Protocol A

An Open-Label Study of Oral Nitrite in Adults with Metabolic Syndrome and Hypertension

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TABLE OF CONTENTS

PROTOCOL SYNOPSIS

Protocol Title:	An Open-Label Study of Oral Nitrite in Adults with Metabolic Syndrome and Hypertension
Protocol Number:	PRO11030131
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Clinical Phase:	Phase II clinical investigation
Investigational Drug:	Nitrogen 14 Sodium Nitrite (^{14} N Sodium Nitrite)
Trial Site:	Single-Center Trial
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Clinical Laboratories:	UPMC Presbyterian University Hospital 200 Lothrop St.

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Study Rationale:	Preclinical and clinical research over the last decade has revealed the important vasoprotective effects of nitrites with regards to reduction in blood pressure, vascular inflammation and endothelial dysfunction. New findings suggest an effect of nitrite therapy in the regulation of glucose-insulin homeostasis. Development of an oral formulation of nitrite salts is attractive, whereby nitrite would ensure rapid acting effects upon absorption.
Study Objectives:	To investigate effects of an oral inorganic nitrite on the cardiometabolic and hormonal disturbances observed in a targeted population of overweight/obese adults with metabolic syndrome and hypertension, at risk for insulin resistance and endothelial dysfunction.
Study Hypothesis:	Oral supplementation with nitrite will result in improvement in insulin sensitivity, reduction in blood pressure, and improvement in endothelial and mitochondrial function, arterial stiffening, and microvascular inflammation, in overweight/obese adult subjects with metabolic syndrome and hypertension with limited toxicity in the dose proposed.
Study Aims:	1. To examine the effect of oral nitrite on insulin sensitivity and mitochondrial function in overweight/obese adults with metabolic syndrome and hypertension. 2. To examine the effect of oral nitrite on blood pressure in overweight/obese adults with metabolic syndrome and hypertension. 3. To examine the effect of oral nitrite on endothelial function, arterial stiffening, and microvascular inflammation in overweight/obese adults with metabolic syndrome and hypertension.
Study Design:	The study aims to assess the safety and feasibility of oral nitrite in adults with hypertension and metabolic syndrome. Subjects will undergo a screening visit to determine that eligibility requirements are met. Prior to receiving the study drug, subjects will be scheduled within 4 weeks of screening for a baseline assessment. Subjects who meet the Inclusion Criteria and none of the Exclusion Criteria will be provided study drug. The study population (n=30) will receive oral sodium nitrite for 12 weeks: **Drug**: oral formulation of sodium nitrite 40 mg three times daily or TID (once in the morning and again in the early afternoon and again in the evening).

	Subjects will be evaluated as an outpatient with the first dose of study drug. Blood pressure, heart rate, methemoglobin levels and plasma nitrate and nitrite will be measured in the first two hours after dosing. Every other week safety visits will occur to assess adverse events (AE), methemoglobin level, interval histories, brief physical exams, medication compliance review and dispensing of study drug. Interval histories and physical exams will be obtained by a study investigator. Subjects will also be contacted by the study investigator and/or the study coordinator for a follow-up assessment on Week 16 by telephone.
Planned Sample Size:	30 subjects
Duration of Treatment:	12 weeks
Major Inclusion Criteria:	• Age 18-60 years • BMI ≥ 30 kg/m^2 • Hypertension: defined as systolic blood pressure ≥130 and/or diastolic blood pressure ≥85 mm Hg at screening • Waist circumference: >102 cm in men, >88 cm in women
Major Exclusion Criteria:	• Positive urine pregnancy test or breastfeeding • Concurrent use of medications affecting glucose (oral hypoglycemics, insulin, atypical antipsychotics) • Recent addition or change in dosing of hormonal contraceptive medications (OCP, IUD, DepoProvera) • Current use of ≥3 anti-hypertensive agents regardless of blood pressure control or normotensive (SBP<130 and DBP<85 mmHg) on a single agent • Current use of PD5 inhibitors or organic nitrates • Not stable on treatments for the prior three months or not planning to remain on current dose of medications for blood pressure, contraception, etc. • Known chronic psychiatric or medical conditions including diabetes, liver or kidney disease or obesity syndromes • TSH >8 mIU/mL • Smoker • Hemoglobin <11 g/dL
Study Endpoints:	<u>Primary endpoint</u>: Primary outcome measure: change in insulin sensitivity (i.e. insulin stimulated glucose disposal) by hyperinsulinemic euglycemic clamp <u>Secondary endpoints</u>: Secondary outcome measures: 1. Change in systolic, diastolic and mean arterial pressures from baseline to 12 weeks

	2. Change in endothelial function by flow mediated dilation (FMD) 3. Change in muscle and platelet reactive oxygen species (ROS) generation, mitochondrial efficiency, and uncoupling in platelet and muscle biopsy sample studies 4. Change in body weight 5. Change in arterial stiffening by carotid-femoral pulse wave velocity (PWV) 6. Change in arterial (intimal) thickening by carotid intima-media thickness (IMT) 7. Change in HbA1C 8. Change in blood markers of inflammation, and vascular dysfunction: ICAM-1, VCAM-1, e-Selectin, CRP, PAI-1, IL-6, TNF-α, lipid profile, adiponectin, and leptin 9. Change in substrate oxidation by indirect calorimetry 10. Change in cardiorespiratory fitness by maximal exercise tolerance test 11. Change in body composition by whole body DEXA for total body fat 12. Safety monitoring with every other week methemoglobin by co-oximetry and plasma nitrate and nitrite concentrations by gas phase ozone-based chemiluminescence detection

1. OBJECTIVE, SPECIFIC AIMS, BACKGROUND, AND SIGNIFICANCE

1.1 OBJECTIVE

The objective of the study is to investigate effects of an inorganic sodium nitrite on the cardiometabolic and hormonal disturbances observed in a targeted population of overweight/obese adults with metabolic syndrome and hypertension, at risk for insulin resistance and endothelial dysfunction.

1.2 SPECIFIC AIMS

Hypothesis:
Oral supplementation with sodium nitrite will result in improvement in insulin sensitivity, reduction in blood pressure, and improvement in endothelial and mitochondrial function, arterial stiffening, and microvascular inflammation, in overweight/obese adult subjects with metabolic syndrome and hypertension with limited toxicity in the dose proposed.

Specific Aims:

Aim 1: To examine the effect of oral nitrite on insulin sensitivity and mitochondrial function in overweight/obese adults with metabolic syndrome and hypertension.

Aim 2: To examine the effect of oral nitrite on blood pressure in overweight/obese adults with metabolic syndrome and hypertension.

Aim 3: To examine the effect of oral nitrite on endothelial function, arterial stiffening, and microvascular inflammation in overweight/obese adults with metabolic syndrome and hypertension.

1.3 BACKGROUND

1.3.1. Insulin Resistance is a Key Link in the Metabolic Syndrome, Type 2 Diabetes & Cardiovascular Disease

Insulin resistance (IR) within muscle is a significant risk factor for the development of T2DM[1,2] and CVD[3]. Weight loss and exercise are effective treatments of IR[4], but compliance and adherence are typically poor. There are effective pharmacologic therapies, e.g., PPARγ receptor agonists (Rosiglitazone), but there are significant side effects. More effective targeted approaches to improve IR would therefore have a major impact on the prevention and treatment of both diabetes and CVD. The potential link between impaired mitochondria function and skeletal muscle IR has been extensively investigated and is disputed[5,6]. In addition, H_2O_2 emission from skeletal muscle mitochondria is an important marker of the balance between oxidative stress and redox state and is associated with IR independently of mitochondrial energy production[7].

1.3.2. Cardiovascular Disease and Inorganic and Dietary Nitrates and Nitrites

Cardiovascular disease remains the leading cause of death in the United States and worldwide[1]. While several large studies including the landmark Dietary Approaches to Stop Hypertension (DASH) study demonstrated that fruit and vegetable rich diets significantly reduced blood pressure[2-4] and reduced the risk of ischemic stroke[5] and cardiovascular disease in general[6], the exact mechanisms remain poorly understood. A new theory is

focusing on dietary nitrate and endogenous nitrite as major players in the cardiovascular protection seen in fruit and vegetable-rich diets. In humans, two major sources of nitrate and nitrite exist in the body: diet and endogenous production from the L-arginine-NO synthase pathway[7]. Green leafy vegetables and beetroots are rich dietary sources of nitrates (NO_3^-). When ingested, they are chemically stable endogenous reservoirs of nitric oxide (NO) and are reduced to nitrites (NO_2^-) by oral bacterial flora in the saliva. Next, the nitrites are swallowed entering the GI tract and accumulate via the enterosalivary circulation pathway. While the classical pathway of vasoprotective NO production predominates from oxidation of L-arginine by endothelial NOS (eNOS) activity, nitrites in the vasculature can also be reduced to generate NO via a reverse or "alternate" pathway under conditions of acidosis and hypoxia. This theory is evidenced by human studies and animal models demonstrating that inorganic and dietary nitrates and nitrites have robust NO-signaling effects. These effects include maintaining basal vascular tone, decreasing blood pressure (BP) by vasodilation, reducing vascular inflammation, preventing endothelial dysfunction and inhibiting platelet aggregation[8,9]. Very recent animal studies suggest nitrates and nitrates may also positively impact blood glucose and insulin sensitivity[10]. Thus, a novel therapeutic strategy targeting the cardiometabolic mechanisms underlying metabolic syndrome and hypertension may lie in the nitrate-nitrite-NO pathway.

1.3.3. Effects of Nitrite/Nitrate on Blood Pressure

Currently, several human studies have examined the effect of single ingestion[11,12], 6 day[13,14] and 15 day[15] supplemental courses of nitrate in the form of beetroot juice on blood pressure in healthy adults. Systolic[11-15] (SBP), diastolic[11,14,15] (DBP) and mean arterial pressures[11,14,15] (MAP) were significantly reduced with beetroot juice in these trials. A traditional Japanese diet is another rich source of dietary nitrates and short term evaluation of this diet in healthy Japanese adults decreased DBP 4.5 mmHg in comparison to a low nitrate control diet ($p=0.0066$) without a change in SBP[16]. Single administration of inorganic potassium nitrate[12] in various mmol concentrations or 3 days of sodium nitrate[17] significantly reduced SBP[12], DBP[12,17] and MAP[17] in healthy adults compared to placebo, with the magnitude of the blood pressure (BP) reduction related to the baseline BP[12]. Sex differences in the reduction of SBP ($p<0.01$) and DBP ($p<0.001$) were also identified and greater in males than females[12]. Single administration of oral and intravenous 0.12mmol sodium nitrite solution/mmol hemoglobin in healthy adults, equivalent to 290-380 mg sodium nitrite, resulted in a median decrease in MAP of 11 and 14 mmHg, respectively. A compensatory increase in HR of 10 and 18 bpm, respectively, was observed[18]. To our knowledge, the effect of longer courses of dietary or inorganic nitrate and nitrite supplementation on blood pressure, particularly in at-risk patients, has not been investigated.

