Carnitine Consumption and Augmentation in Pulmonary Arterial Hypertension

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Abstract

Right ventricular (RV) failure is the most common cause of death in pulmonary arterial hypertension (PAH). No RV-specific therapies are available, in part because the underlying mechanisms of RV failure are poorly understood. A growing body of evidence suggests that metabolic abnormalities may underlie RV dysfunction in PAH. Interventions against metabolic dysfunction in PAH may protect against RV failure. We have identified abnormalities in fatty acid (FA) metabolism in PAH that overlap considerably with disorders of carnitine deficiency. Carnitine links to an acyl group, which is required to transport FAs across the mitochondrial membrane to undergo beta-oxidation, the predominate source of ATP production in the human heart. Inborn errors of carnitine metabolism and acquired carnitine deficiency are associated with cardiomyopathy. Acquired deficiency primarily occurs via binding of carnitine to excess circulating fatty acids or renal wasting. Carnitine deficiency and PAH are both associated with insulin resistance, myocardial lipotoxicity, and mitochondrial oxidative stress. Carnitine supplementation in humans and animal models of cardiometabolic dysfunction reverses these abnormalities but has not been studied in PAH. In published work, we found that in RV samples from humans with PAH, there is a marked (up to 300-fold) reduction in acylcarnitines along with increased long-chain fatty acids. We also a found a two-fold increase in circulating fatty acids FAs in humans with PAH, indicating increased delivery to the myocardium. As a consequence of unchecked fatty acid accumulation, we observed 7-fold higher RV lipid content and markers of lipotoxicity. These observations suggest there is inadequate carnitine substrate to bind fatty acids and facilitate their transport across the mitochondrial membrane in the human PAH RV.

Our overarching hypothesis is that in human PAH, RV function can be improved by augmenting carnitine substrate availability to improve outcomes. In preparation for a future mechanistic study, we now propose to test the specific hypothesis that carnitine consumption is not reduced in PAH, that plasma carnitine levels are stable over time in PAH and that carnitine supplementation in PAH can increase plasma carnitine and thereby delivery of carnitine to the RV and possibly improve RV function. We propose three aims in humans to test this mechanistic hypothesis, 1) Measure the oral consumption of carnitine in human PAH. This aim will use food diaries and carnitine supplement use questionnaires in PAH patients to test the hypothesis that carnitine supplementation is uncommon in PAH and food consumption is adequate. Aim 2) Measure the stability over time in plasma carnitine levels in PAH patients. This aim will test the hypothesis that plasma carnitine is not affected by disease severity and is stable over time in PAH patients. We will measure plasma carnitine concentration and markers of fatty acid oxidation at Visit 1 and Visit 2. 3) Perform a mechanistic pilot study using carnitine supplementation to enhance circulating carnitine in PAH. This small pilot study will test the hypothesis that carnitine supplementation increases plasma carnitine (primary endpoint) and will test for physiologic effects using six minute walk testing, echocardiography and plasma markers of lipid metabolism.

Protocol Summary

OBJECTIVES:

The primary objective of this study is to evaluate the effect of standard L-carnitine supplementation in PAH patients on plasma carnitine levels.

Secondary objectives include:

- to measure the oral consumption of carnitine in human PAH
- to measure the stability over time in plasma carnitine levels in PAH patients

STUDY DESIGN:

This is a single-center, prospective study enrolling 10 PAH patients. All eligible participants will be given carnitine supplements for 2 weeks.

STUDY POPULATION:

Inclusion criteria:

- Adults aged 18 or older.
- Diagnosed with idiopathic, heritable, simple congenital heart disease, or drug- or toxin-associated pulmonary arterial hypertension (PAH) according to World Health Organization consensus recommendations.
- Stable PAH-specific medication regimen for three months prior to enrollment. Subjects with only a single diuretic adjustment in the prior three months will be included. Adjustments in IV prostacyclin for side effect management are allowed.
- FEV1> or = 60% predicted and no more than mild abnormalities on lung imaging
- WHO Functional Class I-III
- Ambulatory

Exclusion criteria:

- Prohibited from normal activity due to wheelchair bound status, bed bound status, reliance on a cane/walker, activity-limiting angina, activity-limiting osteoarthritis, or other condition that limits activity
- Pregnancy
- Diagnosis of PAH etiology other than idiopathic, heritable, simple congenital heart disease, or associated with drugs or toxins
- Drug and toxin associated PAH patients with active drug use

- Prior diagnosis of cirrhosis
- Malignancy
- eGFR by MDRD <60mL/min
- Known allergy to carnitine

PRIMARY ENDPOINT:

Difference in plasma carnitine concentration from Visit 1 to Visit 2 and Visit 3 to Visit 4.

SECONDARY ENDPOINTS:

- Quantify the prevalence of carnitine supplement use and measure oral ingestion of carnitine in PAH patients.
- Correlation of carnitine ingestion with six-minute walk test.
- Correlation of carnitine ingestion with functional class.
- Correlation of carnitine ingestion with plasma BNP.
- Difference in plasma carnitine concentration from Visit 1 to Visit 2.
- Correlation of plasma carnitine concentration with TMAO concentration.
- Difference in plasma acylcarnitines from Visit 1 to Visit 2.
- Difference in lipid metabolites from Visit 1 to Visit 2.
- Difference in RV function with L-carnitine supplementation.
- Difference in plasma carnitine concentration from Visit 1 to Visit 2.
- Difference in plasma acylcarnitine profile from Visit 3 to Visit 4.
- Difference in lipid metabolites from Visit 3 to Visit 4.
- Correlation of change in plasma carnitine with change in Functional class.
- Correlation of change in plasma carnitine with change in six-minute walk distance.
- Correlation of change in plasma carnitine with change in BNP.
- Correlation of change in plasma carnitine concentration with TMAO concentration
- Correlation of change in plasma carnitine with change in markers of RV function including TAPSE and RV fractional area change.
- Markers of tolerability including presence of side effects, adverse events and serious adverse events.
- Pill counts at Visit 4 for adherence.

