The Endogenous Renin-Angiotensin-Aldosterone System and Glucose Metabolism

NCT02034435

October 4, 2016

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Study Protocol
Statistical Analysis Plans

Date: 10/4/16

1.0 Inclusion/Exclusion Criteria

Inclusion criteria (Aims 1, 2)

1) Ambulatory subjects, 18 to 70 years of age, inclusive
2) For female subjects, the following conditions must be met:
    a. postmenopausal status for at least 1 year, or
    b. status-post surgical sterilization, or
    c. if of childbearing potential, utilization of adequate birth control and willingness to undergo urine beta-hcg testing prior to drug treatment and on every study day.
3) Either Metabolic Syndrome as defined by the presence of ≥ 3 of the following:
    a. Systolic Blood Pressure > 130 mm Hg OR Diastolic Blood Pressure > 85 mm Hg.
    b. Glucose Intolerance (Fasting Plasma Glucose ≥ 100 mg/dL)
    c. Increased triglyceride level ≥ 150mg/dL (1.7mmol/L)
    d. Decreased levels of HDL cholesterol
        - For males, less than 40 mg/dL
        - For females, less than 50 mg/dL
    e. Waist circumference
        - For males, greater than 40 inches (102 cm)
        - For females, greater than 35 inches (89 cm)

    OR impaired fasting glucose (fasting glucose greater than 100 but less than 150 mg/dL)

Exclusion criteria (All Aims)

Subjects presenting with any of the following will not be included in the study:

1) Previously diagnosed type 1 Diabetes
2) Type II Diabetes, as defined during a 75g oral glucose tolerance test performed after an 8-hour fast, by ADA criteria:
    a. Hemoglobin A1C ≥6.5%
    b. Fasting plasma glucose ≥126mg/dl (7.0mmol/l)
    c. 2-hour glucose ≥200mg/dl (11.1 mmol/l)
    d. current treatment with anti-diabetic medication(s)

3) Impaired renal function [estimated glomerular filtration rate (eGFR) of <60ml/min] as determined by the four-variable Modification of Diet in Renal Disease (MDRD) equation, where serum creatinine (S_{cr}) is expressed in mg/dl and age in years:

eGFR (ml/min/1.73m^2)=175 • $S_{cr}^{-1.154}$ • age$^{-0.203}$ • (1.212 if black) • (0.742 if female)

4) Prior allergies to medications used in the study protocol (e.g., Amlodipine, Eplerenone, L-arginine, potassium chloride, insulin), or to drugs within the same class.

5) Screening plasma potassium >5.5 mmol/L or sodium <135 mmol/L

6) Cardiovascular disease such as recent (<6 months) myocardial infarction, presence of angina pectoris, significant arrhythmia, congestive heart failure (LV hypertrophy acceptable), deep vein thrombosis, pulmonary embolism, second or third degree heart block, mitral valve stenosis, aortic stenosis or hypertrophic cardiomyopathy

7) Breast-feeding

8) Treatment with anticoagulants

9) History of serious neurologic disease such as cerebral hemorrhage, stroke, seizure, or transient ischemic attack

10) History or presence of immunological or hematological disorders

11) Diagnosis of asthma requiring use of inhaled beta agonist >1 time per week

12) Clinically significant gastrointestinal impairment that could interfere with drug absorption

13) Impaired hepatic function [aspartate amino transaminase (AST) and/or alanine amino transaminase (ALT) >2.0 x upper limit of normal range]

14) Hematocrit <35%

15) Any underlying or acute disease requiring regular medication which could possibly pose a threat to the subject or make implementation of the protocol or interpretation of the study results difficult, such as arthritis treated with non-steroidal antiinflammatory drugs

16) Treatment with chronic systemic glucocorticoid therapy (more than 7 consecutive days in 1 month)

17) Treatment with lithium salts

18) History of alcohol or drug abuse

19) Treatment with any investigational drug in the 1 month preceding the study

20) Mental conditions rendering the subject unable to understand the nature, scope and possible consequences of the study

21) Inability to comply with the protocol, e.g., uncooperative attitude, inability to return for follow-up visits, and unlikelihood of completing the study

2.0 Enrollment/Randomization

Ninety one subjects will be consented in **AIM1** (57 subjects) and **AIM2** (34 subjects). We estimate fifty per cent of subjects will be female. The study population will reflect the demographics of our area: 27% of subjects will be African American, 70% Caucasian, 1.6% Asian

The protocol and rationale for assignment to treatment group will be randomized and blinded to the investigators and subjects. Subjects will be randomly assigned to low/high dietary sodium order in **AIM1** and to drug order in **AIM2** using a permuted-block randomization algorithm. Dr. Yu, study biostatistician, will provide an allocation schedule. The Vanderbilt Investigational Pharmacy will be responsible for the storage, preparation, and labeling of all investigational agents, and for maintaining accurate drug storage and dispensing logs. The Clinical Research Pharmacist will devise standard operating procedures for the pharmacy to follow with regard to preparing, labeling, blinding, and dispensing study drug.