1.3.4. Effects of nitrite/nitrate on glucose-insulin homeostasis

The PPARγ receptor is known to regulate glucose uptake, lipid metabolism/storage and cell proliferation/differentiation. Potential benefits of a particular drug in the class targeting this receptor, rosiglitazone, has been touted, but with an increased risk for adverse cardiovascular events[19]. In contrast, nitro-fatty acids, endogenous products of nitric oxide and nitrite-mediated redox reactions, were shown in a recent study to act as partial PPARγ receptor agonists with profiles distinct from rosiglitazone and activate the PPARγ receptor at nanomolar concentrations[10]. In the same paper, obese diabetic insulin resistant ob/ob mice were treated with nitro-fatty acids by subcutaneous pump for 4 weeks with reduction of insulin plus normalized blood glucose levels, ie. improvement of insulin sensitivity, without the side effect of weight gain seen with rosiglitazone. A recent animal study examined the effects of dietary supplementation of inorganic nitrate on various features of metabolic syndrome in mice lacking the gene for endothelial NO synthase (eNOS)[20]. These mice are obese, hypertensive, insulin resistant and have dylipidemia, much like humans with metabolic syndrome or a polymorphism in the eNOS gene. With chronic (10 weeks) nitrate treatment in eNOS -/- mice,

significant reductions in blood pressure, fasting blood glucose, HbA1C, proinsulin to insulin ratio, visceral fat, circulating triglycerides, and body weight were demonstrated. To our knowledge, however, no human data is available on the effects of inorganic nitrate or nitrite supplementation on insulin sensitivity, especially in an adult overweight/obese population with metabolic syndrome and hypertension.

1.3.5. Effects of nitrite/nitrate on endothelial function and vascular inflammation

One of the earliest pathophysiological responses to cardiovascular injury is a reduction in NO bioavailability. This has been implicated in the resulting endothelial dysfunction, inflammation, platelet activation, and smooth muscle proliferation and migration[9]. Recent evidence has suggested the alternate NO pathway involving nitrate and nitrite may attenuate arteriolar endothelial dysfunction and microvascular inflammation through increased NO bioavailability. Stokes and colleagues fed male C57 mice for 3 weeks with either a high fat or normal diet. Those mice on the high fat diet were supplemented with nitrite free or nitrite containing water. Nitrite supplementation with high fat feeding significantly increased plasma and tissue nitrite levels, inhibited leukocyte adhesion and emigration in venules and prevented impairment of endothelium-dependent vascular relaxation while MAP remained unchanged. Additionally, nitrite supplementation was associated with a reduction in triglycerides ($p<0.01$) and CRP without altering total cholesterol[9]. In human models, dietary nitrate in the form of beetroot juice[11,12] and inorganic nitrate supplementation[12] prevented I/R-induced endothelial dysfunction ($p<0.05$) assessed by FMD when compared to no treatment. Collectively, evidence from these animal models and human studies suggest a potential therapeutic role of exogenous nitrates and nitrites to enhance NO bioavailability, reduce microvascular inflammation, and preserve endothelial function.

1.3.6. Effects of nitrite/nitrate on mitochondrial function

Mitochondria are critical for oxidizing fuels (i.e., glucose, fatty acids, amino acids) for cellular energy production. There is also accumulating evidence that mitochondria dysfunction is associated with insulin resistance within skeletal muscle[21,22]. Larsen et al recently reported in healthy volunteers that dietary inorganic nitrate supplementation increased ATP synthesis in mitochondria isolated from muscle biopsy specimens concomitant with a reduced whole-body oxygen cost during steady-state exercise[23]. This suggests that dietary nitrate enhances the efficiency of energy generation through direct effects on muscle mitochondria. Taken together, these studies raise important questions regarding the potentially positive benefits of inorganic nitrate and nitrite supplementation on mitochondrial energy metabolism and links with insulin resistance and the metabolic syndrome. The current study will extend these observations significantly by examining the effects of dietary nitrate/nitrite supplementation on energy efficiency, mitochondria function, oxidative stress and insulin resistance in subjects with key features of insulin resistance and the metabolic syndrome.

1.4 SIGNIFICANCE

Important vasoprotective effects of inorganic nitrates and nitrites have been reported during the past decade, as evidenced by reduced BP, vascular inflammation and endothelial dysfunction. More recent findings suggest that inorganic nitrate and nitrite therapy may be involved in the regulation of glucose-insulin homeostasis. This proposal hypothesizes that oral supplementation with sodium nitrite will result in improvement in insulin sensitivity, reduction in BP, and improvement in endothelial and mitochondrial function, arterial stiffening, and microvascular inflammation, in overweight/obese adult subjects with metabolic syndrome and hypertension with limited toxicity in the dose proposed. First, we utilize a combination of powerful *in vivo* techniques (hyperinsulinemic euglycemic clamp, pharmacokinetic testing, muscle biopsy and platelet

analysis of mitochondrial function, cardiorespiratory fitness and various vascular function studies). Second, this is the first human study to investigate the inorganic nitrate or nitrite effects (in any form) on insulin sensitivity in any subject population. Additionally, this study is the first to design inorganic nitrite capsules for cardiovascular applications.

This study is an open-label, single-center non-randomized drug treatment study of overweight adults with metabolic syndrome and hypertension.

Adults (N=30) who fulfill the inclusion and exclusion criteria will be treated with 40 mg 14N sodium nitrite three times daily or TID (once in the morning and again in the early afternoon and again in the evening).

2. RESEARCH DESIGN AND METHODS

2.1 CLASSIFICATION AND METHODOLOGICAL DESIGNS

The study is a 12 week open-label, single-center, non-randomized drug treatment study of overweight adults with metabolic syndrome and hypertension.

2.2 DETAILED DESCRIPTION OF STUDY DESIGN

Potential subjects will undergo a screening visit to determine that eligibility requirements are met prior to being enrolled in the study. Subjects who meet the Inclusion Criteria, none of the Exclusion Criteria, and prior to receiving the study drug will be scheduled within 4 weeks of screening for baseline assessments. The study population (n=30) will receive oral sodium nitrite as follows:

Drug: oral formulation of sodium nitrite 20 mg or 40 mg capsules, for a dose of 40 mg three times daily or TID (once in the morning and again in the early afternoon and again in the evening).

Subjects will complete a 12-week outpatient treatment period. During the treatment period, subjects will be evaluated at weekly intervals. Throughout the study period, subjects will be asked to remain diligent in recording the exact date and time of study drug dosing with any associated symptoms.

2.2.1. Study Drug Preparation and Distribution

The nitrite formulation will be prepared at and obtained from the Investigational Drug Pharmacy Service at the National Institutes of Health (NIH-IDS). The University of Pittsburgh Medical Center Investigational Drug Pharmacy Service (UPMC-IDS) will be utilized for dispensing of the study drug.

The daily study drug is:

^{14}N Sodium nitrite

Standard sodium nitrite will be supplied as 20 or 40 mg capsules for three times daily oral administration at the dose strength of 40 mg.

2.2.2. Dose Selection

The Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) proposed an Acceptable Daily Intake (ADI) for nitrate in 1990 based on harmless daily intakes of ≤500 mg of sodium nitrate/per kg of body weight in rats

and dogs. They divided this dose by an uncertainty factor of 100 to yield an ADI of 5 mg of sodium nitrate or 3.7 mg nitrate ion per kg of body weight. An ADI for nitrite ion was derived and revised in 2003 based on intakes of 10 mg of sodium nitrite or 6.7 mg nitrite ion per kg of body weight in rats. They divided this dose by an uncertainty factor of 100 and rounded to yield an ADI for sodium nitrite of 0.1 mg/kg of body weight[24].

Using the ADI for sodium nitrite (0.1 mg/kg of body weight) and a body weight of 95 kg, the calculated ADI of sodium nitrite would be 9.5 mg.

A recent modeling of the fruit and vegetable consumption patterns of The Dietary Approaches to Stop Hypertension (DASH) diet was done choosing foods that were low or high in nitrate. The "high nitrate" DASH diet pattern would result in consumption of 1,222 mg nitrate ion and 0.351 mg nitrite ion daily. The "low nitrate" DASH diet would result in consumption of 174 mg nitrate ion and 0.41 mg nitrite ion daily. The hypothetical high-nitrate DASH diet pattern exceeds the WHO's ADI for nitrate ion by 550% for a 60-kg adult[26]. Subjects on this diet exhibited lower blood pressure with no adverse effects.

In a recent study[12], single dose potassium nitrate (KNO_3) capsule ingestion in healthy adult volunteers in doses of 2,424 mg KNO_3 (or 1,488 mg nitrate) and 1,212 mg KNO_3 (or 744 mg nitrate) resulted in dose dependent reductions in BP over a 24 hour period without adverse effects.

Hunault et al. reported an open-label pharmacokinetic crossover study in 9 healthy adult subjects who received two single high dose oral sodium nitrite solutions equivalent to 290-380 mg and 140-190 mg sodium nitrite, respectively, and one intravenous sodium nitrite dose of 290-380 mg[18]. Mild headache occurred in 44-55% and up to 22% experienced nausea, which subsided within half an hour during each treatment session. Lowered blood pressure accompanied by increased heart rate were observed after each higher dose treatment, whereas the lower dose did not induce notable changes.

Chronic dietary supplementation of inorganic sodium nitrate in obese, hypertensive, insulin resistant mice lacking the gene for endothelial NO synthase (eNOS)[20] was studied. With a 10 week treatment of a modest dose of sodium nitrate of 0.1 mmol/kg/day (8.5 mg/kg/day) in the drinking water, significant reductions in blood pressure, fasting blood glucose, HbA1C, and proinsulin to insulin ratio were demonstrated, indicating reduced demands on the β-cell for insulin secretion.

Sodium nitrate drug is considered a source of nitrite through salivary nitrate reduction (rise in plasma nitrite after single oral sodium nitrate dose - see attached Fig 2A attached). Despite the plasma nitrite rise following a single oral sodium nitrate dose, we recently demonstrated in our phase I PK/PD study in 10 normal volunteers that no persistent blood pressure reduction with sodium nitrate 1,000 mg (see attached Fig 3A). In contrast, in the same phase I PK/PD study in 10 normal volunteers, we showed blood pressure decline in systolic, diastolic and mean arterial pressures over 3 hours with sodium nitrite 20 mg (Fig 3B). The oral sodium nitrite half-life was about 40-minutes and the effects of nitrite only lasted ≤6 hours. This was demonstrated by the plasma nitrite levels returning to baseline levels between 3 and 6 hours after nitrite dosing (i.e. the drug was given and gone out of the circulation within 6 hours). Specifically, mean baseline (pre-nitrite dosing) blood nitrite concentrations were 0.11 micromolar with a peak at 30 minutes of 5.5 micromolar, and return to baseline levels between the 3- (0.18 micromolar) and 6-hour (0.11 micromolar) monitoring time points (Fig 2B). In addition, there were no serious adverse events when these normotensive individuals received sodium nitrite including our 2 safety endpoints of methemoglobin levels and mean arterial pressure.

Additionally, we demonstrate lack of blood pressure lowering in our first 5 subjects on this current protocol's combined regimen of sodium nitrate 1,000 mg daily and sodium nitrite 20 mg BID during 2-hour post-dose BP monitoring at baseline vs. 12 weeks post (see attached Fig 1A), weekly seated blood pressures (Fig 1B) or 24-hour ambulatory blood pressures (ABP) at baseline vs. 12 weeks post (Fig 1C) and possible concern the sodium load within the 1,000 mg sodium nitrate dose may be blunting any potential BP effect.