STUDY OBSERVATIONS:

- Subjects will be evaluated in person at Visit 1, Visit 2, Visit 3, and Visit 4.
- BNP, carnitine assay, lipid metabolites, carnitine, acylcarnitine, and other biomarkers will be assessed at Visit 1, Visit 2, Visit 3, and Visit 4.
- Subjects will have six-minute walk testing at Visit 1, Visit 2, and Visit 4.
- Subjects will have a transthoracic echocardiogram at Visit 2 and Visit 4.
- Subjects will submit food and supplement diary at Visit 1, Visit 2, and Visit 4.

SAMPLE SIZE AND POWER:

In a study of 28 PAH patients[6] the mean ±SD of plasma carnitine data was 38.6±11.1 uM. The data appeared to be normally distributed. In a pilot study[21], L-carnitine supplementation increased free plasma carnitine concentration by 22% (Table 3 in[21]). A sample size of 10 will have 80% power to detect a within subject change in means of 8.5 (increase from Visit 1 mean of 38.6 to 47.1 after 2 weeks of treatment, representing a 22% increase) using a paired t-test with a 5% two-sided significance level. The standard deviation of within-subject differences is assumed to be 8.6 and it is calculated using SD of 11.1 assuming a moderate within-subject correlation 0.7. We plan to study 10 PAH patients in this proof-of-concept study. The study has 80% power to detect a within-subject change of effect size 1, i.e. change measured as 1 times of the SD.

DATA ANALYSIS:

The primary endpoint and other continuous endpoints will be summarized using the mean, standard deviation, the median, and interquartile range. We will use either the paired t-test or the Wilcoxon-signed rank test to assess the change from Visit 1 on these endpoints. Estimate of mean change along with its 95% confidence interval will be reported to quantify the treatment effect. To assess tolerability of carnitine, we will closely monitor any adverse events. For these binary events, proportion of subjects who experience the event will be calculated along with its 95% confidence interval. We will use the open-source statistical package R[48] for analyses.

1 Background & Significance

1.1 RV Failure in Pulmonary Arterial Hypertension

PAH is a progressive and incurable disease characterized by obliteration of the pulmonary vasculature, elevated pulmonary vascular resistance (PVR), and eventual right ventricular (RV) failure often within 7-10 years of diagnosis[1]. Mortality in patients with PAH predominantly results from RV failure and its complications[2]. However, no RV-specific therapy exists because the underlying determinants of RV failure are poorly understood. The importance of targeting RV function directly is highlighted by the observation that outcomes in PAH more closely mirror changes in RV function than pulmonary hemodynamics[3]. Identification of additional molecular contributions to RV failure in PAH would advance the pursuit of RV-specific therapies and carnitine supplementation holds promise to improve RV function in this deadly disease.

1.2 PAH As A Systemic Metabolic Disease with RV Manifestation

PAH is associated with systemic abnormalities in glucose and lipid metabolism. The first clue in humans was a study showing that patients with PAH have an elevated triglyceride to high density lipoprotein ratio, a marker of insulin resistance. Subsequently, we found a prevalence of almost 60% of unrecognized glucose intolerance in our PAH patients, independent of body mass index (BMI)[4]. The lack of an association with BMI suggests that the mechanism of insulin resistance in PAH may be unrelated to increased adiposity, per se**.** In addition, we found elevated plasma free fatty acids and long-chain acylcarnitines in humans with PAH compared with matched controls (see Preliminary Data)[5]. More recently, we showed that insulin resistance in PAH specifically involves the lipid axis and is detected through peripheral blood abnormalities in triglycerides and lipid metabolism[6]. While many organs may be affected by insulin resistance, the RV is highly reliant on fat metabolism and thus susceptible to metabolic disorders. We have previously published that the RV in humans with PAH, both alive and at autopsy, has enhanced lipid content[5,7]. We have also demonstrated that this lipotoxicity is due to increased lipid import via CD36 and reduced fatty acid oxidation in cardiomyocytes[8]. We also demonstrated in human samples an increase in plasma free fatty acid delivery to the PAH RV and a marked (100-300 fold) reduction in myocardial long-chain acylcarnitine concentration, suggesting reduced fatty acid oxidation[5]. These findings provide strong evidence that metabolic dysregulation, and specifically lipid metabolism, is an important feature of PAH and that the RV appears impacted by this altered metabolism.

1.3 Carnitine Metabolism in Health and Disease: Focus on the heart

Carnitine is a nutrient required for the transport of long-chain fatty acids as acylcarnitines across the mitochondrial membrane to undergo beta-oxidation and subsequent ATP production[9]. Carnitine is particularly abundant in skeletal muscle and the myocardium, where fatty acids are the predominant substrate for energy production[10]. The presence of adequate carnitine in myocardial cells is essential for normal fatty acid metabolism. Reduced availability of carnitine from any cause leads to impaired fatty acid oxidation and muscle dysfunction. When carnitine binds fatty acids, acylcarnitines are formed and then normally undergo beta-oxidation. In conditions of excess circulating free fatty acids (e.g. diabetes and heart failure), acylcarnitines are formed in excess of mitochondrial oxidative capacity. This results in a depletion of carnitine, reduced fatty acid oxidation, oxidant stress, and accumulation of toxic lipids. Excess acylcarnitines are excreted from cells and elevated levels can be detected in the plasma in conditions of abnormal