After subjects have been consented and screened, the investigator or research nurse will fax a copy of the consent form and a prescription containing check boxes for the inclusion and exclusion criteria and race to the Vanderbilt Investigational Pharmacy. Once the pharmacy has confirmed that consent was obtained and the subject met entry criteria, the pharmacist will assign the subject a randomization number and will provide the investigator with labeled study drug. An extra label containing the randomization number will be attached to each bottle of study drug. The investigator will affix this extra label to the subject's records. The Investigational Pharmacy will retain secure documentation containing the treatment assignment. This will be opened in the event of a clinical scenario which necessitates unblinding, as determined by the PI and the safety reviewers. Subjects randomized in **Specific Aims 1** and **2** who do not complete the whole protocol for any reason will be replaced and their data will be analyzed separately.

3.0 Study Procedures

General Protocol

SPECIFIC AIM 1: Test the hypothesis that activation of the endogenous renin-angiotensin-aldosterone system impairs glycemic control via effects on insulin sensitivity and secretion.

Rationale. We previously demonstrated that insulin secretion is deceased during low salt intake in healthy individuals; however, fasting glucose was not affected. We hypothesize that insulin-sensitive subjects can compensate for impaired insulin

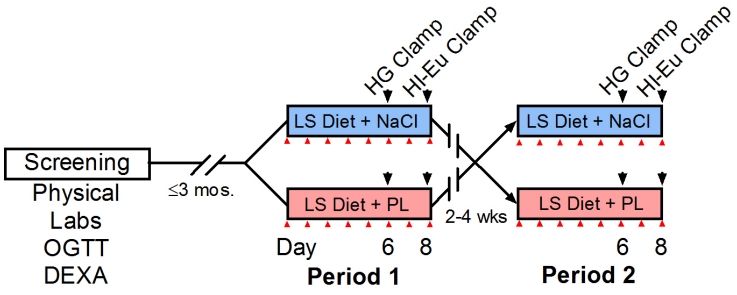

Figure 5. AIM 1 Study design. OGTT, Oral Glucose Tolerance Test; HG, hyperglycemic; HI-Eu, hyperinsulinemic euglycemic; LS, low sodium diet (50mmol/d). NaCl tablets=150mmol/d.

secretion, whereas insulin-resistant subjects such as those with the MetSyn are susceptible to adverse effects of LS diet. Because our animal data and studies in the literature suggest that Ang II and aldosterone also affect insulin sensitivity, we will also assess glucose turnover during hyperinsulinemic Euglycemic (HI-Eu) clamps. Because other compensatory effects may also alter glucose tolerance (decreased gut absorption, incretin signaling), we will also monitor home blood glucose serially at home to increase sensitivity to determine the timing of this effect. We will avoid confounding effects of different diets by using the same LS diet in both arms and providing either a sodium chloride supplement or matching placebo to increase dietary sodium intake.

Study Protocol. We will complete the study in 37 subjects (of the 57 consented) with MetSyn using the study protocol depicted in **Figure 5**. Eligible individuals must meet WHO criteria for MetSyn without diabetes. Detailed inclusion and exclusion criteria appear in **Human Subjects**. Time from initial screening to study completion for each subject will be 2 months (plus an additional 1 month, if drug washout needed).

Screening visit: Subjects will be recruited by the study coordinator with use of the Vanderbilt GCRC volunteer database via ResearchMatch.org, Vanderbilt computerized medical record, e-mail, and paper flyer advertisement. After informed consent is obtained, a study physician will conduct a complete history and physical exam and obtain screening labwork (fasting electrolytes, liver enzymes, creatinine, complete blood count, lipid panel, urine beta-hcg [if female], and EKG). Subjects who meet ≥3 criteria for MetSyn will return to the CRC for baseline lab assessment (plasma EETs, aldosterone) and formal glucose tolerance testing (75g OGTT) between the screening day and day one diet. Body composition measurement by Dual Energy X-ray Absorptiometry (DEXA, see Standard Techniques), DEXA scan will be done within 3-5 days of the hyperglycemic clamps in AIMS 1 and 2. A total of 4 DEXA scans will be performed on each subject who completes the study (AIMS 1 and 2.) If subjects are taking anti-hypertensive medications, they will wash out of these medications for 3 weeks prior to OGTT as outlined in **Standard Techniques** to avoid their confounding metabolic effects. Subjects with a fasting glucose >126mg/dL will be excluded. Subjects will be started on the study protocol within 3 months of completing the screening visits.

Study Periods: After completing initial screening/baseline measurements, subjects will be randomized to either low sodium (LS, 50mmol/day) or high sodium (HS, 200mmol/d) arm. To avoid differences in diet, HS intake will be achieved by addition of sodium chloride supplements (150mmol/d) to achieve a total intake of 200 mmol/d; matching placebo will be provided during the LS period. Subjects will be provided a diet containing 50mmol/d sodium, 100mmol/d potassium, and 1000mg/d calcium for 7 days. We chose the low dietary sodium target of 50 mmol/d to minimize blood pressure perturbations and reduce the risk to subjects during RAAS blockade in subsequent aims. A high sodium diet of 200 mmol/d suppresses the endogenous RAAS[99] and maintains the 150 mmol/d difference between diets used in our prior study. The duration of these diets was determined by our previous studies which demonstrate that sodium balance is achieved after 5 days on LS diet and ~3 days on HS diet. The calorie content of the diet will be calculated for weight maintenance (with 55% of calories from carbohydrates, 25% from fats, 20% from proteins). After proper instruction by the research nurse, subjects will monitor glucose each day two hours after lunch and dinner during each study period. An 8-hour urine collection will be collected overnight prior to the hyperglycemic clamps in AIMS 1 and 2 for isolation of urinary exosomes. Each morning of the clamp studies, subjects will report to the Vanderbilt CRC in the fasting state. Indwelling catheters will be placed in an antecubital vein and a hand vein. After the subject has remained supine