Next, a recently published study (Mohler III ER, Hiatt WR, Gornik HL, Kevil CG, Quyyumi A, Haynes WG, Annex BH. Sodium nitrite in patients with peripheral artery disease and diabetes mellitus: safety, walking distance and endothelial function. Vascular Medicine 2014; 19(1):10-17) randomized adults 1:1:1 to oral sodium nitrite 40 mg BID vs. 80 mg BID vs. placebo for 10 weeks in >50 adults (age 35-85 years) with peripheral arterial disease +/- diabetes. The dose was escalated after the 10 weeks for 1 additional week in all 3 groups (dose-doubling) to 80 mg BID vs. 160 mg BID vs. doubling of placebo, respectively. The dosing was well tolerated, especially in the 40 mg BID and 80 mg BID randomized dosings. Our proposed study dosing of 40 mg TID (120 mg daily) falls in between the above two total daily doses of 80 mg and 160 mg daily. Safety was monitored throughout all aspects of the study. No serious adverse events occurred in the two sodium nitrite groups. There were no imbalances in GI, musculoskeletal, respiratory, vascular or skin disorders nor shifts in liver function or creatinine levels. Pertinent safety outcomes (also measured in our study) revealed that methemoglobin changes never exceeded 3% in any nitrite dose regimen and no clinically significant change in resting blood pressure.

Finally, a current phase II study of inhaled nitrite in patients with pulmonary arterial hypertension (PAH) at the University of Pittsburgh uses a dose of 80 mg, and is approved by the University of Pittsburgh IRB and FDA. Also, phase II studies went as high as 90 mg three times a day, approved by the FDA. So, our dose of 20 mg or 40 mg TID will not be out of line of approved dosing.

Based on the above PK and the differential BP effects with single doses of sodium nitrate vs. nitrite, the published 10-week oral nitrite trial and preliminary findings in this combined trial, we propose to remove sodium nitrate, which is the second potential depot of nitrite. Therefore, we need to administer a greater nitrite load since the previous two sources combined (sodium nitrate and nitrite) in the first 5 subjects of this study did not result in any BP lowering (Fig 1A-C). Similar to our prior dosing modification (when we titrated from sodium nitrite 20 mg once daily to 20 mg twice daily), we propose a step-up dosing in the first 3 subjects enrolled whereby we will begin with sodium nitrite 20 mg TID daily and after 2 weeks on this dosing and if no SAE, increase to 40 mg TID for the remaining 10 weeks of the study, given the PK of the drug and to induce blood pressure lowering in our hypertensive subjects. After three subjects have completed this regimen, then we propose that from the start, all remaining subjects start on sodium nitrite 40 mg TID and continue for all 12 weeks.

Although we are increasing the dose/frequency of sodium nitrite, since we are discontinuing sodium nitrate (which is reduced to nitrite in our body as was confirmed in our Phase I PK study), we are continuing our in-person safety monitoring visits to assess for adverse events at every other week. We will continue alternating every other week phone visits to assess compliance and for adverse events. Our two safety outcomes are methemoglobin by non-invasive co-oximetry and mean arterial pressure (MAP) obtained through 3 automated resting BP measurements 30-45 min following morning nitrite dosing and averaging the final 2 of 3 MAPs.

Based on the Joint FAO/WHO ADI for sodium nitrate and nitrite, the above DASH diet patterns, and the recently observed human and rodent effects of nitrate and nitrite on blood pressure and

glucose homeostasis, including our PK/PD trial of single doses of oral nitrate and nitrite, the existing inhaled nitrite trial, and this open label trial safety and lack of BP reduction findings following a total of 5 subjects' completion, the study investigators believe that a safe and likely therapeutically effective dose of sodium nitrite will be 40 mg three times daily (~1.26 mg/kg body weight per day for a 95 kg subject). Our sodium nitrite dose selection is equivalent to 12.6 times the ADI.

2.2.3. Treatment Period
Subjects will undergo study treatment duration of 12 weeks of ^{14}N sodium nitrite three times daily. This is considered appropriate to demonstrate the potential effects of nitrite on the cardiometabolic and hormonal disturbances in overweight/obese adults with metabolic syndrome and hypertension.

2.2.4. Safety Monitoring:
Subjects will be evaluated with outpatient two hour post-dose safety assessments at baseline and at 12 weeks following completion of the 12-week treatment. Every other week safety visits will occur to assess adverse events (AE) and interval histories in the Endocrinology and Metabolism Research Center (EMRC) at Montefiore Hospital) for visits 6, 8, 10, 12 and 14. Visits 5, 7, 9, 11, 13 and 15 will occur by phone. The in person safety visits will include brief physical exams, methemoglobin level, interval histories medication compliance review, symptom review and dispensing of study drug. The phone visit will include symptom review, assessing adverse events and interval histories.

In addition to these formal evaluations, subjects will be encouraged to immediately contact the study investigator and/or the study coordinator with questions, concerns, or to report new symptoms that occur during their study participation. If there are particular concerns that need addressed sooner than the every other week in-person visit, the subject will be asked to return to the EMRC as soon as possible for evaluation. If appropriate, based upon the evaluation, medical treatments will be provided to subjects, including appropriate referral to physicians or other services at the UPMC.

2.2.5. Medication Compliance
Subjects will self-administer their first dose of study medication under supervision of the physician investigator in the outpatient CTRC during Day 1 (Visit 4- First Drug Dose visit), following completion of all baseline clinic assessments. Subjects will also be dispensed their study medication for home use. Subjects will return the previously dispensed study medication at each every other week study visit thereafter, and medication compliance will be assessed. The study investigator and/or the study coordinator will review the daily diary. In the event that the compliance rate is < 80%, subjects will be re-educated on medication compliance. If medication compliance repeatedly falls outside of the acceptable range, the study investigators will discuss subject eligibility for continued participation in the study.

2.2.6. Medication Accountability
The study investigators or the study coordinator will document the amount of study drug dispensed from UPMC-IDS. The study drug accountability records will be maintained at UPMC-IDS throughout the course of the clinical trial.

2.3 STUDY ASSESSMENT

The complete study assessment and procedures are outlined in Table 1. During the treatment period, a variance of 7 days will be allowed to accommodate for scheduling needs.

2.3.1. Screening Procedures

Visit 1 (Screening)

The outpatient fasting screening will take place in the Montefiore Clinical and Translational Research Center (CTRC) or the Endocrinology Metabolism Research Center (EMRC) at the UPMC Montefiore Hospital. Subjects will be asked to fast for 8 hours before coming in for the screening blood work.

- Obtain written informed consent.
- Obtain waist and hip circumferences.
- Complete history and physical examination to include body weight, height, and menstrual history in females.
- Metabolic syndrome screening blood draw: triglycerides, HDL and fasting glucose.
- TSH level to rule out uncontrolled hypothyroidism (TSH >8 mIU/mL)
- Hemoglobin and hematocrit to assess for anemia (hemoglobin <11.0); If the value is within IRB guidelines, the subject may proceed with full protocol implementation. If the hemoglobin is less than required, a stat CBC will be sent to chemistry. If the central lab value is within range or the subject is determined to have thalassemia trait per the IRB "Blood Withdrawal Guidelines for Research Subjects", the subject may proceed with full protocol implementation.
- Blood pressure and other vital signs.

Screening evaluations will include triglycerides, HDL, glucose, TSH, and hemoglobin and hematocrit by the Presbyterian Hospital clinical automated testing lab and are to be covered by research funds. The total volume of blood drawn is approximately 1 Tablespoon (15 mL). If laboratory results exclude elevated TSH (>8 mIU/mL) and low hemoglobin (<11.0 g/dL) and blood pressure measurement confirms hypertension, the subject will be called to arrange the baseline assessments.

Subjects who fail to meet applicable inclusion/exclusion criteria based upon the results of the screening assessment will be excluded from further study participation.

Visit 2 (First Baseline Assessment Visit)

Subjects will be scheduled for an outpatient fasting Vascular CTRC and EMRC visit within 4 weeks of screening. Subjects will be asked not to drink or eat anything other than plain water for 8 hours prior to their arrival for this visit.

- Body weight
- Pulse Wave Velocity (PWV)
- Flow Mediated Dilation (FMD)
- Carotid Intima-Media Thickness (IMT)
- Urine pregnancy test in females of child-bearing potential (i.e. women who are not at least 1 year post-menopausal or who have not undergone a surgical sterilization procedure)
- Light breakfast snack in CTRC
- Whole body DEXA scan
- Exercise testing

- 24-hour ambulatory blood pressure monitor (ABPM) placement *This may also be placed at Visit 3 if a monitor is not available when a participant comes in for Visit 2.*
- AE assessment

Visit 3 (Second Baseline Assessment Visit)
Visit 3 involves a percutaneous muscle biopsy. To prevent acute effects of exercise on muscle mitochondria function, the visit will occur 2-7 days after Visit 2. This visit will take place at the MUH-CTRC. Subjects will be asked not to drink or eat anything other than plain water for 8 hours prior to their arrival for this visit.

- History and physical examination including vital signs, body weight, and menstrual history in females
- Urine pregnancy test in females of child-bearing potential (i.e. women who are not at least 1 year post-menopausal or who have not undergone a surgical sterilization procedure)
- Fasting blood draw to include: estradiol level in females sent to Esoterix; HbA1C, CBC, creatinine, AST and ALT and lipid profile sent to Presbyterian Hospital clinical automated testing lab; research blood for ICAM-1, VCAM-1, e-Selectin, CRP, adiponectin, leptin, uric acid, PAI-1, IL-6, TNF-α, and platelet sample (approximately 3 Tablespoons of blood)
- 72-hour dietary food recall
- Indirect calorimetry
- Percutaneous muscle biopsy
- Hyperinsulinemic euglycemic clamp (22 Tablespoons of blood)
- Meter blood sugar, blood pressure, breathing rate, and heart rate will be performed 30 minutes after completion of the clamp test to make sure they are returning to baseline before discharge home.
- Regular meal after completion of clamp
- Return blood pressure monitor and cuff for download
- AE assessment

The total volume of blood drawn is approximately 25 Tablespoons (375 mL).

2.3.2. Treatment Period and Monitoring

Visit 4 (Day 1: First Drug Dose Visit)
Administration of the first dose of study drug will occur within 1-4 weeks following completion of all baseline assessments. Subjects will be asked not to drink or eat anything other than plain water for 8 hours prior to their arrival for this visit. The fasting visit will take place in the outpatient CTRC.

- Body weight and temperature in all subjects and menstrual history in females
- Urine pregnancy test in females of child-bearing potential (i.e. women who are not at least 1 year post-menopausal or who have not undergone a surgical sterilization procedure)
- Subjects will receive their first dose of study drug as follows:
  - ^{14}N sodium nitrite 40 mg
- Blood pressure, MAP and heart rate will be measured in triplicate after approximately 30 minutes of rest and quiet in the CTRC to ensure blood pressures are at a steady state before taking the study drug (baseline) and then every 15 minutes for the 2 hours post-drug administration
- Methemoglobin level by finger probe at 0 (trough prior to sodium nitrite), 0.5, 1 and 2 hours post-drug administration

- Research blood samples for: plasma nitrate and nitrite concentrations, red cell iron-nitrosyl-hemoglobin and platelet studies at 0 (trough), 0.5 and 2 hours post-drug administration
- AE assessment
- Light breakfast snack
- Provide dosing diary cards and a 11-17 day supply of study drug so there is enough daily drug to get them to their next scheduled Visit 6
- Return completed 72 hour dietary recall forms

The total volume of blood drawn is approximately 3 Tablespoons (or 48 mL).