carnitine metabolism[9]. Oleoylcarnitine and three other long-chain acylcarnitines are strongly associated cardiovascular mortality in a dialysis population[11]; the same four acylcarnitines were most elevated in our PAH population compared to healthy subjects[5]. Cardiomyocytes cannot synthesize carnitine, which must be acquired exogenously. Thus, deficiencies, even relative, in carnitine have particularly adverse effects in cardiomyocytes. Inborn errors of carnitine metabolism involve abnormal transporter function and present with severe, often fatal, cardiomyopathy[12]. Secondary carnitine deficiency occurs because the available carnitine is bound to excess fatty acids or due to renal wasting[13]. Acquired carnitine deficiency has been described in dialysis therapy, diabetes, and heart failure, among other conditions[14-16]. Chronic carnitine deficiency is associated with insulin resistance, lipid-induced cardiomyopathy, and increased mitochondrial oxidative stress[16]. Carnitine supplementation in humans and experimental models appears to mitigate these defects. In humans with heart failure, carnitine improves diastolic and systolic dysfunction and may reduce cardiovascular events[15,17]. In diabetics, carnitine reduces myocardial free FA uptake and circulating long-chain acylcarnitines. In mice exposed to doxorubicin, carnitine reduces ceramide production and apoptosis. Thus, multiple lines of evidence suggest that maintenance of intracellular carnitine may be beneficial for cardiometabolic function.

1.4 Background Ingestion of Carnitine Varies by Diet.

A typical Western diet is high in meat and dairy intake and thus provides adequate carnitine ingestion to prevent depletion[18]. The Food and Nutrition Board has not established a recommended dietary allowance for carnitine and it is not considered an essential nutrient[19]. Nonetheless, relative carnitine deficiency develops in numerous inborn and chronic diseases, indicating that background ingestion is not sufficient to prevent depletion in some states[13,18,20] **and that normal plasma carnitine concentration may not be adequate for tissue needs depending on the demand**[21]. This observation suggests that supplementation may be required to prevent or reverse depletion in at risk individuals despite normal dietary consumption. There are no published data on frequency of vegetarian or vegan diet in PAH that may predispose to deficiency or use of carnitine supplementation in this disease state.

1.5 Carnitine Supplementation in Humans

Given its safety and role in energy production, carnitine has been studied extensively and even touted as a performance enhancer for professional athletes. Although the liver and kidneys in a healthy human can metabolize the amino acids lysine and methionine to meet daily carnitine needs[19], carnitine supplements are widely available. Carnitine supplementation has primarily been in the form of L-carnitine, or levocarnitine, which is the active form in the body[22], that has a bioavailability of approximately 25%[22,23]. After an oral dose of 30-100mg/kg body weight, peak plasma carnitine concentration occurs at 3-6 hours[24]. In a study of the effect of long-term supplementation with L-carnitine, healthy adult men were fed similar doses (2g/day) of L-carnitine per day or placebo for 15 days. Subjects in the supplementation group maintained total plasma concentrations that were about 25 umol/L higher than in the control group, thus demonstrating the ability of carnitine supplementation to increase plasma carnitine over two weeks[22]. While there are no data on the effect of oral carnitine supplementation on cardiac carnitine content, a 2g/day oral L-carnitine supplement can modesty increase the skeletal muscle carnitine concentration by about 13% over four weeks[25]. Carnitine supplementation has been tried in ageing, where it has had no effect on muscle strength[21], however after acute myocardial infarction, a meta-analysis demonstrated that L-carnitine supplementation

reduced mortality, ventricular arrhythmias and angina over two months[26]. L-carnitine can be metabolized by gut microbiota to proatherogenic trimethylamine-N-oxide (TMAO) which is associated with increased cardiovascular risk[27]. However, limited prospective data exist to support this notion and none related to PAH or RV failure, which is not an atherosclerotic disease. Moreover, survival in PAH is generally fewer than 10 years[28], thus the relevance of TMAO to PAH is unclear.

2 Objectives and Specific Aims

2.1 Objectives

This is a single-center, prospective study enrolling 10 PAH patients. All eligible participants will be given carnitine supplements for 2 weeks.

2.2 Specific Aims

Primary Aim:

1. The primary objective of this study is to evaluate the effect of standard L-carnitine supplementation in PAH patients on plasma carnitine levels.

Secondary Aims:

1. to measure the oral consumption of carnitine in human PAH
2. to measure the stability over time in plasma carnitine levels in PAH patients

3 Screening and Subject Selection

3.1 Recruitment of Study Sample

Patients will be recruited from the Center for Pulmonary Vascular Disease (CPVD) at Vanderbilt University Medical Center (Nashville, TN). Potentially eligible subjects will be pre-screened by study personnel through medical record review and informed about that study to determine if they have an interest in enrolling. After the initial conversation by telephone, the subject will be provided informed consent and a link to the e-consent will be emailed directly to them. Informed consent will occur before any study procedures are performed.

3.2 Inclusion/Exclusion Criteria

Inclusion criteria:

- Adults aged 18 or older.
- Diagnosed with idiopathic, heritable, simple congenital heart defect, or drug- or toxin-associated pulmonary arterial hypertension (PAH) according to World Health Organization consensus recommendations.
- Stable PAH-specific medication regimen for three months prior to enrollment. Subjects with only a single diuretic adjustment in the prior three months will be included. Adjustments in IV prostacyclin for side effect management are allowed.
- FEV1> or = 60% predicted and no more than mild abnormalities on lung imaging

- WHO Functional Class II-IV
- Ambulatory

Exclusion criteria:

- Prohibited from normal activity due to wheelchair bound status, bed bound status, reliance on a cane/walker, activity-limiting angina, activity-limiting osteoarthritis, or other condition that limits activity
- Pregnancy
- Diagnosis of PAH etiology other than idiopathic, heritable, simple congenital heart defect, or associated with drugs or toxins
- Drug and toxin associated PAH patients with active drug use
- Prior diagnosis of cirrhosis
- Malignancy
- eGFR by MDRD <60mL/min
- Known allergy to l-carnitine supplements

3.3 Supplement Dosing

A 14-19-day supply of Carnitine supplements will be provided to the subject at Visit 2. The VUMC Pharmacy for Research will supply Nature's Bounty L-Carnitine 500mg caplets to be taken by mouth. For subjects weighing <90kg, l- carnitine will be taken at 1.5g twice a day. For subjects weighing >90kg, l-carnitine will be taken at 50 mg/kg/day split between 2 doses. Dosing for these participants will be rounded to the next whole pill to achieve approximately 50 mg/kg per day. Dosing will be incrementally titrated by 25- 50% in the event of intolerance and/or side effects limiting subject's compliance to prescribed regimen.