for 30-45 minutes, blood pressure and heart rate will be measured three times over ~15 minutes with a Dinamap oscillometric sphygmomanometer. Then blood will be obtained through the venous catheter for measurement of glucose, insulin, PRA, and aldosterone. Subjects will then undergo a hyperglycemic clamp as described under **Standard Techniques**. Subjects will return on Day 8 for HI Eu clamp to assess overall insulin sensitivity and hepatic glucose production (as outlined in **Standard Techniques**) while continuing study diet. A 24-hour urine for measurement of electrolytes, aldosterone, and creatinine will be collected before HI-Eu clamps in AIMS 1 and 2 to assess protocol compliance and achievement of steady-state sodium balance. After completion of Period 1, subjects will resume their normal diet (wash out) for ≥2 weeks, and then cross over to the remaining dietary sodium condition

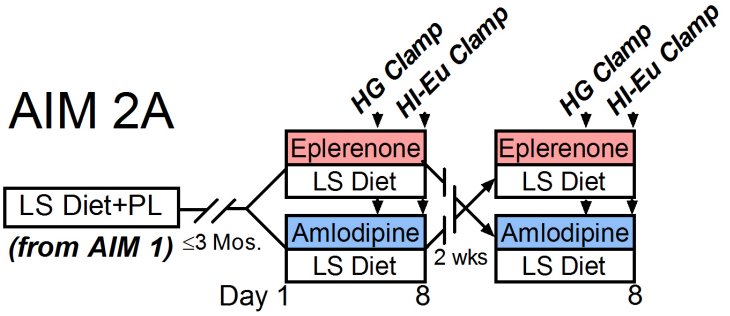

Figure 6. AIM2 Study design. OGTT, DEXA, Oral Glucose Tolerance Test; HG, hyperglycemic; HI-Eu, hyperinsulinemic euglycemic; LS, low sodium (50mmol/d)

SPECIFIC AIM 2: Test the hypothesis that endogenous aldosterone impairs insulin secretion and insulin sensitivity via the mineralocorticoid receptor.

Rationale. Hyperaldosteronism is the most common endocrine cause of hypertension, present in 10-20% of resistant hypertension patients, usually caused by either idiopathic hyperaldosteronism ***(IHA)*** or aldosterone producing adrenal adenomas (APAs).[105-107] Hyperaldosteronism is also associated with diabetes, and aldosterone decreases insulin sensitivity. Our *in vitro* studies in islets demonstrate that aldosterone impairs insulin secretion via oxidative stress independent of the MR. We hypothesize that MR antagonism will improve insulin sensitivity and insulin secretory capacity. We will test this hypothesis in a group of subjects using the specific MR antagonist eplerenone (**AIM 2A**)

Study Protocol. In Aim2, we will complete the study in 20 subjects. After screening and characterization, subjects will be randomized to either eplerenone 50mg daily, or amlodipine 5mg daily in a 2x2 crossover study as shown in **Figure 6**. Subjects will be provided LS diet (50mmol/d) and study drug concurrently for 8 days. Subjects will report to the Vanderbilt CRC after an overnight fast on the 6th day for hyperglycemic clamp and on the 8th day for HI-Eu Clamp. During the LS study period, we will measure seated blood pressure and assess for hypotensive symptoms every day when the subjects report to the CRC kitchen to obtain their food. We will provide subjects with a 24-hour contact number for the study coordinator and/or PI. Based on previous studies of RAAS inhibition during LS diet,[103] we anticipate that this will be well tolerated over the short period of the study. We will further monitor plasma potassium, plasma sodium, and creatinine on day 4 and both study days during each period as safety measures. 24-hour urine collections will be obtained before each study day and plasma creatinine will be measured to assess creatinine clearance, dietary sodium balance, and protocol adherence. After completion of the first study period, the subject will stop the study medication, wash out for ≥2 weeks, and then begin the next treatment period.

STANDARD TECHNIQUES

Data management and quality control. We will use the web-based Vanderbilt REDCap system to design an electronic data collection form. This form will be pilot-tested before use. Data will be input into a protected web-based case report form (which can be readily downloaded into SAS, STATA, R, or SPSS). The form allows for direct data entry by investigators across multiple institutions and is designed to minimize errors and erroneous values. Results from the Vanderbilt Clinical Laboratory can also be directly imported to REDCap, which further reduces typographical data entry errors. Expected ranges are pre-specified to prevent errors such as the shifting of decimal points. The program includes a computerized audit trail so that the identity of individuals entering or changing data and, in the case of changes, both original and revised data are saved. Data are backed up daily. Clinical data, including clinical laboratory, will be entered by the research nurse. Research laboratory data will be entered by a research assistant. A unique identification case number will be used to protect the confidentiality of the study participants. The case numbers and participants' names will be included in the protected source database but only case numbers will be included in any exported data files used for the statistical analysis. Before analysis, the data manager will independently and blindly assess all raw data for accuracy and completeness. Dr. Chang Yu, biostatistician, will check for potential outliers and resolve them with the investigators before unblinding the data and performing statistical analyses with input from Dr. Luther based on the methods specified in the data analysis plan. We will use the web-based case report form to monitor quality performance. We will generate monthly reports to track recruiting and safety.