There is a rare possibility that a subject will have a high methemoglobin level or will have a low blood pressure from their study drug dose during this visit or future study visits. If this happens, their study nitrite drug dose will be lowered so that they receive half the dose of the nitrite (20 mg sodium nitrite three times daily). They will be asked to undergo a repeat of safety monitoring for blood pressure, breathing rate and heart rate before taking the medicines and then every 15 minutes for 2 hours after taking the study medications, methemoglobin level by a finger probe, research blood and storage samples and blood samples for platelet mitochondrial function that will be drawn before, 0.5, 1 and 2 hours after the lowered dose of study medication are administered (as above). The total volume of blood drawn is approximately 3 Tablespoons. If they still have a high methemoglobin or low blood pressure on the lower dose, their participation in the study will be discontinued.

Visits 5-15 (Weeks 1 through 11):

Participants will have every other week in-person EMRC safety visits and phone visits on alternate weeks, starting with week 1 (Visit 5) if no dose limiting adverse events occur (as defined in protocol based on MAP and MetHb levels). Phone visits will take place on Visit 5, 7, 9, 11, 13 and 15.

During Visits 6, 8, 10, 12, and 14 (Weeks 2, 4, 6, 8, and 10 on study drug), the subjects will return every other week ± 7 days to the EMRC in Montefiore Hospital of UPMC for a safety visit, which will take approximately 1-1 ½ hours for:

- Subjects will be seated in the EMRC and given their daily dose of morning study drug or second/midday dose (depending on time of scheduled visit)
- Brief physical exam
- 0.5 hours after taking their study drug, 3 automated blood pressure, HR and MAP measurements will be taken by the subject with 5 minutes intervals between measurements
- Assess adverse events (AE) and medication compliance
- Obtain methemoglobin by co-oximetry and research blood for plasma nitrate and nitrite levels and a sample for storage
- Provide a 14 +/- 3 day supply of study drug

During Visits 5, 7, 9, 11, 13, and 15 (Weeks 1, 3, 5, 7, 9, and 11 on study drug or every other week), we will contact the subject by phone for a safety visit to assess interval histories and assessment of adverse events (AE), which will last about 10 minutes.

The total volume of blood drawn at the in-person safety visits is approximately 1 1/8 Tablespoons (or 16 mL). At the Week 6 (Visit 10) visit, body weight, and menstrual history in females and urine pregnancy test in females of child-bearing potential (i.e. women who are not at least 1 year post-

menopausal or who have not undergone a surgical sterilization procedure) and a blood sample for platelet studies will be performed. Total blood volume for Visit 10 is about 1 2/3 Tablespoons (or 24 mL).

At Week 10 (Visit 14), a hemoglobin and hematocrit will be drawn to screen for anemia. If hemoglobin is less than 11 g/dL, iron supplementation (ferrous sulfate 325 mg twice daily) will be recommended until study completion at Visit 18. A repeat hemoglobin and hematocrit will be repeated at Visit 18. If hemoglobin is > or = 11 g/dL, participation in this visit will occur. If hemoglobin <11 g/dL, continuation of iron supplementation will occur and the visit will be rescheduled within the permitted 7 day scheduling variance when the hemoglobin and hematocrit will again be repeated. Total blood volume for Visit 14 is about 1 1/3 Tablespoons (or 20 mL).

Visit 16 (Week 12: First Completion Assessment Visit)
Subjects will be scheduled for an outpatient fasting CTRC and Vascular CTRC visit for:

- Body weight in all subjects and menstrual history in females
- Waist and hip circumference measurements
- Pulse Wave Velocity (PWV)
- Flow Mediated Dilation (FMD)
- Carotid Intima-Media Thickness (IMT)
- Urine pregnancy test in females of child-bearing potential (i.e. women who are not at least 1 year post-menopausal or who have not undergone a surgical sterilization procedure)
- Whole body DEXA scan
- Exercise testing
- Light breakfast snack
- 24-hour ambulatory blood pressure monitor placement*This may also be placed at Visit 17 if a monitor is not available when a participant comes in for Visit 16.*
- 72-hour dietary food recall
- Assess AEs, review diary cards and medication compliance
- Provide remaining supply of study drug until scheduled Visit 18 Third Completion Assessment Visit (Week 12).

Visit 17 (Week 12: Second Completion Assessment Visit)
This fasting visit will take place at the CTRC or EMRC. Subjects will be asked not to drink or eat anything other than plain water for 8 hours prior to their arrival for this visit.

- Body weight and temperature will be measured in all and menstrual history in females
- Urine pregnancy test in females of child-bearing potential (i.e. women who are not at least 1 year post-menopausal or who have not undergone a surgical sterilization procedure)
- Subjects are asked to bring their morning dose of study drug to this visit for dosing as follows:
    - ^{14}N sodium nitrite 40 mg
- Blood pressure, MAP, and heart rate will be measured in triplicate after approximately 30 minutes of rest and quiet in the CTRC to ensure blood pressures are at a steady state before taking the study drug (baseline) and then every 15 minutes for the 2 hours post-drug administration
- Methemoglobin level by finger probe at 0 (trough prior to sodium nitrite), 0.5, 1 and 2 hours post-drug administration
- Research blood samples for plasma nitrate and nitrite concentrations, red cell iron-nitrosyl-hemoglobin and platelet studies at 0 (trough), 0.5 and 2 hours post-drug administration

- Assess for AEs during study testing
- Standard meal following completion of 2 hour post-drug administration blood draw
- 72-hour food recall forms will be collected if already completed
- Return blood pressure monitor and cuff for download

The total volume of blood drawn is approximately 4 Tablespoons (or 48 mL).

Visit 18 (Week 12: Third Completion Assessment Visit)
This visit involves a percutaneous muscle biopsy. To prevent acute effects of exercise on muscle mitochondria function, the visit will occur 2-7 days after Visit 16 (the exercise visit). Subjects will be asked not to drink or eat anything other than plain water for 8 hours prior to their arrival for this visit.

- History and physical examination, weight, and vital signs
- Urine pregnancy test in females of child-bearing potential (i.e. women who are not at least 1 year post-menopausal or who have not undergone a surgical sterilization procedure)
- Fasting blood draw to include basic labs of estradiol level (in females), HbA1C, and lipid profile; research blood for ICAM-1, VCAM-1, e-Selectin, CRP, adiponectin, leptin, uric acid, PAI-1, IL-6, TNF-α and a sample for storage (approximately 3 Tablespoons of blood)
- Subjects are asked to bring their morning dose of study drug to this visit for dosing prior to start of indirect calorimetry
- Indirect calorimetry
- Percutaneous muscle biopsy
- Hyperinsulinemic euglycemic clamp (22 Tablespoons of blood)
- Meter blood sugar, blood pressure, breathing rate, and heart rate will be performed 30 minutes after completion of the clamp test to make sure they are returning to baseline before discharge home.
- Regular meal after completion of clamp
- Medication compliance review
- AE assessment
- 72-hour food recall forms will be collected if not already returned
- All remaining study drug will be collected since the last dispensing of drug earlier in the week (at Visit 16)

The total volume of blood drawn is approximately 25 Tablespoons (370 mL).

2.3.3. End of Trial and Follow-Up Period

Visit 19 (Week 16: Post Study Drug Telephone Assessment)

- Telephone assessment for interval histories and AEs.

Table 1. Study Assessment and Procedures

	Screening	Baseline			Treatment Period and Monitoring														FU
Visit	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Week of Treatment	**-**	**-4~-1**	**-4~-1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**12**	**12**	**16**
Day of Treatment	**-**	-28~-7	-28~-7	1	8	15	22	29	36	43	50	57	64	71	78	85	86-91	87-92	113
Time Window			V2 +2-7d		±7	±7	±7	±7	±7	±7	±7	±7	±7	±7	±7	±7		V16 +2-7d	±3
Visit Type	OP	OP	OP	OP	Phone	OP	Phone	OP	Phone	OP	Phone	OP	Phone	OP	Phone	OP	OP	OP	Phone
Informed Consent / Eligibility Criteria	x																		
History & Physical Exam	x																		
Body Weight	x	x	x	x						x						x	x	x	
Waist and hip circumference	x															x			
Vital signs	x	x	x			x		x		x		x		x		x		x	
Menstrual History (in females)	x		x							x								x	
Screening Laboratory Profile	x																		
Urine Pregnancy Test (in females)		x	x	x						x						x	x	x	
Methemoglobin %				x		x		x		x		x		x			x		
Estradiol Level (in females)			x															x	
Research Labs			x	x		x		x		x		x		x			x	x	
Two Hour Safety Blood Sampling				x													x		
Two Hour HR, MAP, and BP Monitoring				x													x		
Hemoglobin & hematocrit														x					
Food Questionnaire			x													x			
Whole body DEXA Scan		x														x			
Pulse Wave Velocity		x														x			
Flow Mediated Dilation		x														x			
Carotid Intima-Media Thickness		x														x			
24-hour ABPM		x														x			
Hyperinsulinemic Euglycemic Clamp			x															x	
Indirect Calorimetry			x															x	
Muscle Biopsy			x															x	
Exercise Testing		x														x			
Meal/snack		x	x	x												x	x	x	
AE Assessment		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Medication Compliance Review					x	x	x	x		x		x		x		x		x	
Interval History Assessment			x		x	x	x	x	x	x	x	x	x	x	x	x		x	x
Brief physical exam			x		x	x	x	x		x		x		x				x	

2.4 STUDY PROCEDURES

2.4.1 Insulin Sensitivity Assessment

Euglycemic-insulin clamp

A 4-hour hyperinsulinemic (40 mU/m^2/min) -euglycemic clamp will be performed at baseline (Visit 3) and Week 12 (Visit 18) to evaluate *in vivo* insulin action in stimulating glucose disposal, oxidation, and nonoxidative disposal as described before[25]. Briefly, HumuLIN® R regular insulin will be infused at 40 mU/m^2/min. Plasma glucose will be clamped between 85-95 mg/dL with a variable rate infusion of 20% dextrose in water. The rate of glucose infusion will be adjusted based on arterialized plasma glucose measurements every 5 minutes. Rates of glucose metabolism will be determined using a non-radioactive glucose isotope dilution method based on a primed, then constant infusion of the stable isotope of D-glucose [6,6-D2] 98+% enriched (0.22 µmol•kg-1, 17.6 µmol•kg-1 prime). This will be continued during the insulin infusion to determine rates of systemic glucose utilization (peripheral insulin resistance) and to determine insulin suppression of hepatic glucose production (hepatic insulin resistance). The deuterium labeled glucose isotope chemical purity is provided by Isotec, Inc and is tested for sterility and pyrogenicity by the manufacturer before being shipped. The maximum amount of blood to be drawn during this test is 22 Tbsp (325 mL).

Basal substrate turnover

At the fasting/baseline and end of the euglycemic clamp, basal postabsorptive glucose and fat turnover will be evaluated using a primed-continuous intravenous infusion of [6,6-D2]glucose[25]. Substrate oxidation will be measured by continuous indirect calorimetry (Parvomedics TrueOne, Sandy, UT).