4 Data Collection

4.1 Consent

Written consent or e-consent will be obtained for enrollment from participants. For each consent process, study personnel will discuss the details of the study, the risks and benefits, and the subject's rights and responsibilities if they choose to participate in the trial and their right to refuse to participate. It will be made clear that their clinical care will not be affected by their decision. Written consent or e-consent will be obtained before Visit 1.

For e-consent, the patient consent process will be conducted using a REDCap-based electronic consent form. The consent form has been developed in REDCap, a secure, web-based, HIPAA-compliant, data collection platform with a user management system allowing project owners to grant and control varying levels of access to data collection instruments and data (e.g. read only, de-identified-only data views) for other users. Potential participants will participate in the consent process by either:

1.) Being approached in-person at a Vanderbilt Clinic and accessing the REDCap survey via iPad or other portable electronic device and/or
2.) Self-initiated access of consent forms on personal portable electronic devices using posted QR codes or web-links on study posters, brochures, or websites. Self-initiated accessing of consent forms may occur in clinic or at home.

During the in-person consent process, patients will be consented by a member of the key study personnel.
For self-initiated consent, contact information will be provided (email and phone) for prospective patients to contact a member of the key study personnel with questions, prior to consent.
Patient signatures will be obtained using a typed signature. Upon completion of the consent, patients will be provided with a copy of their version of the consent document by printing a pdf copy of the consent form in clinic or at their Visit 1 visit.

4.2 Study Visits and Contacts

This study will consist of four visits: Visit 1 (0 week), Visit 2 (Week 12), Visit 3 (Week 12+1 day) and Visit 4 (Week 14). The Visit 1 and Visit 3 are both estimated to take approximately 2 hours each. Visit 2 and Visit 4 are both estimated to take approximately 4 hours each. Patients will be consented by the PI, a clinical research coordinator, or a research nurse. Patient's written consent and study procedures will take place at the Pulmonary Vascular Disease Clinic (CPVD), the Clinical Research Center (CRC), there will be no changes to a patient's medication regimen.

4.2.1 Pre-visit Consent Telephone Contact (minimum -4 days)

Prior to subject's first CRC visit, the subject will consent by REDCap e-consent process and complete a 3-day ASA24 food and supplement diary.

4.2.2 Visit 1 Visit (0 Weeks)

This visit will last approximately 2 hours and occur at the Clinical Research Center at VUMC. At Visit 1, written consent will be obtained from subjects after all questions are adequately answered. The following procedures will be performed:

- written informed consent
- review inclusion/exclusion criteria
- review of medical history
- demographic data
- review of current medications
- orientation to food and supplement diary (ASA24)
- WHO functional class
- blood collection
- symptom assessment
- vital signs
- physical exam
- six-minute walk test

After fasting research labs have been drawn, the subject will have the opportunity to eat a snack. The investigator or research nurse will take a medical history, perform a physical examination including checking vital signs, and review current medications. The subject will perform the six-minute walk test.

4.2.3 Telephone Contact (Week 11)

The research coordinator will call the subject 1 week prior to Visit 2 to remind them to complete the 3-day ASA24 food and supplement diary. Study compliance will be assessed and reinforced.

4.2.4 Study Day – Visit 2 (Week 12)

All tests done at the Visit 1 visit will be repeated at the Visit 2 Visit. The following procedures will be performed:

- review of interim medical history
- review of current medications
- WHO functional class
- blood collection
- urine pregnancy test (as necessary)
- symptom assessment
- vital signs
- physical exam
- review of food and supplement diary (ASA24)
- six-minute walk test
- transthoracic echocardiography
- Dosing of Carnitine supplement

After fasting research labs have been drawn, the subject will have the opportunity to eat a snack. The investigator or research nurse will take an interim medical history, perform a physical examination including checking vital signs, and review current medications. The subject will perform the six-minute walk test. An experienced research sonographer will perform the transthoracic echocardiography.

4.2.5 Study Day- Visit 3 (Week 12+1 day)

The day following Visit 2, a brief visit will be performed with the following procedures:

- blood collection
- symptom assessment
- vital signs
- review of food and supplement diary (ASA24)
- 2 weeks supply of carnitine supplement provided

4.2.6 Telephone Contact (Week 13)

The research coordinator will call the subject a week after beginning the carnitine supplement to review and record symptoms, potential side effects, and changes in medications. Study compliance will also be assessed and reinforced.

4.2.7 Study Day- Visit 4 (Week 14)

All tests done at the Visit 2 visit will be repeated at the Visit 4 Visit. The following procedures will be performed:

- review of interim medical history
- review of current medications
- WHO functional class
- blood collection

- symptom assessment
- vital signs
- physical exam
- review of food and supplement diary (ASA24)
- six-minute walk test
- transthoracic echocardiography
- pill count of carnitine supplement

After fasting research labs have been drawn, the subject will have the opportunity to eat a snack. The investigator or research nurse will take an interim medical history, perform a physical examination including checking vital signs, and review current medications. The subject will perform the six-minute walk test. An experienced research sonographer will perform the transthoracic echocardiography.

4.3 Study Schedule of Endpoints and Procedures

The table below summarizes the study endpoint assessments and procedures.