Baseline measurements. Waist (horizontal umbilicus) and hip (at the largest horizontal span) circumferences will be measured to 0.5 cm precision in triplicate with a spring-loaded tape measure (Gulick II by Country Technology, Gay Mills, WI). Body composition will be determined using the Bod Pod Composition System (Life Measurements Instruments, Concord, CA).

Assays. Clinical laboratory assays such as potassium concentrations will be run locally in the CLIA-approved laboratories. All blood samples drawn for research assays will be centrifuged at 0 ºC for 20 minutes immediately after blood drawing, and plasma or serum will be divided into two aliquots, labeled with the case identification number, and logged and stored separately at -80 ºC until sampling. Standard quality assurance measures will be applied to all laboratory analyses including, but not limited to, tracking of inter-assay variability and the reanalysis of blinded internal controls at different times.

Glucose, C-peptide, and Insulin: Plasma glucose will be measured by the glucose oxidase method with a YSI glucose analyzer (YSI Life Sciences, Yellow Springs, OH). 6,6-dideuterated glucose will be assessed using mass-spectrometry. Plasma insulin concentrations will be determined by radioimmunoassay (RIA; Millipore, St. Charles, MO).[11,113] The insulin assay cross-reacts with 38% intact human pro-insulin and with C-peptide, ≤0.01%. Samples for C-peptide and glucagon will be drawn into heparinized tubes containing 250 KIU Trasylol (Aprotinin) per mL of whole blood. This will result in a final concentration of approximately 500 KIU Trasylol per mL of serum or plasma. C-peptide will be measured using RIA (Millipore).[11] Urinary catecholamines will be analyzed by HPLC as previously described.[75]

Lipid Analysis: Total cholesterol, triglycerides, and HDL will be determined using automated techniques (UniCel® DxC 800 Synchron® Clinical System, Beckman Coulter). We will measure non-esterified free fatty acids using a commercially available enzymatic assay (Wako Chemicals, Dallas, TX).

Aldosterone will be determined using a radioimmunoassay utilizing ^{125}I-

aldosterone (MP Biomedicals, Irvine, CA), a primary antibody to aldosterone (NIDDK National Hormone & Peptide Program, Torrance, CA), and a secondary anti-rabbit gamma globulin antibody (Linco Research, St. Charles, MO).[11,75,114-116] Plasma renin activity (PRA) will be determined by radioimmunoassay (DiaSorin, Stillwater, MN).

DNA samples: A DNA sample will be drawn only once, at the beginning of the study, and will be storage for genotypification of SNPs related to impaired β-cell function. The DNA samples analysis will be undertaken in a different study, given that it goes beyond the scope of the present study. Genotyping will be performed for research purposes only, and subjects will not be informed of results.

Medication withdrawal (hypertensive subjects). Anti-hypertensive medications will be discontinued three weeks prior to initiation of study drug. Because we have found that spironolactone has a prolonged duration of effect requiring a longer washout period, this medicine will be discontinued 4 weeks prior to initiation of study drug. Whenever appropriate, medications will be tapered. During this period, blood pressure will be measured every 1 to 3 days. If the subject does not have a suitable home blood pressure monitor, we will provide one for temporary use during the study. Home blood pressure monitors will be confirmed to vary ±<5mmHg for both SBP and DBP in comparison to the Dinamap reading. If any seated systolic pressure is >180 mmHg or the seated diastolic pressure is >110 mmHg or if a hypertensive subject develops symptoms of high blood pressure (e.g. headache, vision disturbance, chest pain, or dyspnea) regardless of the pressure, that subject will be discontinued from the study and his or her anti-hypertensive medications will be restarted. In our experience, approximately 5 per cent of enrolled subjects may be excluded by this blood pressure criterion. Excluded subjects will be replaced.

Blood pressure measurements. During office visits and studies, blood pressure will be measured with an automated oscillometric recording device (Dinamap, Critikon, Carlsbad, CA). At the time the cuff is placed, its readings will be compared against those of the mercury sphygmomanometer. In our experience these two methods give readings within 2-4 mm Hg of each other for both systolic and diastolic pressures. In our experience, this method correlates with average blood pressure obtained during 24-hour ambulatory blood pressure monitoring ($r=0.51$, $P<0.001$).

Oral Glucose Tolerance Tests (OGTT). Oral glucose tolerance tests will be administered according to guidelines by the American Diabetes Association to determine the baseline metabolic characteristics of the subjects. An IV catheter will be inserted for measurements. Baseline glucose and insulin will be drawn after an overnight 8-hr fast, then 75g glucose solution will be administered orally. Blood samples will be collected every 30 minutes for a total of 120 minutes for glucose and insulin. Subjects will be classified according to **Table 1**.