2.4.2. Systemic Arterial Vascular Function Assessments

Prior to when vascular measurements begin, subjects will be asked to abstain from tobacco and caffeine and only to have a light breakfast that is low in fat. The subject's height and weight will be measured and BMI calculated. The studies will be performed at baseline (Visit 2) and at Week 12 (Visit 16) in a quiet, temperature-controlled clinic room in the Vascular Clinical and Translational Research Center (CTRC) at Montefiore University Hospital. The subject will be asked to rest for approximately 10 minutes before the testing so that they are in a relaxed state.

Pulse Wave Velocity (PWV)

Pulse wave velocity will be performed non-invasively using SphygmoCor machine (AtCor Medical, Sydney, Australia. Model MM3. Software version 7.1) equipped with a single tonometric transducer SPT-304.

Blood pressure, pulse, and oxygen saturation will be measured on the same side as the peripheral pulse wave sampling using an automated digital oscillometric device and an average of three readings will be recorded.

Transit distances for PWV will be assessed by surface measurements from the suprasternal notch to each pulse recording site (carotid and femoral arteries). Three ECG leads will be attached on the chest. Participants will be studied in the supine position after 10 minutes of rest. Tests will be performed in duplication and the study will take 30 minutes to conduct.

Blood velocity will be calculated from the pulse wave time difference, simultaneous ECG recording and distance between the two locations with the integral software. The results will be reported as

the Pulse Wave Velocity (V=distance/time), which are a composite measure of the magnitude of pulse pressure and arterial stiffness in all the conduit arteries which affects wave reflection.

Higher values indicate increased wave reflection from the periphery due to increased pulse wave pressure at stiffer arteries. Those techniques have been validated for their reproducibility and used extensively.

Carotid Intima-Media Thickness (IMT)

For assessment of early stages of atherosclerosis and cardiovascular risk, the carotid artery wall is examined to identify thickness of arterial wall constitute. Dedicated ultrasound imaging of the arterial wall produces double echogenic lines. Intima-media thickness (IMT) measurement is an averaged distance between two parallel lines representing the luminal intima and media-adventitia interface. Measurements are performed approximately two centimeters proximally to bifurcation divider at the posterior (far) vessel wall using automated edge detection software.

An ultrasound machine GE VIVID-7 (GE Healthcare, Milwaukee, WI), equipped with the high resolution 8L linear transducer will be used for imaging. Three ECG leads will be attached on the chest. A dedicated IMT setting and protocol will be used for scanning. Imaging will start with transversal carotid arteries sweep for general assessment. Longitudinal B-mode ultrasound cine-loop (5 beats) of the distal common carotid artery, optimally with bifurcation, will be taken bilaterally. Horizontal image with double lines at the end of T wave will be selected. Measurement sample box will be placed approximately two centimeters proximally to bifurcation at the posterior (far) vessel wall. Semi-automated edge detection software, which traces the luminal intima and hypoechoic boundary between media and adventitia, calculates the distance between them in fast, accurate and repeatable manner. Averaged IMT values from each side in mm will be reported. The study will take 15 minutes to conduct.

Flow Mediated Dilation (FMD)

The assessment of endothelial function via Flow Mediated Dilation (FMD) has been proposed to represent a functional bioassay for endothelium - derived NO bioavailability in humans. Vascular reactivity will be quantified noninvasively using vascular ultrasound imaging. Flow and diameter changes of the brachial artery will be assessed with Duplex ultrasound images captured by a high-resolution linear array transducer.

Blood pressure will be measured on the control arm using an automated blood pressure monitor (Model CONTEC08A) and an average of three readings will be recorded. Systolic BP <100 mmHg will be considered as cut off for performing the study.

A long 5 cm wide occlusive cuff (Hokanson SC 5) with rapid release sphygmomanometer (Hokanson DS400) will be attached to an upper forearm approximately 5 cm below an elbow. Three ECG leads will be attached on the chest and lower forearms to capture ECG. Participants will be studied in the supine position after 10 minutes of rest.

Brachial artery diameter and flow velocities will be examined by high resolution ultrasound machine GE VIVID-7 (GE Healthcare, Milwaukee, WI), equipped with the 9-L linear transducer preset on specifically dedicated scanning protocol.

Cine loop 2D baseline images at B mode (15 cycles) will be obtained at the highest optimal resolution following with spectral velocity Doppler imaging (20 cycles) with scale set approximately at 200 cm/s and sweep speed at 12.5 mm/s. The occlusion cuff will be manually

inflated to 200 mmHg (or 50 mmHg above SBP) for 5 minutes and occlusion will be controlled by spectral Doppler mode. Endothelium-dependent flow mediated vasodilation of the artery will be captured in Duplex mode immediately after the cuff deflation by spectral Doppler waveform (20 cycles) approximately at 200 cm/s and sweep speed at 12.5 mm/s. Cine loop 2D vasodilation images at B mode (15 cycles) will be captured through 3 minutes after deflation during hyperemic period. Heart rate and blood pressure will be measured once by automated blood pressure monitor (Model CONTEC08A) after testing.

Images will be saved at ultrasound machine hard drive and transferred in archive format for analysis on separate equipment. The study will take 45 minutes to complete.

2.4.3. Body Composition Assessment

Whole body DEXA (Dual Energy X-ray Absorptiometry) Scan

Body composition is very important for overall health. An excessive amount of body fat is a risk factor for heart disease, type II diabetes, and other diseases. DEXA scan is considered a gold standard for measuring bone density and regional (arms, trunk, and legs) as well as total body %fat, fat mass, bone mass, and lean mass. This regional, three compartment analysis is superior not only for an initial assessment, but also for tracking change of the total increase in muscle mass and the increase in muscle mass in the arms, trunk, and legs. A DEXA scan will take approximately 8-12 minutes in the Clinical and Translational Research Center (CTRC) at the Montefiore Hospital of UPMC and will be performed at baseline (Visit 2) and Week 12 (Visit 16).

2.4.4. Ambulatory Blood Pressure Monitor (ABPM)

This lightweight (12 oz) and compact device (~1x4.5x3.5 inches) can fit in a pant pocket or worn on a belt and attaches to a blood pressure cuff that measures blood pressure every 20 minutes during the waking hours and every 30 minutes during sleeping hours across a 24 hour time period. The ABPM will be placed at the completion of Visits 2 or 3 and 16 or 17 and worn for 24 hours. The subject will be instructed on how to remove and turn off the device at completion of the 24 hours. They will then return the device at Visit 3 or 4 and Visit 17 or 18, 2-7 days later. Verbal and written instructions will be provided to the subjects at this visit before discharge home. It can be worn to work, while sleeping and driving. The cuff and monitor should only be removed when showering/bathing or swimming.

2.4.5. Mitochondrial Function Studies

Platelet mitochondrial studies

In these studies, fresh platelets will be isolated from the blood and mitochondrial function and platelet activation measured. The analyses of mitochondrial function and platelet activation will potentially provide mechanistic information about the metabolic and thrombotic state of the patients at baseline as well as information about the mechanism by which nitrite modulate vascular and mitochondrial function in the subjects.

At Visits 4 and 17, 8 mL each of blood will be taken at time 0, 0.5 and 2 hours (24mL total per visit) and will be used for platelet isolation and measurement of mitochondrial function. Fresh platelets will be isolated and analyzed by extracellular flux analysis and platelet activation by flow cytometry in the lab of affiliated investigator, Dr. Sruti Shiva, at the University of Pittsburgh. A single sample will also be collected at Visit 10 (week 6) to examine the temporal relationship of whether there is a dissipating effect over time on platelet mitochondrial function.

Muscle mitochondrial studies

Percutaneous muscle biopsy: The muscle biopsy will be performed early in the morning of Visit 3 and Visit 18. The biopsy site will be monitored over 6 hours until completion of all of the visit testing. An ice pack will be applied for 20 minutes after the biopsy. The subject will be given instructions about how to care for the incision site and provided with the telephone numbers of the investigators to call if they have any problems.

The EMRC group has performed more than 800 muscle biopsies during the past 16 years, and more than 200 just in the past year. In the CTRC on the morning prior to the glucose clamp, i.e., during fasting conditions, a percutaneous muscle biopsy of the vastus lateralis will be performed. High-resolution respirometry: Mitochondrial respiratory capacity will be measured in permeabilized muscle fibers from vastus lateralis muscle biopsies (Oroboros Oxygraph 2K, Innsbruck, Austria). The instrument has two chambers, which permits high-resolution respiration measurements to be made in duplicate with low amounts of muscle tissue (2mg). The O2K assay allows a comprehensive evaluation of mitochondrial respiratory function upon titration with electron transport chain (ETC) complex-specific inhibitors and substrates[28,29]. Measurement of H_2O_2 emission by mitochondria will be measured from permeabilized muscle fiber bundles by real time monitoring of Amplex Red oxidation using HPLC detection[30]. Maximal State 3 (coupled) respiration and maximal State 4 (uncoupled) respiration will be measured as the primary mitochondrial function parameters. As a measure of mitochondria inner membrane content in muscle biopsies, we will measure cardiolipin content; a phospholipid unique to mitochondrial inner membrane. A HPLC assay with fluorescence labeling will be used for measurements of the content and specific species of cardiolipin as an index of mitochondrial remodeling, in <10mg of biopsy material.

2.4.6 Exercise Testing

Subjects will perform an exercise test on a stationary bicycle ergometer (Lode Excaliber, Gronigen, The Netherlands) in the Exercise Physiology Laboratory in the EMRC. The test will begin with a warm-up of light pedaling. Then each subject will perform two, 4-min standardized workloads of 25 and 50 Watts so that steady-state VO2 can be achieved. This will provide important information about whole body metabolic efficiency that may be linked with the intervention. After the submaximal workloads are completed, they will continue with 2-min stages of incrementally greater workloads of 25 Watts until a maximal voluntary effort is attained to assess maximal O2 consumption as a measure of maximal cardiorespiratory fitness. The EMRC has performed similar tests in more than 1,000 volunteers ranging from elderly women in their 80's to highly trained athletes.

2.4.7. Laboratory Profiles

Basic Laboratory Profiles

Basic laboratory profile of peripheral blood will be analyzed through the clinical laboratories at Presbyterian Hospital of UPMC clinical automated testing lab and at Esoterix. Presbyterian Hospital of UPMC testing will include:

- Triglycerides, HDL, fasting glucose, TSH, hemoglobin and hematocrit at screening (Visit 1)
- CBC, creatinine, AST and ALT at baseline assessment visit (Visit 3)
- HbA1C and lipid profile at baseline assessment visit (Visit 3) and week 12 follow-up assessment visit (Visit 18)
- Methemoglobin % by co-oximetry (Visits 4 through 15 and 17)

Esoterix testing (in females) will include estradiol at baseline assessment visit (Visit 3) and week 12 follow-up assessment visit (Visit 18).

Research Laboratory Profiles
Samples will be analyzed for nitrite and nitrate by gas phase ozone-based chemiluminescence detection[27]. Blood samples will be processed and analyzed in the Core NO (Nitric Oxide) Metabolomics Facility at the University of Pittsburgh, under the supervision of the Sponsor, Dr. Gladwin.

Fasting laboratory testing at baseline (Visit 3) and week 12 follow-up assessment visit (Visit 18) will include hsCRP, uric acid, ICAM-1, VCAM-1, e-Selectin, adiponectin, leptin, PAI-1, IL-6, and TNF-α for a total of approximately 2 Tablespoons (30 mL).