	Phone Call	**Visit 1**	**Phone Call**	**Visit 2**	**Visit 3**	**Phone Call**	**Visit 4**
		Observational Period		*Supplement Period*			
Day	*-3*	*0*	*80*	*84 (+/- 5 days)*	*85*	*92 (+/- 5 days)*	*99 (+/- 5 days)*
Week	*-1*	*0*	*11*	*12*	*12*	*13*	*14*
Informed Consent	x						
History and Physical							
Vital Signs		x		x	x		x
Medical History		x		x			x
Medication review		x		x		x	x
Physical Exam		x		x			x
Functional Class		x		x			x
Symptom Assessment		x		x	x	x	x
Testing							
Phlebotomy							
CBC		x		x	x		x
CMP		x		x	x		x
BNP		x		x	x		x
Carnitine assay		x		x	x		x
Lipid metabolites		x		x	x		x
Carnitine		x		x	x		x
Acylcarnitine		x		x	x		x
6MWTD		x		x			x
Echo				x			x

Urine Pregnancy Test				x			
Study Procedures							
ASA-24 Dietary Assessment	x	x	x	x			
Carnitine supplement (3g/day x 2 weeks)				x	x	x	
Adverse events				x	x	x	x
Supplement compliance					x	x	x

Table 1. Study Procedures

5 Assessments of Efficacy and Outcome Measures

5.1 Assessments of Efficacy

Primary Endpoint: Difference in plasma carnitine concentration from Visit 1 to Visit 4.

Secondary Endpoints:

- Quantify the prevalence of carnitine supplement use
- Measure oral ingestion of carnitine in PAH patients.
- Difference in six-minute walk test from Visit 1 to Visit 2.
- Difference in functional class from Visit 1 to Visit 2.
- Difference in plasma BNP from Visit 1 to Visit 2
- Difference in plasma carnitine concentration from Visit 1 to Visit 2.
- Correlation of plasma carnitine concentration with TMAO concentration.
- Difference in plasma acylcarnitines from Visit 1 to Visit 2.
- Difference in lipid metabolites from Visit 1 to Visit 2.
- Change in plasma carnitine from Visit 2 to Visit 3.
- Difference in RV function from Visit 2 to Visit 4.
- Difference in plasma acylcarnitine profile from Visit 2 to Visit 4.
- Difference in lipid metabolites from Visit 2 to Visit 4.
- Correlation of change in plasma carnitine with change in Functional class.
- Correlation of change in plasma carnitine with change in six minute walk distance.
- Correlation of change in plasma carnitine with change in BNP.
- Correlation of change in plasma carnitine concentration with TMAO concentration
- Correlation of change in plasma carnitine with change in markers of RV function including TAPSE and RV fractional area change.
- Markers of tolerability including presence of side effects, adverse events and serious adverse events.

- Pill counts at Visit 4 for adherence

5.2 Secondary Outcome Measures

5.2.1 Six Minute Walk Distance

Walking is the most basic form of exercise and is integral to daily activities. The 6MWT is a standardized, times submaximal test of unencouraged, self-determined distance walked which is reliable and valid[54]. Standardized test methods and scripted and timed statements have been established in prior studies of PAH[28,29-35]. The 6MWT will be administered according to the Thoracic Society guidelines[36,54]. The 6MWT will be performed at Visit 1, Visit 2 Visit 3 and Visit 4. The subject will be instructed to wear comfortable clothing and shoes. The test will be performed at approximately the same time of day at each visit. In addition, Borg dyspnea Score, oxygen saturation, and heart rate will be recorded at the beginning and conclusion of each test.

5.2.2 The Borg Dypsnea Score

In order to assess exercise capacity, the Borg score for dyspnea and overall fatigue will be recorded before and after subjects complete the six-minute walk, performed at Visit 1, Visit 2 visits and Visit 4 visits. This score is a measure the physical activity intensity level based on the subject's perceived exertion. Subjects will rate at resting and peak exercise.

5.2.3 WHO Functional Class

The WHO functional classification for PAH has been modified from the well-known New York Heart Association functional classification. This functional classification is based on symptoms, with Class I being defined by no symptoms, Class II as having mild limitation in physical activity, Class III as having markedly limited physical activity and Class IV as being unable to perform any physical activity. The WHO functional class will be assessed at Visit 1, Visit 2 visits and Visit 4 visits.

5.2.4 Blood Biomarkers

5.2.4.1 Lipid Metabolism

Fasting plasma lipid metabolites will be evaluated via non-targeted metabolomics screen. Mass spectroscopy metabolites such as LDL, HDL, and triglycerides will be performed at Visit 1, Visit 2, Visit 3 and Visit 4.

5.2.4.2 Fatty Acid Metabolism

Elevated circulating fatty acids and acylcarnitines contribute to RV lipotoxicity. To evaluate the effect of this intervention on fatty acid metabolism, we will measure fasting long-chain fatty acid and acylcarnitine profiles using mass spectrometry at Visit 1 and Visit 2.

5.2.4.3 Plasma NT-proBNP

Plasma NT-proBNP reflects right ventricular function in PAH and is a strong predictor of outcome in PAH. BNP will be assessed at Visit 1, Visit 2, Visit 3, and Visit 4.

5.2.4.4 TMAO

Trimethylamine-N-oxide (TMAO) is associated with increased cardiovascular risk[27] and presents as a detrimental carnitine effect. As a safety endpoint, TMAO will be measured via mass spectroscopy.

5.2.5 Transthoracic Echocardiographic Measures

We will assess RV free wall longitudinal strain and other parameters as secondary endpoints via transthoracic echocardiography. The ultimate determinant of outcome in subjects with PAH is right ventricular function, so we have focused on metrics, which are predictors of survival in PAH, including RV strain and qualitative RV function. The following metrics will be obtained: 1) tricuspid annular plane systolic excursion (TAPSE); 2) RV & LV ejection fraction[51]; 3) RV fractional area change; 4) tricuspid annular velocity (S'); 5) tricuspid regurgitant (TR) velocity; 6) RV systolic pressure (estimated from the equation: RV systolic pressure = 4(maximum TR velocity)[61] + right atrial pressure); 7) right atrial pressure[59]; 8) RV & LV diastolic function[53]; 9) RV free wall longitudinal strain[54,60]. All measurements will be performed according to the American Society of Echocardiography guidelines[51,53,59].