Table 1 OGTT Classification (75g)

Time Point	Glucose (mg/dL)	Classification
0 hours (Fasting Glucose)	<100	Normal Fasting Glucose
	100-125	Impaired Fasting Glucose
	≥126	Diabetes
2 hours	<140	Normal Glucose Tolerance
	140-199	Impaired Glucose Tolerance
	≥200	Diabetes

Home blood glucose monitoring. Subjects will be trained to properly measure home blood glucose by a trained research nurse, and will be provided a logbook for recording results, time of day, and timing of standardized meal ingestion. Subjects will be provided a Freestyle Freedom Lite glucose monitor (Abbott), alcohol wipes, single-use lancets, and test strips. Subjects will be directly observed performing the initial measurement to verify proper sterile technique, and printed manufacturer instructions will be provided for future reference. Between subjects, glucose monitors will be sterilized with alcohol pads and battery status will be checked and replaced near the end of life. Dedicated clinical glucose monitors will be kept in the Vanderbilt GCRC.

Body Composition Measurement. Body composition will be measured by Dual Energy X-ray Absorptiometry (DEXA). The computer software permits measurements of the whole body as well as individual segments, this is an accurate, validated method with minimal radiation exposure (Mazess et al., 1990;Svendsen et al., 1993). A Lunar model DPX Absorptiometer (Lunar DPA software 3.6; GE Medical System, Madison, WI), a research scanner located in the Vanderbilt GCRC will be used to measure total and regional adiposity.

Resting Energy Expenditure measurement. Resting energy expenditure will be measured using indirect calorimetry with a ventilated canopy device (CPX/D system, Medical graphics Corporation, St Paul, MN), this is based on the calculation of heat production through the measurements of gas exchange, specifically oxygen consumption and carbon dioxide production. Both measurement are converted to energy expenditure by application of deWeir's formula (deWeir, 1949). This method has been validated previously (Isbell et al., 1991) and proved to be accurate for resting energy expenditure determination. Only the last 20 minutes of a 40-minute measurement period will be analyzed. The respiratory quotient RQ will be obtained by dividing $\dot{V}CO_2$ by $\dot{V}O_2$. The RQ will be used as an indicator of quality control because in humans it resides in a fairly narrow range (0.67-1.3) (Branson, 1990).

Assessment of Insulin secretion by Hyperglycemic Clamp. After an overnight fast, primed continuous glucose will be infused for 2 hours via an antecubital vein, per the methods of DeFronzo et al, and Elahi et al. Arterialized blood will be obtained by inserting a venous cannula into a hand vein in a retrograde fashion and then placing the hand into a heated box or under a heating pad at 60ºC. If a retrograde hand vein catheter is not obtained, an antecubital vein may be used instead. Blood sampling will be done according to **Figure 7**, and the exogenous infusion of 20% dextrose will be varied to maintain the plasma glucose at a target glycemia of 200 mg/dL for 150 minutes, using a standardized algorithm as described by DeFronzo.[93] Blood samples will be collected every 5 minutes, immediately centrifuged, and plasma glucose will be determined with a YSI glucose analyzer (average time ~2 minutes to result). Samples for insulin will be collected (t= -20, -10, 0, 2.5, 5, 7.5, 10, 15, 20, 30, 60, 90, 105, 120, 122.5, 125, 127.5, 130, 135, 140, and 150) and stored at -80ºC until assay. At 120 minutes, an intravenous L-arginine hydrochloride bolus (5 g) will be administered over 5 minutes to assess non-glucose stimulated insulin release.[94] After completion of the study, dextrose will be tapered, and plasma glucose will be monitored for signs of hypoglycemia for 2-3 hours. The acute insulin response (AIR) will be defined as the incremental change for insulin for time 0-10 minutes following initial glucose bolus. Second phase insulin response ($\Delta Insulin_{120}$) will be calculated as the incremental change in plasma insulin during steady state (time period 90 to 120) compared to baseline values. L-Arginine stimulated insulin secretion will be calculated as the incremental

change 0-10 minutes after injection compared to steady state (average during time period 90-120 min.). To ensure that changes of insulin concentration are not due to altered metabolism, C-peptide will be assessed simultaneously.

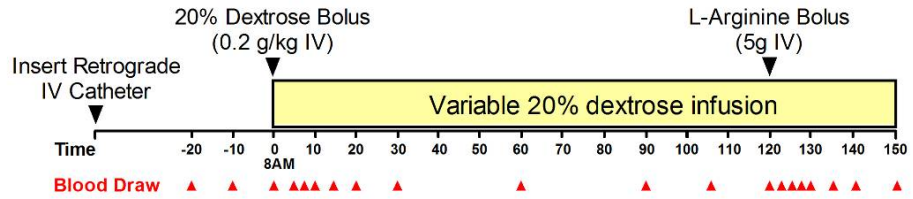

Figure 7. Hyperglycemic clamp protocol. Plasma glucose is measured every 5 minutes throughout the study, and dextrose infusion rate is adjusted. Blood sampling for insulin and C-peptide (▲).