2.4.8. 72 hour dietary food recall
Subjects will be asked to complete dietary food recalls of the prior 72 hours. These will be collected at baseline visit (Visit 3) and week 12 follow-up visit (Visit 15 or 16) to assess for dietary nitrate and nitrite consumption.

2.5 STUDY ENDPOINTS

2.5.1 Primary End-Points
Primary outcome measure: change in insulin sensitivity (ie. insulin stimulated glucose disposal) by hyperinsulinemic euglycemic clamp

2.5.2. Secondary End-Points
Secondary outcome measures:

1. Change in systolic, diastolic and mean arterial pressures from baseline to 12 weeks
2. Change in muscle and platelet reactive oxygen species (ROS) generation, mitochondrial efficiency, and uncoupling in platelet and muscle biopsy sample studies
3. Change in endothelial function by flow mediated dilation (FMD)
4. Change in body weight
5. Change in arterial stiffening by carotid-femoral pulse wave velocity (PWV)
6. Change in arterial (intimal) thickening by intima-media thickness (IMT)
7. Change in HbA1C
8. Change in blood markers of inflammation, and vascular dysfunction: ICAM-1, VCAM-1, e-Selectin, CRP, PAI-1, IL-6, TNF-α, lipid profile, adiponectin, and leptin
9. Change in substrate oxidation by indirect calorimetry
10. Change in cardiorespiratory fitness by maximal exercise tolerance test
11. Change in body composition by whole body DEXA for total body fat
12. Safety monitoring with every other week methemoglobin by co-oximetry and PK obtained plasma nitrate and nitrite concentrations by chemiluminescence and red cell iron-nitrosyl-hemoglobin

2.6 STATISTICAL ANALYSIS

Primary endpoint sample size estimates: To develop sample size estimates, we hypothesize that inorganic nitrite will have significant but perhaps slightly less robust effects compared to a 4-month supervised exercise weight loss intervention. We anticipate an effect size (improvement) in insulin stimulated glucose uptake of ~1.68 mg/min/kg fat free mass (FFM) with dietary nitrite. This represents just 60% of the effect size we have observed with diet and exercise (based on a mean

improvement in insulin sensitivity from 6.70 ± 2.0 (SD) to 9.51 ± 2.5 mg/min/kg FFM; n=25). This would provide 80% power to detect this conservatively-estimated smaller effect size in 30 subjects at a p value of 0.05.

Primary and secondary endpoint analyses: Repeated measures ANOVA with visit (pre-, post-treatment) as the within-subject effect will be used to evaluate response to treatment for primary and secondary endpoint measures of insulin sensitivity, mitochondrial function (muscle and platelet ROS generation, mitochondrial efficiency, and uncoupling by various platelet and muscle biopsy samples), BP, methemoglobin %, endothelial function (FMD), body weight, arterial stiffening (PWV), arterial thickening (IMT), glucose tolerance (HbA1C), blood markers of inflammation and vascular dysfunction, substrate oxidation (indirect calorimetry), cardiorespiratory fitness (maximal exercise tolerance test), and body composition (whole body DEXA for total body fat). Data will be presented as the mean ± standard error for continuous variables. Transformations will be considered for data demonstrating non-normal distributions.

An interim statistical analysis is planned following completion of the 10^{th} subject. If there is a statistically significant change in the primary outcome measure of insulin sensitivity with $p<0.001$, discontinuation of the trial will occur. If $p>0.001$, the trial will continue. Based on an extremely low p value to stop the trial, there will be no need to adjust final significance level.

3. HUMAN SUBJECTS

3.1 SUBJECT POPULATION

The anticipated age range for the study population will be 18-60 years of age based upon the target disease population. Twenty overweight/obese subjects (BMI ≥ 30 kg/m^2) with any ethnic background who have a confirmed diagnosis of hypertension and meet waist circumference criteria will be eligible for enrollment. All subjects must provide written informed consent prior to participation.

3.1.1 Inclusion of Women and Minority

Women who meet the inclusion criteria, and have none of the exclusion criteria, will be enrolled without restriction as dictated by the study protocols. Because of the use of a study medication, woman of child bearing potential must meet specialized inclusion/exclusion criteria to minimize this risk. We will make efforts to enroll participants in this research in a distribution which mirrors the study population of the Pittsburgh area.

3.1.2 Inclusion of Children

This investigation will not enroll children based upon the target disease population and lack of safety data in adults.

3.2 INCLUSION CRITERIA

Potential study subjects must satisfy the following criteria to be enrolled in the study:

- Age 18-60 years old
- BMI ≥ 30 kg/m^2
- Hypertension: defined as systolic blood pressure ≥130 and/or diastolic blood pressure ≥85 mm Hg at screening
- Waist circumference: >102 cm in men, >88 cm in women

The age and blood pressure criteria are specific to baseline screening at Visit 1. If subjects for instance have a birthday after Visit 1 or develop BPs outside of the screening criteria after drug dosing at Visit 4 or at subsequent visits, they will not be excluded. For safety purposes, subjects however can be excluded based on low mean arterial pressures (MAP) that persist on repeat PK testing on lower dose of study drug.

3.3 EXCLUSION CRITERIA

Subjects meeting any of the following exclusion criteria at baseline will be excluded from participating in the study:

- Positive urine pregnancy test or breastfeeding
- Recent addition or change in dosing of hormonal contraception medications (OCP, IUD, DepoProvera)
- Concurrent use of medications affecting glucose (oral hypoglycemics, insulin, atypical antipsychotics)
- Current use of ≥3 anti-hypertensive agents regardless of blood pressure control or normotensive (SBP<130 and DBP<85 mmHg) on a single agent
- Current use of PD5 inhibitors or organic nitrates
- Not stable on treatments for the prior three months or not planning to remain on current dose of medications for blood pressure, contraception, etc.
- Chronic psychiatric or medical conditions including diabetes, liver or kidney disease or obesity syndromes
- TSH >8 mIU/mL
- Smoker
- Hemoglobin <11 g/dL

4. RECRUITMENT AND INFORMED CONSENT PROCEDURES

4.1 RECRUITMENT METHODS

The potential study subjects will be recruited from university and community advertisements and the University of Pittsburgh Nutrition and Obesity Research Center registry.

Potential subjects will also be identified by the physician investigator who is also the primary care or treating physician. The physician investigator who already has knowledge of and access to subjects' information will review the subjects' records to identify potential research subjects for

the study. After identifying potentially eligible subjects, the physician investigator will then approach these subjects to discuss the research opportunity.

To minimize the possibility that subjects will feel obligated to participate, investigators will reinforce with their subjects that participation is voluntary, that they do not have to participate, and the decision not to participate will not affect their care, now or in the future. The physician investigator will also allow subjects to make further inquiries if they are interested.

4.2 INFORMED CONSENT PROCEDURES

Subjects must provide informed consent. The information about this study will be given to the subject in language understandable to them. Either the physician investigator or a non-physician member of the research team will present the study. They will verbally present a general outline of the research plan, including inclusion and exclusion criteria, to the prospective participant. The consent form, outlining the design of the study, will include the risks and benefits of participating, and will be reviewed and the physician investigator and/or non-physician member of the research team will answer any questions. Prospective participants may take as much time as required to make an informed decision. Written informed consent will be obtained from each participant and the investigator prior to performing any research study procedures.

5. POTENTIAL RISKS AND BENEFITS

5.1 POTENTIAL RISKS

5.1.1 Risk of Experimental Drug Intervention
Numerous studies have evaluated acute, subacute and chronic drinking water exposures of nitrite in laboratory animals and drinking water and dietary exposures in humans. Recent studies are available using a high dose of nitrite by oral route in the form of beet root juice. Recent studies have evaluated acute exposures of oral preparations of nitrite on PK and blood pressure and are characterized below. More extensive human data is available on parenteral sodium nitrite as it is currently available and approved by the FDA for use in the emergency treatment of cyanide poisoning[31,32]. It is also notable that neutrapharmaceutical preparations are currently being sold with levels of nitrite (12.7 mg per tablet).

Nitrite:
Sodium nitrite has been used commercially as a food preservative, an anti-corrosive agent, a coloring agent, and an anti-anginal agent, with additional uses in laxatives, burn ointments, and liniments. Amyl nitrite has been inhaled or ingested as an euphoric stimulant. Nitrite has also been found as a contaminant in well water. Literature searches generated case reports of nausea, vomiting, abdominal pain, dizziness, headache, flushing, cyanosis, tachypnea, dyspnea, hypotension and death attributed to excess nitrite (high-dose) exposure from these sources as a consequence of methemoglobinemia due to oxidation of heme-iron in oxyhemoglobin. Normal background methemoglobin production is 1-3%. If levels of methemoglobin rise above approximately 30% of total hemoglobin, a subject may appear cyanotic and experience dyspnea, due to the reduced oxygen carrying capacity of hemoglobin (methemoglobin cannot bind oxygen). Levels above 50% can cause seizures, hypotension, coma and death. Sodium nitrite

administration for cyanide poisoning at the labeled dosage of 300 mg causes methemoglobinemia, a desirable effect, as methemoglobin binds to cyanide, thus protecting cellular mitochondria. A standard dose of nitrite used for cyanide poisoning is 300 mg up to 600 mg. Note that methemoglobin levels have never risen higher than 3% at the currently used therapeutic doses (< 75 mg) in 80 volunteers in phase I studies at the NIH.

The Sponsor of this study proposal, Dr. Gladwin, has previously held an IND for sodium nitrite (IND # 70,411) for cardiovascular applications and currently has an approved IND for the use of sodium nitrite for lung transplant recipients (IND # 111,643). The cardiovascular IND involved the administration of sodium nitrite to 69 normal volunteers in 4 phase I-II clinical trials without observed adverse effects. He has also treated 11 subjects with sickle cell disease on this IND without observed adverse effects. The lower doses of nitrite used in these investigational treatment regimens – 60-120mg daily or 20-40% of the dose (300mg) used in the emergency treatment of cyanide poisoning – do not produce methemoglobin levels greater than 3% and have not been associated with clinically significant hypotension. There have been no adverse events noted in the 80 treated normal human volunteers and patients with sickle cell disease disease[33-35]. In another study by Gladwin et al., 18 healthy adults received an infusion of sodium nitrite totaling 75 mg (15 minutes each x 2 infusions). This was associated with a 7 mmHg decrease in mean arterial pressure, a peak methemoglobin of less 3% and no other significant effects[34]. Note this single dose is 1.9 times the single dose per time of day we plan to use in this trial.

In an open-label three-way crossover study, 9 healthy adult subjects received two single high dose oral sodium nitrite aqueous solutions (0.12 and 0.06 mmol $NaNo_2$/mmol Hb, equivalent to 290-380 mg and 140-190 mg sodium nitrite, respectively, depending on the total body hemoglobin level of the person) and one intravenous sodium nitrite dose (0.12 mmol $NaNo_2$/mmol Hb)[18]. Note that this is 1.2-3.2 times the daily dose we plan to use in this trial. There was a washout period of at least 7 days between each of the treatments. Mild headache occurred in 44-55% of subjects and was the most frequent complaint during each treatment session, which the authors ascribed to the sodium nitrite, not methemoglobinemia, as the percentage of methemoglobinemia stayed below clinically toxic levels (<15%). By report, up to 22% experienced nausea, which subsided within half an hour[18]. The pharmacokinetic analysis of this study indicated similar bioavailability of oral and IV delivery of nitrite, as well as similar side effect and safety profiles.