5.2.6 Other Feasibility Endpoints

a) Drop Out Rates

b) Side effects and tolerability of supplementation including carnitine

5.2.7 Hospitalization

A hospital admission due to PAH progression and/or right-sided heart failure will be defined as a hospitalization because of lower extremity edema or dyspnea and/or PAH symptoms (e.g., syncope) refractory to outpatient increases in dose or frequency of diuretics or specific PAH medications.

We will record all hospitalizations during the time of the study. Records from each hospitalization will be obtained by the local study coordinator. These records will be reviewed by the Data and Safety Monitoring Board (DSMB), which is unrelated to the study.

5.2.8 Death

Cardiovascular Death: We define as cardiovascular death:

1) sudden death
2) death preceded by either one of the following:
   a) cardiogenic shock defined by either:
      i. hypotension resulting in a failure to maintain normal renal
      ii. cerebral function for >15 minutes prior to death)
   b) heart failure symptoms or signs requiring one of the following:
      i. intravenous therapy or oxygen in the hospital

ii. confinement to bed in the absence of secondary causes (such as systemic infection or dysfunction of intravenous or subcutaneous medication delivery devices) or alternative causes of death.

Non-Cardiovascular Death: A death which does not meet the criteria above will be considered a non-cardiovascular death.

6 Statistical Considerations

6.1 Study Design

In a study for 28 PAH patients[6] the mean ± SD of plasma carnitine data was 38.6±11.1 uM. The data appeared to be normally distributed. In a pilot study[21], L-carnitine supplementation increased free plasma carnitine concentration by 22% (Table 3 in[21]). A sample size of 10 will have 80% power to detect a within subject change in means of 8.5 (increase from Visit 1 mean of 38.6 to 47.1 after 2 weeks of treatment, representing a 22% increase) using a paired t-test with a 5% two-sided significance level. The standard deviation of within-subject differences is assumed to be 8.6 and it is calculated using SD of 11.1 assuming a moderate within-subject correlation 0.7. We plan to study 10 PAH patients in this proof-of-concept study. The study has 80% power to detect a within-subject change of effect size 1, i.e. change measured as 1 times of the SD.

6.2 Statistical Analysis

The primary endpoint and other continuous endpoints will be summarized using the mean, standard deviation, the median, and interquartile range. We will use either the paired t-test or the Wilcoxon-signed rank test to assess the change from Visit 1 on these endpoints. Estimate of mean change along with its 95% confidence interval will be reported to quantify the treatment effect. To assess tolerability of carnitine, we will closely monitor any adverse events. For these binary events, proportion of subjects who experience the event will be calculated along with its 95% confidence interval. We will use the open-source statistical package R[57] for analyses.

6.3 Subjects' retention and supplement compliance

We will enforce subject retention in several ways. We will record extensive contact information for each subject at their enrollment in the trial. This will include home, work, and cellular telephone numbers. The research coordinator will call before each study visit to remind the subject to attend. Subjects will be reimbursed for time at the clinic for research procedures and reasonable travel expenses necessary for their participation in the study. Subjects will receive $75 each for Visit 1 and Visit 2, $100 for Visit 3, and $150 for Visit 4. The total reimbursement for completing all study procedures will be $400.

The research coordinator and physician will explain the importance of compliance with the study protocol at each subject contact. If a subject fails to comply with a study visit, the coordinator will contact him or her by telephone. If this fails, the coordinator will send two letters, one week apart, to request follow-up.

We have considered how to minimize difficulties with adherence to the supplement. We will strongly emphasize the importance of complying with the supplement regimen. Nonetheless, we will perform pill counts at visits and record episodes when the supplement is withheld for any reason. If a subject wishes to drop-out from the supplement phase of the study or has a serious adverse event (SAE) (whether related to study drugs or not), we will continue to follow-up with the subject for study assessments to assist with safety monitoring and to avoid the problems introduced by missing data. The inclusion of such follow-up data will allow for analysis by intention-to-treat.

If a subject is withdrawn from the supplement portion of the study for any reason, the subject will be strongly encouraged to continue with the remainder of the study assessments, as scheduled.

6.4 Interim Monitoring Guidelines

We have not planned formal interim analyses for efficacy and therefore there are no stopping rules for efficacy for this trial. This project will have an independent safety officer who may consider whether to stop the trial or not if there is an increased risk of adverse events or toxicity.

6.5 Protocol Violations

Serious protocol violations such as discontinuation of the intervention unrelated to AEs will be carefully recorded and regularly reviewed by the Principal Investigator. Remedial changes in procedure will be recommended where feasible to reduce the incidence of such violations. When known, the causes and circumstances of all violations will be documented for future secondary analyses and interpretation. Because all primary analyses will be intent-to-treat, it is essential that violations be kept to a minimum especially where it is possible to influence their rate of occurrence.

6.6 Safety and masking analysis

All subjects will be assessed for toxicity and included in the safety analysis. This analysis will include summaries of the incidence and grade of toxicities. Safety interim analyses will be performed and reported to the DSMB. Subjects will be evaluated for SAEs.

7 Study Risks and Discomforts

7.1 Echocardiography

There are no risks to this ultrasound procedure aside from minor discomfort related to placement of the ultrasound probe on the chest.

7.2 Venipuncture

The protocol requires patients to have blood drawn for research purposes. The risks of drawing blood are uncommon and may include bleeding, minor infection, and bruising. Commonly, having blood drawn is painful, and rarely can lead to infection at the site of the blood draw. The amount of blood drawn is small and represents an exceedingly small percentage of the amount of the total blood volume and will not represent a significant risk to the patient.