Assessment of Insulin sensitivity by hyperinsulinemic-euglycemic clamp. An intravenous catheter will be placed in the antecubital vein of the non-dominant arm, and a retrograde catheter will be inserted into the contralateral hand vein. If a hand vein catheter cannot be maintained, a one will be inserted into a forearm or antecubital vein. Following the placement of catheters, subjects will be allowed to rest at least 30 minutes before baseline measurements are made. Insulin will be infused in the arm contralateral to the sampling venous catheter. Insulin infusion rates were chosen to partially suppress hepatic glucose production during the low dose infusion, while completely suppressing hepatic glucose production and maximally stimulating peripheral glucose utilization during the high dose infusion in insulin resistant subjects.[119-122] Resting Energy Expenditure (REE) measurement will be performed during the equilibration period of HI-Eu clamp in both AIMS 1 and 2. A priming insulin dose will be given at the time of each insulin infusion change. Plasma glucose will be measured every 2.5 to 5 minutes and an exogenous infusion of 20% glucose will be adjusted to control the rate of fall of glucose and maintain the plasma glucose at a target glycemia of 90 mg/dL by a modification of the glucose clamp technique (**Figure 8**).[123,124] Potassium chloride (KCl) 40mEq will be administered orally prior to initiation of glucose/insulin infusion during each hyperinsulinemic test period to maintain plasma potassium levels. Serum potassium will be measured prior to insulin initiation,during, and at the end of the study, and KCl will be administered to keep K^+>3.8. Heart rate and ECG will be recorded continuously during all glucose clamp studies.

Glucose Turnover: During hyperinsulinemic-euglycemic clamps, endogenous glucose appearance ($EndoR_a$) and utilization (R_d) will be determined by stable isotope

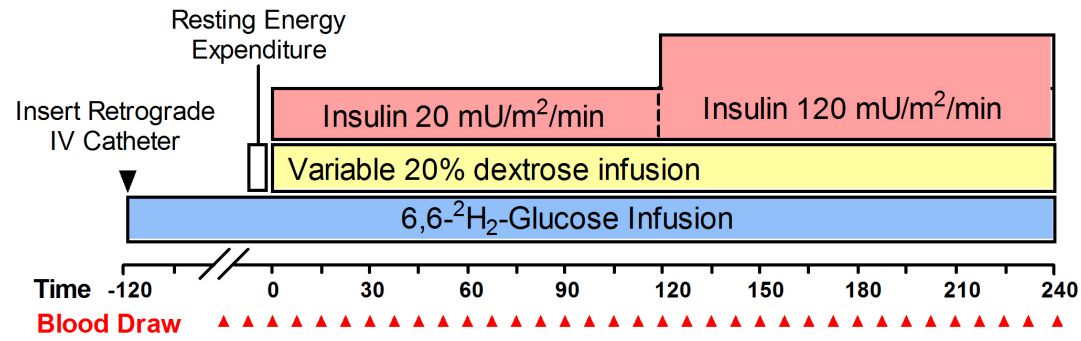

Figure 8. Two-step hyperinsulinemic euglycemic clamp protocol. Plasma glucose is measured every 5 minutes throughout the study, and dextrose infusion rate is adjusted. Blood sampling for glucose (▲).

enrichment with 6,6-dideuterated glucose.[125] In the proposed experiments, total rates of appearance (R_a) and R_d will be calculated based on the equations of Steele et al.[126] as modified by Debodo.[127] During hyperinsulinemic glucose clamps, R_a and R_d, calculated using this approach, may be underestimated.[128] The error appears to be greatest in states of high glucose flux.[129] We will use 6,6-dideuterated glucose as the glucose tracer to avoid calculation errors due to glucose contamination. To minimize the effect of rapidly changing enrichment[130], conclusions based on glucose turnover will only be made when steady state conditions are proven to exist. This will include the end of a 120 min tracer equilibration period and during the last 30 min of the hyperinsulinemic-euglycemic clamps, when a steady state exists. We define steady state as a period when the coefficient of variation of the plasma glucose and enrichment are stable at <3%. Insulin sensitivity will be estimated by the rate of glucose infusion (M) necessary to maintain euglycemia during steady-state (terminal 30 minutes of the study). To control for inter-individual variations in plasma insulin concentration, the insulin sensitivity index will be calculated by dividing M by average steady state insulin concentration. Tracer delivery will be varied to ensure that plasma enrichment changes very little. In experiments in which the change in glucose flux is so minimal that no exogenous glucose is required, the tracer will be administered as a primed continuous infusion (3.6 mg/kg bolus followed by 0.06 mg/kg/min constant infusion. Using this methodology, Dr. Ikizler has determined that endogenous glucose disposal rate is greatly diminished in obese chronic hemodialysis patients. The Disposition Index will be calculated as the product of insulin secretion during hyperglycemic clamps ($\Delta Insulin_{0\text{-}10}$) and insulin sensitivity during hyperinsulinemic euglycemic clamps (M/I-high dose).

4.0 Risks

Inconveniences:

- Not eating or drinking after 10p.m. on the night before each study
- Collecting urine in a jug for 24 hours
- Eating a special bland diet for 7 days
- Traveling to the GCRC and picking up meals and medications

Risks:

- Frequent blood draws can lead to anemia.
- Putting a catheter into a vein may cause bleeding, bruising, or infection (uncommon). We will use careful and sterile techniques to minimize these side effects.
- Withholding blood pressure medications may result in elevation in blood pressure. We will monitor blood pressure at home every 1-3 days after stopping or decreasing these medications. If home blood pressure is reported to be >180/110 mmHg, the study nurse and physician will re-evaluate them. During this evaluation, if any seated systolic pressure is >180 mmHg or the seated diastolic pressure is >110 mmHg or if a hypertensive subject develops symptoms of high blood pressure (e.g., headache, vision disturbance, chest pain, or dyspnea) regardless of the pressure, that subject will be discontinued from the study and his or her anti-hypertensive medications will be restarted.
- Glucose: Infusion of glucose may cause hyperglycemia. Symptoms of high blood sugar are hunger, thirst, frequent urination, dry mouth, and dry skin. Blood will be checked after the study completion until blood sugar returns to the normal range. IV Glucose may cause irritation of the vein, including redness and pain. We will give the glucose through a bigger vein to decrease this risk.