A recent study determined the safety and feasibility of prolonged intravenous nitrite infusion. Twelve adult volunteers received increasing starting doses of sodium nitrite, 4.2 to 533.8 µg/kg/hr for 48 hours. Dose limiting toxicity occurred at 445.7 µg/kg/hr (10.6 mg/kg/day) and was limited to asymptomatic transient decreases of arterial blood pressure of up to 20 mmHg and asymptomatic increases of methemoglobin levels above 5%. No tolerance or clinically significant rebound was observed[36]. Note, this is 8.2 times the daily dose we plan to use in this trial (based on an adult body weight of 95 kg).

For nitrite, two retrospective case-control studies have shown that high maternal dietary nitrite intake from cured meat or drinking water during pregnancy might be associated with risk of childhood brain tumors and possibly gastric and esophageal cancer. This evidence is only based on retrospective case-control studies; cohort studies have found no significantly increased risks[24].

In the 2001 National Toxicology Program (NTP) Report summarizing 2-year rodent drinking water studies, there was no evidence of carcinogenic activity of sodium nitrite in male or female F344/N rats exposed to up to 130 mg/kg/day in males and 150 mg/kg/day in females, or in male B6C3F1 mice exposed to up to 220 mg/kg/day. There was equivocal evidence of carcinogenic activity of

sodium nitrite in the highest dose of 165 mg/kg/day in female B6C3F1 mice based on the positive trend in the incidences of squamous cell papilloma or carcinoma (combined) of the forestomach. Exposure to sodium nitrite in drinking water resulted in increased incidences of epithelial hyperplasia in the forestomach. However, no chromosomal damage (genetic toxicity) was observed in three studies conducted in rats and mice *in vivo*[37]. Taken together, these findings suggest minimal carcinogenic nitrite-mediated risk.

In the current study, we will use much lower doses of nitrite than used safely in any of these studies. Our nitrite dose of 20 mg three times daily stepping up to 40 mg three times daily is lower than the dose used on the cardiovascular IND and <40% of the dose for cyanide poisoning.

To summarize, we anticipate the following symptoms by organ system and likely frequency of risk:

Gastrointestinal
- Common: none
- Frequent: none
- Infrequent: dry mouth
- Rare: nausea, abdominal pain, and vomiting

Hematologic
- Common: none
- Frequent: none
- Rare: methemoglobinemia

Cardiovascular
- Common: none
- Frequent: none
- Rare: flushing, tachycardia, hypotension

Neurologic
- Common: none
- Frequent: none
- Rare: headache, dizziness, seizure, coma

Respiratory
- Common: none
- Frequent: none
- Rare: tachypnea, dyspnea, cyanosis

5.1.2 Risk of Study Procedures

The collection of the research data from history and physical examination such as body weight, height and vital signs carries minimal risk. Intravenous blood sampling risks of bruising, infection, anemia, and fainting are common. The clamp study involves likely risk of discomfort associated from two venipunctures. The infusion of 20% sugar water during the clamp may cause local tissue damage if it leaks outside the vein. However, the chances for this happening are minimal because of very close observation by the nurse and doctor. Hematoma formation and hypoglycemia are rare complications, which we have not had since clamp experiments were initiated 16 years ago. The stable isotope glucose tracer in the amount used has no hazardous effects.

The risks of percutaneous muscle biopsy are bleeding and infection (both rare), bruising (infrequent) and discomfort (likely). With the use of a local anesthetic, our subjects describe the

discomfort of muscle biopsy as about twice that of intravenous cannulation. Some residual stiffness in the vastus lateralis can persist for 1-2 days (common). An elastic wrap and ice bag are applied post-biopsy to decrease the risk of bruising and for an anesthetic effect. In our experience using similar protocols, subjects have not experienced any adverse effects from these procedures other than a small amount of residual localized soreness at the biopsy site. Additional risk includes any unusual reaction to the elastic bandage wrap and ice, i.e. leg numbness which would indicate the elastic bandage had been applied too tightly or the ice left on too long; or any skin redness, irritation, and chafing from the applied antibiotic ointment and/or steri-strips.

The exercise test (VO2max) and subsequent exercise training sessions may cause muscle soreness or fatigue, but in adults without a known history of heart disease, the risk of heart attack or death from maximal or sub-maximal exercise bouts is rare. The relative risk of exercise testing for obese adults has not been clearly defined. However, a survey of more than 2,000 clinical exercise testing laboratories, in which more than 600,000 tests were performed, showed a death rate of approximately 1 per 20,000.

American College of Sports Medicine (ACSM) criteria will be used to halt exercise testing. In addition to ACSM criteria, the exercise test will be stopped if the subject has either signs or symptoms of cardiovascular compensation, e.g. hypotensive response to exercise. These exercise tests will be interpreted by a physician at the University of Pittsburgh Medical Center. The staff conducting maximal exercise tests are ACLS certified. The Exercise Physiology Laboratory has a crash cart with emergency equipment (including a defibrillator-monitor, airway equipment, IV sets and fluids, syringes, needles, Lidocaine, Epinephrine, Nitroglycerine, etc.) and will be present during maximal exercise testing.

The radiation dose of the whole body DEXA is approximately 3 mrems. For comparison, adult radiation workers are permitted, by Federal regulation, to receive a maximum whole body radiation dose of 5000 mrems/year and a maximum single organ radiation dose of 50,000 mrem/year. There is no minimum level of radiation exposure that is considered totally free of the potential risk of causing genetic changes (cellular abnormalities) or cancer. However, the risk associated with the amount of radiation exposure that the subject will receive from participation in this research study is considered to be low and comparable to everyday risks. Menstruating females in the reproductive age group must have a negative pregnancy test within 24 hours prior to each of these studies in order to reduce the potential for exposing a fetus to radiation.

During the FMD test, the occlusion cuff will be placed on a subject's arm and will stay inflated for 5 minutes. The cuff could cause minor pain, possible bruising, numbness, tingling and irritation to the patient's skin. The subject will be asked to lie still on their back for 30 minutes during the test; this could cause stiffness or dizziness.

The risks associated with carotid Intima-Media Thickness (IMT) and Pulse Wave Velocity measurements are rare and include mild discomfort at the sites on the neck or groin where the ultrasound probe or tonometer are placed or sites on the neck, chest or groin where the skin is marked with a marker. The ECG electrodes will stick to the subject's skin and sometimes, when the electrodes are pulled off, the subject can experience slight irritation of the skin.

The risks associated with the ambulatory blood pressure monitor (ABPM) are rare and include a feeling of pressure, bruising, or irritation at the site where the blood pressure cuff is worn.

There is a potential risk of stress and anxiety while participating in this study. It is likely that at least mild anxiety will occur while the subject is hospitalized to participate in this study. There is a potential of developing an infection (viral or bacterial) while in the hospital because of exposure to sick patients. We will try to not pair participants to share a hospital room with a sick patient. Participants will not have tests that have more than a minimal risk of causing an infection. In our experience, the risk of developing an infection while at the CTRC is minimal.

5.2 ALTERNATIVE TREATMENTS

The alternative treatments for the subjects participating in this investigation are to continue their medical care under the direction of their primary physicians.

5.3 POTENTIAL BENEFITS

Participation in the proposed research may or may not provide a direct benefit to participants in this research. This research involves an intervention or procedure that presents greater than minimal risk to the involved adults, but which holds out the potential for direct individual benefit. The risk is justified by the extent of potential benefit to the involved adults; which includes possibly revealing previously-undiagnosed morbidities commonly seen in obesity such as hyperlipidemia, impaired fasting glucose, or type 2 diabetes mellitus. In addition, they will have their percentage and distribution of body fat assessed by DEXA, an important piece of information should the individuals choose to enter a weight management program after the completion of the study. This data has also been shown to predict cardiovascular and diabetogenic risk, information that is important for an overweight/obese subject. Also, assessments of baseline cardiovascular health will be made. This data is important for these overweight/obese subjects. Information obtained from the proposed research will provide information about the relationship between metabolic syndrome and hypertension treatment and patient outcome.

Based on the preceding assessment of risks and potential benefits, the risks to subjects are reasonable in relation to anticipated benefits. The research presents a balance of risks and expected direct benefits similar to that available in the clinical setting.

5.4 DATA SAFETY MONITORING PLAN

5.4.1 Data Safety Monitoring Board

The local Data Safety Monitoring Board (DSMB) will be comprised of members including experts in endocrinology, exercise physiology, clinical research, and clinical trial design, biostatistics, and research ethics. The DSMB will conduct interim monitoring of accumulating data from research activities to assure the continue safety of human subjects, relevance and appropriateness of the study, and the integrity of research data.

5.4.2 Data Safety Monitoring Plan

Assuring patient safety is an essential component of this protocol. The study Investigator has primary responsibility for the oversight of the data and safety monitoring. The study sub-investigators will evaluate all adverse events. All subjects who have AEs, whether considered associated with the use of the study medication or not, must be monitored to determine the

outcome. The clinical course of the AE will be followed up according to accepted standards of medical practice, even after the end of the period of observation, until a satisfactory explanation is found or the Investigator considers it medically justifiable to terminate follow-up.

The study coordinators must view patient records for possible adverse events until 72 hours after the last dose of study drug. All untoward medical occurrences observed in subjects receiving the study drug will be recorded on the participants' adverse event case report forms (CRF) by the study coordinator under the supervision of the Investigator. The CRFs will then be reviewed for completeness and internal consistency. Subsequently, the CRFs will be recorded on an electronic password-guarded study database. The electronic database that is being used for the purpose of this study has not been fully validated to be in compliance with the FDA regulations at 21 CFR Part 11; i.e. taking into account the limited scope of this clinical investigation. In addition to internal safeguards built into a computerized system, external safeguards will be put in place to ensure that access to the computerized system and to the data is restricted to authorized personnel. Training conducted by qualified individuals on a continuing basis will be provided to individuals in the specific operations with regard to computerized systems that they are to perform during the course of the study.

The Investigator will work with the reporting sponsor and sub-investigator to prepare a detailed written summary of serious, unexpected, and treatment related adverse events, and will compare, and contrast the event with prior events. The detailed written summary will be provided to the local DSMB and the IRB.

In addition, the DSMB Report addressing the following information will be submitted to the IRB at the time of continuing review annually or more often as required:

- A list of the research personnel who participated in the data and safety monitoring.
- The frequency of monitoring that took place during the renewal intervals and/or the dates that data and safety monitoring was conducted.
- A summary of cumulative data related to unanticipated problems (including adverse events) including a determination of causality and whether the risk to benefit assessment has changed.
- If appropriate, a summary of pertinent scientific literature reports, therapeutic developments, or results of related studies that may have an impact on the safety of study participants or the ethics of the research study.
- A summary of the outcome of reviews conducted to ensure subject privacy and research data confidentiality.
- Final conclusions regarding changes to the anticipated benefit-to-risk assessment of the study participation and final recommendations related to continuing, changing, or terminating the study.