7.3 Carnitine

Given its safety and role in energy production, carnitine has been studied extensively and is widely available. In long-term administration of oral L-carnitine, mild gastrointestinal side effects have been reported; these include transient nausea and vomiting, abdominal cramps, and diarrhea. Notable, are some reported cases of a “fishy” body odor while taking carnitine supplements. Mild myasthenia is reported in uremic patients; however, this patient population has been excluded from enrolling in our study. In rare cases, patients with or without pre-existing seizure activity have reported seizures to occur, in addition to increase in frequency and/or severity of seizures.

8 Quality Control

Design strategies and monitoring activities throughout the study will ensure the integrity and high quality of the data. Design strategies include training and certification of personnel. The rigorous monitoring program includes data queries and performance monitoring over the time of the trial.

8.1 Personnel training

Prior to enrolling the first subject in the study protocol, study coordinators and research assistance who will perform the outcome assessments will have completed appropriate training. The purpose of training is to ensure that study personnel are carrying out the protocol in a consistent way and are adhering to a good clinical practice guidelines. Staff will have current Human Subjects Training Certification on file.

8.2 Data Quality

The PI and study coordinator will constitute the first line of monitoring of the safety of the human participants. They will perform continuous monitoring of data quality and completion of CRFs. Surveillance for AEs will consist of questioning subjects about potential AEs at every study contact, having subjects report any adverse event to the study team, and having subjects undergo vital sign checks and physical exams during each study visit.

Project team members at Vanderbilt listed as Key Study Personnel with existing electronic health record (HER) system access rights will make use of REDCap Clinical Data Pull (CDP) tools. These tools are designed to enable transfer of relevant study-related data from the Vanderbilt Research Derivative and/or directly from the HER into REDCap.

8.3 Independent Safety Officer

Dr. Meredith Pugh will serve as an independent safety officer and will monitor the trial. The aims of the officer are to safeguard the interests of the trial’s participants, potential participants, and investigators, to ensure the safety of the trial’s interventions, to monitor the trial’s overall conduct, and protect the trial’s validity and credibility. Dr. Pugh serves as an attending physician in the Vanderbilt Medical Intensive Care Unit, the inpatient consult service, and the pulmonary hypertension service. Dr. Pugh has outpatient clinics where she sees a variety of general pulmonary diseases and pulmonary vascular disease.

She will be invited to attend weekly meetings with the PI and study personnel. She will be available to discuss adverse events with the PI and respond to safety concerns from subjects or study personnel.

9 Participant Safety and Confidentiality

9.1 Consent

Written consent will be obtained for enrollment for participants. For each consent process, study personnel will discuss the details of the study, the risks and benefits, and the subject's rights and responsibilities if they choose to participate in the trial and their right to refuse to participate. It will be made clear that their clinical care will not be affected by their decision. Subjects will be permitted to provide verbal consent over the phone prior to being scheduled for a screening visit. A consent script will be provided, and documentation of verbal consent will be noted. Written or e-consent will be obtained at the Visit 1 visit or before.

9.2 Safety and Adverse Event

9.2.1 Definitions of Adverse Events

9.2.1.1 **Unanticipated Problem (UP):** any incident, experience, or outcome that meets **all** of the following criteria:

1) unexpected (in terms of nature, severity, or frequency) given a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and b) the characteristics the subject population being studied;

2) related or possibly related to participation in the research (Possibly related to participation in the research means there is a reasonable possibility that the AE, experience, or outcome may have been caused by the procedures involved in the research); and

3) suggests that the research places subjects or others at a greater risk of harms (including physical, psychological, economic, or social harm) that was previously known or recognized.

9.2.1.2 **Adverse Event (AE):** Any symptom, sign, illness, or experience that develops or worsens in severity during the course of the study. Intercurrent illnesses or injuries should be regarded as AEs. Abnormal results of diagnostic procedures are considered to be AEs if the abnormality:

- Results in study withdrawal
- Is associated with a serious AE
- Is associated with clinical signs or symptoms
- Leads to additional treatment or to further diagnostic tests
- Is considered by the investigator to be of clinical significance

9.2.1.3 **Serious Adverse Event (SAE):** Adverse reactions are classified as serious or non-serious. A serious adverse event is any AE that is:

- Fatal
- Life-threatening
- Requires or prolongs hospital stay
- Results in persistent or significant disability or incapacity
- A congenital anomaly or birth defect
- An important medical event

Important medical events are those that may not be immediately life threatening but are clearly of major clinical significance. They may jeopardize the subject and may require intervention to prevent one of the other serious outcomes noted above. For example, drug overdose or abuse, a seizure that did not result in in-subject hospitalization or intensive treatment of bronchospasm in an emergency department would typically be considered serious. All AEs that do not meet any of the criteria for serious should be regarded as **non-serious AEs.**

9.2.1.4 **Suspected Adverse Reaction:** any adverse event for which there is a reasonable possibility that the drug caused the adverse event. For reporting purposes, “reasonable possibility” means there is evidence to suggest a causal relationship between the drug/investigational product and the adverse event.

9.2.2 Classifying AEs

9.2.2.1 Severity: the intensity of the AE is classified according to the CTCAEv4.0. Grade refers to the severity (intensity) of the AE: If the intensity of an AE worsens during study drug administration, only the worst intensity should be reported on the AE page. If the AE lessens in intensity, no change in the severity is required.

CTCAEv4 Grade 1: mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention is not indicated.

CTCAEv4 Grade 2: moderate; minimal, local, or noninvasive intervention is indicated; limiting to age-appropriate instrumental activities or daily living (ADL; instrumental ADL refers to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc).

CTCAEv4 Grade 3: severe or medically significant but not immediately life threatening; hospitalization or prolongation of hospitalization is indicated; disabling; limiting to self-care ADL (self-care ADL refers to

bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden)

CTCAEv4 Grade 4: life-threatening consequences; urgent intervention is indicated.

CTCAEv4 Grade 5: death due to an AE.

In this grading system, severity is not equivalent to seriousness. For example, a SAE would be any events which is life-threatening or disabling (Grade 4) or fatal (Grade 5) or was moderate-severe (Grade 2-3) and required or prolonged hospitalization.

