-30 minutes (baseline)	x
60 minutes (intervention given) – blood draw immediately before intervention	x
90 minutes *(start of echo)*	x

105 minutes *(mid-echo)*	x
120 minutes (start of maximal effort exercise)	*x*
Every 3 minutes during maximal effort exercise	x
Standardized draw at 25 Watts (if not already drawn)	x
Time of exhaustion after maximal effort exercise	x
Following 15 minutes of rest after completion of peak VO_2 study recovery and prior to lunch	x
~160 minutes (1/2 dose of intervention prior to submax CPET – blood draw immediately prior to intervention)	*x*
~190 minutes (submaximal CPET): At rest, 4 minutes (standardized draw), and time of exhaustion	x

Total blood drawn at Visit 2 and 3: ~180 mL/visit

Stable Isotope Infusion

A secondary goal of this proposal is to understand the metabolic changes induced by acute ketone administration. As described above, ketones have been shown to reduce glucose utilization in both healthy[10, 24, 44] and disease populations.[45] Systemic substrate utilization can be estimated through indirect calorimetry, using the data from gas exchange (carbon dioxide production rate and oxygen uptake rate) to derive rates of systemic carbohydrate and fatty acid oxidation.[46, 47] Yet systemic measurements do not identify the source of the substrate utilized (i.e. plasma-derived versus intramuscular), which may vary with exercise intensity, gender, and possibly disease state.[48-50] Knowing whether there is a deficit in substrate import, leading to alterations in utilization, could lead to new therapeutic opportunities.

Stable isotopes, in which the molecular weight of an atom is slightly increased due to additional neutrons within the nucleus, are naturally-occurring, non-radioactive, and are handled similarly by the body to their more abundant counterparts.[51] The higher molecular weight of the compound allows this 'label' to be traced throughout the body and the plasma using mass-spectometry. When infused into the blood, rates of endogenous production and utilization can be computed. Comparison of systemic oxidation rates with those derived from plasma measurements allows for an estimation of oxidation rates from intramuscular stores.[48]

To define how exogenous ketones affect endogenous glucose utilization, we may infuse [6,6-$^2H_2$] glucose during the submaximal exercise in a subset of participants. Participants will be administered stable isotope pending ability to place a second, contralateral IV. In such cases, infusions will be started ~1.5 hour prior to submaximal exercise, and initiated with a 5 mg/kg priming dose (MW 182.17 g/mole; 27.45 umol/kg; delivered over 5 minutes) followed by a continuous infusion at 0.05 mg/kg/min (0.2745 umol/kg/min), leading to a 100:1 prime/continuous infusion ratio.[52] We note that this prime/infusion protocol is well established and is used on campus by other investigators.[53] The stable isotope will be infused intravenously, with sampling done from a peripheral vein on the contralateral extremity. A typical blood sampling schema may be as follows: (a) prior to start of infusion, (b) every ten minutes following the start of the continuous infusion, (c) densely around 1 hour after delivery of the prime and start of the continuous infusion (for example, at 55, 60, and 65 minutes), (c) every ten minutes following administration of the study intervention and immediately prior to the start of exercise, (d) every 2 minutes during exercise, (e) at the time of exhaustion, (f) every 2 minutes during recovery for 10 minutes **(Table 3)**. Plasma samples will be stored for later mass-spec analysis. Additional blood samples may also be taken. It is anticipated that the addition of stable isotopes to the submaximal study will only require ~30 mL/ of additional blood/endpoint assessment visit, as mass spectrometry measurements can be made on a small volume (<2 mL).

We estimate that sampling blood for the stable isotope measurements during submaximal exercise will increase the amount of blood drawn by approximately 30 mL/visit (total of 60 mL for Visits 2 and 3).

Table 3. Approximate Timing of Laboratory Draws during Study Protocol for Visit 2 and 3 WITH Stable Isotope Infusion

Study Time	Plasma and/or Serum Tube Draw
0-30 minutes *(baseline)*	x
60 minutes *(intervention given)*	x
90 minutes *(start of echo)*	x
105 minutes *(mid-echo)*	x
120 minutes (start of maximal effort exercise)	*x*
Every 3 minutes during maximal effort exercise	x
Standardized draw at 25 Watts (if not already drawn)	x
Time of exhaustion after maximal effort exercise	x
Following 15 minutes of rest after competion of peak VO_2 study	x
Immediately prior to start of stable isotope infusion	x
10, 20, and 30 min, 40, 50, 55, 60, 65 min after start of the prime/continuous infusion	x
*~200 minutes (1/2 dose of intervention given) *lunch is provided after submaximal CPET*	*x*
Every 10 min prior to the start of the submaximal CPET (e.g. 10, 20, 30 min after intervention)	*x*
~230 minutes (submaximal CPET): At rest, every 2 minutes during exercise, and every 2 min of recovery for 10 minutes	x
Standardized draw at 4 minutes and at the time of exhaustion (if not already drawn)	x

Stable Isotope Studies: Total blood drawn at Visit 2 and 3 ~ 210 mL/visit (each visit). Note, if stable isotope infusion being performed, additional blood draws will be performed prior to the start of the infusion and every 10 minutes following infusion. Additionally, after ~1 hour of infusion dense sampling will be done (55 minutes, 60 minutes, and 65 minutes) prior to administration of the intervention. After the intervention, samples will be drawn every 10 minutes until the start of submaximal exercise at which time, blood samples will be drawn approximately every 2 minutes of exercise, at the time of exhaustion, and every 2 min of recovery for 10 minutes. The stable isotope infusion will then be discontinued.