Stopping Rule:
For safety reasons, we propose to discontinue this study protocol once 2 subjects enrolled experience one of two of the following on repeat PK testing despite appropriate nitrite dose reduction as follows:

1. Methemoglobin greater than 5%. A nitrite dose reduction is proposed from 40 mg three times daily to 20 mg three times daily if this methemoglobin % is reached at any study visit. Two hour PK testing will be repeated on a separate day at the lower nitrite dose once two subjects have reached this initial methemoglobin threshold. If methemoglobin is greater than 5% on the lower nitrite dose, participation in the study for the subject will be discontinued.
2. Mean arterial pressure (MAP) decrease <55 mmHg, or if subject's baseline MAP is <60, and there is a decrease greater than or equal to 20% from their baseline (on the morning of receiving study drug). A nitrite dose reduction is proposed from 40 mg to 20 mg three times daily if this MAP threshold is reached. Two hour PK testing will be repeated on a separate day at the lower nitrite dose once two subjects have reached this initial MAP threshold. If MAP remains <55 mmHg or in the case of the subject with a baseline MAP <60, then if MAP decrease is greater than or equal to 20% from baseline on the lower dose, subject participation in the study will be discontinued.
3. Positive pregnancy test.
4. In the event that the medication compliance rate is <80%, subjects will be re-educated on medication compliance. If medication compliance repeatedly falls outside of the acceptable range, the study investigators will discuss subject eligibility for continued participation in the study.

If an unexpected fatal event or any of the above three life-threatening events occur despite dose reduction, the study will be halted, until data review by investigators and the Data Safety Monitoring Board has rendered a final recommendation about study continuation.

5.4.3 Parameters to be Monitored

The following progress will be monitored throughout the course of the research to ensure the safety of subjects as well as the integrity and confidentiality of their data.

- An evaluation of the progress of the research study, including subject recruitment and retention, and an assessment of the timeliness and quality of the data.

- A review of collected data (including adverse events, unanticipated problems, and subject withdrawals) to determine whether there is a change to the anticipated benefit-to-risk assessment of study participation and whether the study should continue as originally designed, should be changed, or should be terminated.

- An assessment of external factors or relevant information (eg. pertinent scientific literature reports or therapeutic development, results of related studies) that may have an impact on the safety and study participants or the ethics of the research study.

- A review of study procedures designed to protect the privacy of the research subjects and the confidentiality of their research data.

The severity of adverse changes in physical signs or symptoms will be classified as follows:

- Grade 1 (Mild): asymptomatic or mild symptoms; clinical or diagnostic observation only; intervention not indicated.

- Grade 2 (Moderate): minimal, local or noninvasive intervention indicated; limiting age-appropriate ADL (Activities of Daily Living).

- Grade 3 (Severe): medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL.

- Grade 4 (Life-threatening): consequences; urgent intervention indicated.

- Grade 5 (Death): event is a direct cause of death.

5.4.4 Frequency of Monitoring

The Investigator will review subject safety data as it is generated. The Investigator, sub-investigators, and the research staff will meet on a two week interval to re-evaluate study goals, subject recruitment, data coding and retention, documentation and identification of adverse events, complaints and confidentiality of subjects. There will be an evaluation of the progress of the research study, including assessments of data quality, time lines, participant recruitment, accrual, and retention. The Investigator will also review the outcome and adverse event data to determine whether there is any change to the anticipated benefit-to-risk ratio of study participation and whether the study should continue as originally designed or should it be re-evaluated and changed.

The DSMB will also be expected to meet as needed, but not less than, every six months to provide an overall summary status report to the regulatory agencies.

5.4.5 Reportable Adverse Events

For this study, a serious adverse event is any untoward clinical event that is thought by the investigator to be study-related, that is also:

1. Fatal or immediately life threatening
2. Permanently disabling, or severely incapacitating.
3. Requires or prolongs inpatient hospitalization.
4. Important medical events that may not result in death, be life threatening, or require hospitalization may be considered serious adverse events when, based upon appropriate medical judgment, they may jeopardize the patient, or subject, and may require medical, or surgical intervention to prevent one of the serious outcomes listed above.

If clinically important and unexpected adverse experiences, or clinically important study-related adverse experiences occur, they will be recorded on the adverse event case report form.

5.4.6 Adverse Events Reporting Timeline

Life-threatening or fatal unexpected adverse events associated with the use of the study drug or procedures must be reported to the DSMB and the IRB within 24 hours of discovery of the incident with subsequent follow-up submission of a detailed written report.

The FDA must be notified by telephone or facsimile transmission of a human adverse event that is fatal or life-threatening no later than 7 calendar days after receiving the respective human adverse event information, followed by the subsequent submission of a written IND Safety Report.

Serious and unexpected adverse events associated with the use of the study drug or procedures must be reported to the DSMB and the IRB with subsequent follow-up submission of a detailed written report in accordance with the respective policies and procedures of the IRB. Written IND Safety Reports will be submitted to the FDA as soon as possible and, in no event, later than 15 calendar days following the investigator-sponsor's receipt of the respective adverse event information.

A summary report of the DSMB's findings will be prepared and submitted to the regulatory agencies.

5.5 RISKS MANAGEMENT PROCEDURES

5.5.1 Protection Against Risks

General Risks of Study Protocol and Procedures

All research interventions/activities will be conducted in private patient care areas. The collection of sensitive information about subjects is limited to the amount necessary to achieve the aims of the research, so that no unneeded sensitive information is being collected.

All demographic and clinical information about the subject will be stored on an electronic password-guarded study database under the supervision of the Investigator for this protocol. The electronic database that is being used for the purpose of this study has not been fully validated to be in compliance with the FDA regulations at 21 CFR Part 11; i.e. taking into account the limited scope of this clinical investigation. The data will be stripped of individual identifiers and stored anonymously with a subject number. Information linking subject identifiers with the coded subject number will be stored under password protection on computers in locked areas, with access only to the database manager. Maintaining records in locked files in locked offices will protect confidentiality of subjects. All staff will sign confidentiality statements. Access to the database will be limited to the data manager and staff under the supervision of the Investigator.

Specimens will be stripped of subject identifiers and stored according to a similar coding protocol as described above. These specimens will be stored safely in the custody of the Investigator responsible for the individual assays. The Investigators will limit future access to any remaining sample to only those investigators with prior IRB approval for their studies.

All staff involved in this study are properly credentialed and instructed in the areas of testing, confidentiality, and safety.

The Investigator will retain the data for the entire period of this study. The Investigator may continue to use and disclose subjects' de-identified information for the purpose of this study for a minimum of seven years after final reporting or publication of the study. If the subject and/or legal representative decide to withdraw or be withdrawn from study participation, they may request that the study data and samples be destroyed.

5.5.2 Protection Against Potential Risks of Experimental Intervention

- Involvement by trained staff / investigators with experience in the administration of the study drug

- Continuous monitoring by the Data and Safety Monitoring Board
- Required Education in the Protection of Human Research Participants

The Investigator and all sub-investigators listed on University of Pittsburgh Institutional Review Board approved protocol are required to participate in a course entitled The Education and Certification Program in Research & Practice Fundamentals (RPF). This web based tutorial is a requirement of the IRB for protocol submission.

6. COSTS AND PAYMENTS

6.1 COSTS

Study drug and all research testing will be supported by ongoing research grants. All medications, lab tests, and any procedures described will not be billed to the subjects and/or their health insurance company.

6.2 PAYMENTS

Subjects will be reimbursed $200 for the baseline study visits (Visits 2 and 3). Subjects will be reimbursed $300 for the week 12 study visits (Visits 16 and 18). Subjects will be reimbursed $25 for the first dose of drug study visit (Visit 4) and Week 12: Second Completion Assessment Visit (Visit 17), for a total of $50. Subjects will be reimbursed $10 for each of the 5 in-person safety visits (Visits 5, 6, 7, 8, 10, 12, and 14) for a subtotal of $50. The total compensation will be received when participation in all aspects of this clinical study are completed and will be $600.00. Subjects will also be reimbursed for outpatient Montefiore parking at all visits except the screening visit (this includes the (2) baseline and (3) week 12 study visits, the 5 in-person safety visits, and the first dose of drug study visit). In the event that a subject drops out due to an AE, the subject will be reimbursed for the individual study visits that they completed to date per the above reimbursement schedule.

7. QUALIFICATIONS AND SOURCE OF SUPPORT

7.1 QUALIFICATIONS OF THE INVESTIGATORS

Sponsor:

Mark Gladwin, M.D., is Professor of Medicine, University of Pittsburgh. Dr Gladwin is the Chief of Pulmonary, Allergy, and Critical Care Medicine and Director of the Vascular Medicine Institute at the University of Pittsburgh. He is an internationally recognized authority in the field of sodium nitrite including both the basic science and a broad range of clinical applications in cardiovascular disease. He is a current IND holder for the investigation of sodium nitrite in lung transplant.

Investigator:

Kara Hughan, M.D., Investigator will provide daily leadership and supervision to all aspects of the clinical trial execution. Dr. Hughan is Assistant Professor of Pediatrics, Division of Pediatric Endocrinology and Diabetes and Division of Weight Management and Wellness, Children's Hospital of Pittsburgh of UPMC.

Sub-Investigators:

Bret Goodpaster, Ph.D., is Senior Faculty Investigator and Scientific Director at the Translational Research Institute Exercise Core in Orlando, FL. He has 22 years of experience designing and conducting exercise studies. He has 16 years of experience with performing clinical investigations involving glucose infusions, muscle biopsies and non-invasive body composition imaging.

Mark Gladwin, M.D., is Professor of Medicine, University of Pittsburgh. Dr. Gladwin is the Chief of Pulmonary, Allergy, and Critical Care Medicine and Director of the Vascular Medicine Institute at the University of Pittsburgh. He is an internationally recognized authority in the field of sodium nitrite including both the basic science and a broad range of clinical applications in cardiovascular disease. He is a current IND holder for the investigation of sodium nitrite in lung transplant.

Maja Stefanovic-Racic, M.D., Ph.D. is faculty in the Department of Medicine, Division of Endocrinology, University of Pittsburgh. She is a board certified endocrinologist with research interests in type 2 diabetes and obesity. She has extensive experience performing muscle biopsies in adults of all ages within the Endocrinology and Metabolism Research Center (EMRC) research team. She will be a study physician available to recruit subjects and to supervise/perform muscle biopsies throughout the study.

Sruti Shiva, Ph.D., is an Associate Professor in the Department of Pharmacology and Chemical Biology and Vascular Medicine Institute (VMI) at the University of Pittsburgh. Her research focuses on the mechanisms by which reactive nitrogen species (particularly nitrite and nitric oxide) regulate mitochondrial function, the factors that influence this regulation and the implications of this regulation on pathology.

Andrea Levine, M.D. is a fellow in the Division of Pulmonary, Allergy, and Critical Care Medicine at the University of Pittsburgh. She will perform muscle biopsies in adults within the Endocrinology and Metabolism Research Center (EMRC) research team. She also performs bench research investigating the role of oral nitrite in the metabolic syndrome using both a mouse model and a skeletal muscle cell line.

Other Personnel:
Nicole Helbling, MS RN, is a registered nurse with extensive clinical and translational research experience in the EMRC. She has been a research coordinator within the EMRC and is well trained to review study consent, obtain medical histories, perform glucose infusions, venipunctures and IV placements and assist with day to day clinical trial execution.

7.2 SOURCES OF SUPPORT

UL1 RR024153 (University of Pittsburgh Clinical and Translational Science Institute), the Vascular Medicine Institute, the Hemophilia Center of Western Pennsylvania, and the Institute for Transfusion Medicine to K.H.

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