- Insulin: Persistently elevated insulin levels may cause hypoglycemia. Symptoms of low blood sugar are nausea, extreme hunger, feeling nervous or jittery, cold, clammy, wet skin and/or excessive sweating not caused by exercise, a rapid heartbeat, numbness or tingling of the fingertips or lips, and trembling. Blood glucose will be checked after the study until it returns to the normal range.
- Anti-hypertensive medications (amlodipine, eplerenone): may lower blood pressure excessively or alter serum potassium levels. We will monitor blood pressure and potassium throughout the study, and supplement with potassium as needed.
- Anti-hypertensive medications (amlodipine, eplerenone): may produce allergic reactions. We will exclude subjects with allergies to these drugs and if allergies to these drugs develop during the course of the study, we will discontinue the subject.
- Drugs that interrupt the renin-angiotensin system (angiotensin receptor blockers, Eplerenone in Aim 2) have been associated with birth defects if used during pregnancy. As a precaution we will exclude pregnant women or those women not using a reliable birth control method. We will check urine pregnancy tests before each study day. If a woman self-reports pregnancy to us, we will immediately discontinue study medication and withdraw that subject. Risk is further minimized by the short duration of eplerenone treatment.
- L-Arginine: May cause irritation of the vein. We will give it through a bigger vein to decrease this risk. It may cause nausea, vomiting, headache, flushing, numbness, or local venous irritation in ~3% of subjects using doses 6 times the dose that will be used in this study (30g over 30 minutes). Post-marketing surveillance of 1,670 infusions has revealed the following: One patient had an allergic reaction which was manifested as a confluent macular rash with reddening and swelling of the hands and face, which subsided rapidly after the infusion was terminated and 50 mg of diphenhydramine were administered. One patient had an apparent decrease in platelet count from 150,000 to 60,000. One patient with a history of acrocyanosis had an exacerbation of this condition following infusion of RGene® 10. We have not experienced any side-effects in our prior studies infusing L-arginine at the dose used in this study.
- Potassium Chloride: Administration of potassium chloride in excessive doses can cause hyperkalemia. We will monitor for signs and symptoms of high blood potassium include weakness, cardiac arrhythmias, and numbness. The dose administered in this study is not expected to cause an excessive rise in potassium, and is necessary to prevent or treat hypokalemia due to our standardized diet, glucose, and/or insulin administration. Oral potassium chloride may cause an unpleasant taste, stomach discomfort, nausea, stomach bleeding or diarrhea. Intravenous potassium may cause discomfort when infused rapidly or in a concentrated solution. Potassium will be diluted by co-administered glucose, decreasing this risk.
- There may be risks that we do not know about at this time. If we should find out any new information, we will notify the participants.

5.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

All protocols have been or will be reviewed and approved by the Vanderbilt IRB before any subject is enrolled. The Principal Investigator and Co-Investigator will closely oversee the protocol in conjunction with the dedicated research nurse.

We will use structured electronic case report forms and the REDCAP Dynamic Data Pull (DDP) within REDCap to assess compliance, concurrent medications and medical conditions, and adverse events.

Any adverse events or toxicities will be reported to the IRB as per IRB guidelines. Any untoward medical event will be classified as an adverse event, regardless of its causal relationship with the study. An adverse event will be classified as serious if it a) results in death, b) is life-threatening, c) requires inpatient hospitalization or prolongation of existing hospitalization, d) results in persistent or significant disability or incapacity, e) is a congenital anomaly or birth defect, or f) is an important medical event based upon appropriate medical judgment. Serious adverse events will be reported to the Safety Monitor, IRB, and NIH within 24 hours. An initial written report will be submitted within 72 hours.

Non-serious, unexpected adverse events will be reported to the IRB and Safety Monitor at the time of annual Continuing Review AEs will be graded as Mild (no limitation of usual activities), Moderate (some limitation), or Severe (inability to carry out usual activities) and attributed according to the relationship to the study drug and/or procedures as definitely, probably, possibly or unrelated to the study intervention and/or procedures.. Summary Reports will be submitted to the IRB at least annually and will contain a) The number of adverse events and an explanation of how each event was handled, and b) The number of complaints and how each complaint was handled, c) The number of subject withdrawals and an explanation of why the subject withdrew or was withdrawn, and d) The number of protocol violations and how each was handled.

Dr. C. Michael Stein, MD, professor of medicine and pharmacology, Dan May Chair in medicine, will serve as the independent Safety Monitor for these studies. Dr. Stein is an experienced physician scientist with extensive experience with human subject research and post-marketing drug surveillance. Dr. Stein will not be involved in the conduct of the studies, as a key personnel on the corresponding NIH grant, or serve in a reporting relationship (boss or subordinate) with Dr. Luther. As the Safety Monitor, Dr. Stein will annually review subject enrollment, retention, compliance, and adverse events in a report prepared by Dr. Luther and the study statistician. The Safety Monitor will have the authority to request necessary protocol changes. Dr. Stein will additionally provide an annual summary report to the IRB and NIH at the time of each annual review.