9.2.2.2 Expectedness: AEs must be assessed as to whether they were expected to occur or were unexpected, meaning not anticipated based on current knowledge found in the protocol, investigator brochure, product insert, or label.

Expected: an AE known to be associated with the intervention or condition under study. OHRP defines an unexpected AE as any AE occurring in one or more subjects participating in a research protocol, the nature, severity, or frequency of which is not consistent with either:

1) the known or foreseeable risk of AEs associated with the procedures involved in the research that are described in a) the protocol-related documents, such as the IRB-approved research protocol, any applicable investigator brochure, and the current IRB-approved informed consent document, and b) other relevant sources of information, such as product labeling and package inserts; or

2) the expected natural progression of any underlying disease, disorder, or condition of the subject(s) experiencing the AE and the subject's predisposing risk factor profile for the AE.

9.2.2.3 Relatedness

1) Definite: the AE is clearly related to the research procedures

2) Probably: the AE is likely related to the research procedures

3) Possible: the AE may be related to the research procedures

4) Unlikely: the AE is doubtfully related to the research procedures

5) Unrelated: the AE is clearly not related to the research procedures

Possibly related to participation in the research: There is a reasonable possibility that the adverse event experience, or outcome may have been caused by the procedures involved in the research.

For each identified AE, an AE entry on the appropriate form will be completed using the above classifications as soon as possible, updating as necessary. Reporting procedures should be started immediately (within 24 hours) upon learning of a SAE or UP.

9.2.3 Interpretation of Definitions

9.2.3.1 AE and UP Reporting Period

The study period during which AEs must be reported is normally defined as the period from the initiation of any study procedures to the end of the study treatment follow-up. For this study, the study treatment follow-up is defined as 14 days following the last administration of study treatment.

9.2.3.2 Expedited Reporting Procedures

Any adverse event that occurs will be reported to Dr. Anna R. Hemnes, MD, or Dr. Evan Brittain, MD, within 24 hours of the event using a required form or as a written report of the event.

9.2.4 Subject withdrawal

A subject has the right to withdraw from the study entirely at any time for any reason without prejudice to future medical care by the investigator or other physician. The investigator also has the right to withdraw subjects from the study in the event of concurrent illness, AEs, or other reasons deemed to be in the subject's best interest. A subject should be withdrawn from the study if there is:

- Withdrawal of consent
- Termination of study
- PI determination that the subject should be withdrawn for safety.

In order to preserve the integrity of the intention-to-treat analysis, even if the subject is withdrawn from the treatment portion of the protocol (either due to subject, physician, or investigator decision), it is imperative to continue with the scheduled follow-up assessments both for the safety of the subject and for completeness of data collection. This will be explained to potential subjects at the time of informed consent. The importance of compliance with study visits will be reinforced throughout the trial.

9.3 Confidentiality

In this study, each patient will be assigned a unique Participant ID number (PID) when his/her demographic and race/ethnicity information is entered for the first time. Follow-up data are subsequently entered as needed when a patient has a clinic visit. The unique PID number remains with each patient permanently and is matched with all new data entered. The PID number and patient identifiers are directly linked in the study database.

Study personnel will also generate a Global Unique Identifier (GUID) for each subject using a NIH tool client. This is an identifying code assigned to a single research participant so that data can be compiled between research studies without using personally identifiable information (PII), even if the data are collected at different locations or by different studies. The GUID is created using PII (including, current name, legal given name given at birth (first, middle, and last), date of birth, city of birth, state of birth, country of birth, and physical sex at birth). Data including the GUID (without other identifiers) is considered

de-identified by the NIH and OHRP. Personal identifying information used to generate the GUID will be erased by the study staff after the GUID is created at the end of the study.

The potential for data sharing has been included in the informed consent. Data releases to investigators for approved research purposes and analyses (after review and approval by the Publications and Presentations Committee and approval by IRB and execution of a Data Use Agreement) will be stripped of identifiers using a "Safe Harbor" approach. If an approved investigator has conducted a separate study in which a shared participant has also consented to use of GUID then this will be retained in the data release; however, for all other data releases the GUID will be removed.

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Those regulations require a signed subject authorization informing the subject of the following:

- What protected health information (PHI) will be collected from subjects in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research subject to revoke their authorization for use of their PHI

9.4 Privacy

Consent forms, medical history data, and study data are stored in secured files, either in locked file cabinets or in a locked room separate from medical records and coded such that all subject identifiers have been removed. As an additional precaution all HIPAA regulated information is stored in an electronic file separate from other study data. Only approved study staff (determined by the PI) will be given authorization to access the database. Bio-specimens are processed and labeled with barcode labels that include the subjects electronically generated study code and date of sample collection. The bio-specimens are stored in locked freezers in the study Laboratory; only approved study staff has access to the keys for each freezer. Access to the electronic freezer inventory of the specimens is kept on a secure password protected computer.

9.5 Follow-up and Record Retention

The duration of this study is estimated to be 1 year. The duration of record retention will be at least 6 years after study completion, but the possibility exists for indefinite archival of study information via the REDCap database. Should the outcome of this study prompt future investigations, the participants may be contacted to obtain follow-up information and invited to participate in additional studies.

9.6 Ethical Considerations

This study is to be conducted in accordance with applicable US government regulations and international standards of Good Clinical Practice, and applicable institutional research policies and procedures.

This protocol and any amendments will be submitted to a properly constituted IRB in agreement with local legal prescriptions for formal approval of the study conduct. The decision of the IRB concerning the conduct of the study will be made in writing to the investigator and a copy of this decision will be provided to the NIH before commencement of this study.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subject to make an informed decision about their participation in this study. This consent form will be submitted with the protocol for review and approval by the IRB for the study. The formal consent of a subject, using the IRB-approved consent form, must be obtained before that subject undergoes any study procedure. The consent form must be signed by the subject or legally acceptable surrogate, and the personnel obtaining the consent.

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