Routine, stress, and strain echocardiography

A transthoracic echocardiogram will be performed in accordance with American Society of Echocardiography recommendations, with special attention to endocardial border and left ventricular outflow tract velocity optimization. Standard views will be obtained at rest and during exercise (though views may be more limited during exercise due to respirophasic motion of the heart). Metrics of cardiac performance and hemodynamics will be measured and may include (pending adequate border definition): left atrial strain, global left ventricular longitudinal strain, and strain rate may be

assessed using TomTec Cardiac Performance Analyses (TomTec Imaging Systems, Unterschleissheim, Germany) or similar commercial software in the apical 4 chamber, apical 3 chamber, and apical 2 chamber views. Myocardial strain is a sensitive marker of subclinical function and is a well validated, reproducible, and highly prognostic marker of systolic function.[54-56] A Doppler-derived LVOT VTI will be acquired at rest, each stage of exercise and at peak exertion to calculate cardiac output. Diastolic function will be assessed at rest and during the exercise study. In total, endpoints may include, but not limited to, left atrial strain, left ventricular global longitudinal strain, ejection fraction, stroke volume, and cardiac output, as well as diastolic indices of tissue relaxation (tissue Doppler E' velocity), filling pressure (E/e' ratio), and pulmonary artery systolic pressure.

Lung Ultrasound

Lung ultrasound will be performed in order to assess extravascular lung water, similar to what has previously been performed during stress echocardiography protocols.[57, 58] B-lines can be summed to give an approximation of the severity of extravascular lung water accumulation.

Arterial tonometry

We will use a commercially available system with a high-fidelity applanation tonometer to assess arterial stiffness at both rest and stress. The tonometer will record radial arterial pressure waveforms with participants in a recumbent position at rest and during exercise. We will pair arterial waveforms with LVOT Doppler profiles to assess ventricular-vascular coupling. Additional pressure waveforms may be obtained at the carotid, femoral, and brachial arteries.

Heart rate variability (HRV)

HRV is a non-invasive surrogate measure of autonomic tone (the balance of the sympathetic and parasympathetic nervous systems), which is a critical determinant of outcomes in many cardiovascular conditions.[59, 60] This analysis will offer insight into mechanisms responsible from a neuromodulatory perspective. A 15-minute single-lead reading will be performed including at rest and after randomization arms, with an ~5 minute segment exported for analysis (e.g. Kubios HRV software). This analysis will only be performed in participants in sinus rhythm. We will measure standard indices in HRV analysis (including time- and frequency-domain indices). The principal index will be standard deviation of the N-N (R-R) intervals. We may also assess high frequency and low frequency power, as well as their ratio.

Potential Pitfalls and Troubleshooting Strategies

Absorption of the compound is brisk and results in serum BHB levels of roughly 3 mmol/L in healthy volunteers at the dose studied here. Among HF patients, gut absorption might be reduced or delayed, leading to different peak effects of the compound. To address this potential problem, we have used a moderate dose of the compound to ensure we achieve therapeutic ketosis. Even if our goal levels of serum BHB are not achieved, recent data has demonstrated the benefits of ketone therapy accrue at even low levels (<1 mmol/L).[61]

Risks and Safety Assessment

KE risks: Rigorous previous analysis of the compound at doses (714 mg/kg) greater than what is administered here revealed a very favorable safety profile when administered as a single dose, with only 1 adverse event reported (transient mild rise in triglycerides that resolved by study completion).[9] While ketoacidosis can induce pH disturbances or neurologic complications, this is seen only at supraphysiologic levels (10-20 mmol/L); our study is anticipated to achieve levels of around 3 mmol/L, similar to the levels observed with fasting for a few days (for example, levels of roughly 2 mmol/L are achieved after 48 hours of fasting).[41, 62] We are also excluding type 1 diabetics, who are at risk of ketoacidosis for other reasons as well as those on SGLT2 inhibitors which can lead to ketoacidosis as well. Given that type 2 diabetics are not generally at risk for diabetic ketoacidosis, we will include

them in our study. Vital signs will be monitored closely during the study visits. Documentation of adverse event monitoring will take place during the study. Given the short half-life of the compound, adverse events will be considered up to the time of the participant leaving the research unit.

We will also review the medical charts of patients with an implantable cardioverter-defibrillator to determine therapy zones prior to exercise. The primary safety outcome will be stress-induced sustained arrhythmias during exercise (atrial arrythmias, ventricular tachycardia). Participants are free to terminate study involvement at any time, at which time they may be asked the reason for study termination.

Stable Isotope Infusion [(6,6-$^{2}H_{2}$)glucose]: There are no known risks associated with the use of stable isotopes as proposed in this study.[51] Stable isotopes are not radioactive. Solutions will be prepared by UPenn IDS and will be tested for sterility and the absence of pyrogens.

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