No early stopping based on efficacy is planned. Interim data will be provided as requested to the Safety Monitor by Drs. Luther and Yu.

6.0 Study Withdrawal/Discontinuation

Subject Withdrawal

Participation in this study is entirely voluntary, and subjects may withdraw from the study at any point by informing the study coordinator or PI. Data that have been collected may be used for analysis, but no further data will be collected after subject withdrawal.

Subject Discontinuation

Subjects may be removed from this study without consent if:

- Staying in the study would be harmful to the subject
- The subject no longer meet the requirements of the study
- The study is stopped

7.0 Statistical Considerations

Sample Size Calculations and Statistical Analysis. Sample size calculations are based on our prior results, using the hyperglycemic clamp first phase insulin response ($\Delta Insulin_{0\text{-}10}$ minutes) in normal and MetSyn subjects. This measure was chosen because it is the first detectable insulin secretory defect in subjects which progress to diabetes,[65] and because it is a highly reproducible measure. In our prior studies in MetSyn subjects, this measure is highly correlated within subjects over the course of 3 months ($r=0.91$, $P<10^{-6}$). The average response is 44±27 μU/ml in MetSyn subjects during HS diet, with an average coefficient of variation of 0.71 (range 0.61-0.80) across multiple studies. Based on our previous observed effect of LS diet (-40% vs HS), a sample size of *20* will have 80% power to detect a difference in means of *11* (*i.e.* effect size of -25%, a First condition mean, μ_1, of 44±32 and a Second condition mean, μ_2, of 33±24, correlation of 0.85), using a paired t-test with a 0.05 two-sided significance level. In our prior studies targeting subjects with MetSyn, ~75% of those we consented met inclusion criteria using our recruitment and screening methods. We estimate 15% dropout.

We will increase enrollment to allow for completion of 20 subjects in Aim2 based on the above assumed drop-out rate and consent rate. Therefore in AIM1, we will screen 57 subjects and enroll 40 subjects in order to complete 34. This would provide 96% power to detect the above difference in Aim1. In a prior study of MetSyn subjects with repeated measurements over 3 months, fasting glucose values were normally distributed with SD of 12mg/dl (CV 12%). Therefore, the detectable change in fasting glucose in this study with 34 subjects is 5.9 mg/dl, with 80% power and Type I error rate of 0.05 assuming a conservatively weak within-subject correlation 0.5 for this endpoint.

Data analysis plan. Analyses in **AIM1** will focus on the primary outcomes of insulin secretion, insulin sensitivity, and plasma glucose and the secondary outcomes of hepatic glucose production and disposition index. The within-subject design avoids inter-individual variation in measures of insulin secretion. The primary measure of the hyperglycemic clamp will be peak insulin secretion in response to glucose and L-arginine. Because the secretion of insulin during the study is time-dependent and a steady state is not achieved, we will assess baseline, acute response (ΔInsulin from 0-10 minutes), second phase (ΔInsulin from 90-120 minute), and L-arginine peak insulin concentrations, insulin sensitivity index (M/I), and disposition index using mixed effects models. A comparison of C-peptide will be performed to determine whether altered insulin metabolism accounts for the difference in insulin concentrations. Secondary analyses will be performed similarly.

Even though we do not expect any carry-over effect based on our prior experience with similar studies, we will nevertheless test for a carry-over effect using the T-test approach proposed by Jones and Kenward.[100] Mixed effect models will be used to analyze the data with a random subject effect and with the treatment (LS vs. HS diet) and the time trend (baseline, 10 min, 30 min, and 60 min into the infusion) as fixed effects. The focus of this study will be the treatment effect and the time trend of the endpoints; however, mixed effect models also provide the flexibility of controlling for and

evaluating covariates such as age, sex, baseline insulin sensitivity, and family history of T2DM. The mixed effect models are robust in the sense that they can include subjects with missing data at some time points but not all time points to estimate the effects of interest. Besides the above evaluation of the treatment effect and the time trend through the regression models, specific inferences regarding effects of interest will be made by reporting a point estimate along with a 95% confidence interval. Hypotheses will be tested at the level of α=0.05. This data analysis plan will be carried out using statistical software SPSS for Windows (Version 19.0, SPSS, Chicago), SAS® release 9 (Cary, North Carolina) and the open source statistical package R (version 2.12).[101]

8.0 Privacy/Confidentiality Issues

A unique identification case number generated by the collection program will be used to protect the confidentiality of the study participants. The case numbers and participants' names will be entered into the secure web-based database, and only the PI or the study nurse will have access to PHI. The GCRC database program will automatically exclude any PHI during export of the data to any external data files used for analysis. Only the PI, co-investigators, and research team listed as Key Study Personnel will have access to any PHI.

9.0 Follow-up and Record Retention

The duration of this study will be ~3 years. The study results will be kept in the subject's research record for at least seven years after the study is finished. At that time, the research data that has not been put into the computerized medical record may be destroyed. Any research data that has been put into the computerized medical record will be kept for an unknown length of time.