Soluble Epoxide Hydrolase Inhibition and Insulin Resistance
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Variation in Soluble Epoxide Hydrolase Activity and Insulin Sensitivity in Humans

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1.0 Background

An estimated 422 million adults are living with diabetes worldwide.[1] The majority (95%) have T2DM. Patients with T2DM are four times more likely to suffer from cardiovascular and renal disease than those without diabetes. Despite the development of new classes of anti-diabetic medications,[4] T2DM is uncontrolled in one-third of patients.[5] In addition, while controlling blood glucose with anti-diabetic agents reduces microvascular disease, many commonly prescribed anti-diabetic agents do not reduce risk of cardiovascular events.[6-9] There is a continued need to develop therapies to prevent and to treat T2DM while decreasing end-organ damage. Data from rodents, outlined below, suggest that the EET/sEH pathway (**Figure 1**) represents a potential new target for the prevention or treatment of diabetes. The well-described beneficial effects of this pathway on endothelial function, inflammation, and end-organ damage make it a particularly attractive target.[10] This proposal expands on preliminary data on the role of EETs and sEH in glucose metabolism in humans.

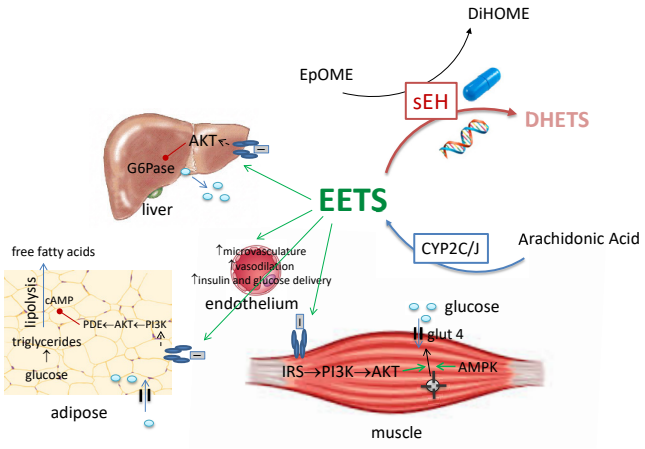


Figure 2: Possible mechanisms through which epoxyeicosatrienoic acids (EETs) improve glucose homeostasis. EETs are produced from arachidonic acid by epoxygenases CYP2C and CYP2J. EETs improve insulin signaling in muscle, liver, and adipose tissue. EETs also increase capillary volume and microvascular blood flow in insulin-sensitive tissues. Actions of EETs are limited by hydrolysis by soluble epoxide hydrolase (sEH) to dihydroxyeicosatrienoic acids (DHETs). sEH activity is often measured as the ratio of dihydroxyoctadecenoic acid (DiHOME) to epoxy-9Z-octadecenoic acid (EpOME). AKT, protein kinase B; IRS, insulin receptor substrate; G6Pase, glucose 6 phosphatase; glut 4, glucose transporter type 4; PDE, phosphodiesterase; PI3K, phosphoinositide 3-kinase

Tissue-specific sEH expression and/or activity are increased in rodent models of diabetes and obesity. sEH activity is increased more than two-fold in microsomes prepared from livers of rats treated with alloxan or streptozotocin (STZ).[30] sEH expression is also increased in the heart and gastrocnemius muscle of Akita mice, a murine model of T1DM, but not in liver.[31] In T2DM, sEH expression is increased 2.6-fold in the liver of C57BL/6 mice after long-term (16 weeks), high-fat (HF) feeding, but not after shorter duration (8 weeks) HF feeding.[26] sEH *activity* is increased approximately 35% in the liver regardless of the duration of HF feeding.[26] Schäfer et al. reported that a HF diet significantly decreases protein expression of the epoxygenases that form EETs in the liver and up-regulates sEH three-fold.[32] sEH activity is also increased in epididymal fat in mice fed a HF diet.[27] In addition, sEH expression increases with differentiation of preadipocytes into mature adipocytes.[27] Conversely, EET concentrations are higher in preadipocytes or mesenchymal stem cells (MSCs) compared to mature adipocytes.[27,33]

Loss of *Ephx2* and pharmacological sEH inhibition enhance insulin sensitivity in rodent models of insulin resistance. *Ephx2* deficiency or pharmacologic inhibition of sEH increases endogenous EETs and enhances insulin signaling in liver and in epididymal adipose tissue in HF mice.[19] Insulin-induced tyrosyl phosphorylation of the insulin receptor

(IR) is increased, as is tyrosyl phosphorylation of insulin receptor substrate (IRS)-1. Insulin-stimulated association of IRS-1 and the P85 subunit of phosphoinositide 3-kinase is enhanced in *Ephx2*-null and inhibitor-treated mice, resulting in increased phosphorylation of Ser473 and mitogen-activated protein kinase (MAPK). *Ephx2* deficiency and sEH inhibition also reduce HF-induced endoplasmic reticulum (ER) stress in the liver and subcutaneous adipose tissue (SAT).[21] Iyer et al. reported that sEH inhibition decreases glucose after an oral glucose load, decreases non-esterified fatty acids and total cholesterol, and decreases steatosis in the liver of high carbohydrate-, HF-fed rats.[20]

Beneficial effects of sEH inhibition are likely due to decreased degradation of endogenous EETs. In heme oxygenase (HO)-2 deficient mice, a model of obesity and insulin resistance, treatment with an EET agonist reduces weight gain, subcutaneous and visceral fat, and glucose.[23] The EET agonist increases vascular adiponectin, phosphorylation of 5' adenosine monophosphate-activated protein kinase (AMPK), nitric oxide synthase (eNOS), and improves endothelial function.[23] The EET agonist increases circulating adiponectin and decreases tissue necrosis factor (TNF)-α and monocyte chemotactic protein (MCP)-1.

EETs and sEH inhibition enhance insulin signaling *in vitro*. In HepG2 cells, sEH inhibition prevents palmate-induced ER stress.[21] sEH inhibition or treatment with EETs or epoxy-12Z-octadecenoic acid (EpOME), the epoxygenase product of linoleic acid, increases insulin-stimulated IR and protein kinase B (AKT) phosphorylation, whereas DHET and dihydroxy-12Z-octadecenoic (DiHOME) reduce insulin signaling.[21] EETs prevent inactivating phosphorylation of IRS-1 at S312 and increase insulin-stimulated AKT phosphorylation and glucose 6 phosphatase (G6P) expression in HepG2 cells.[34] Schäfer et al. also observed an effect of EETs on insulin-stimulated phosphorylation of AKT in primary mouse hepatocytes but did not detect an effect on phosphorylation of IR or IRS-1.[35]

In cultured MSCs, multi-plural stromal cells that can differentiate into adipocytes, EETs suppress adipogenesis and induction of fatty acid synthase and increase phosphorylation of AKT as well as adiponectin levels.[36] These effects are enhanced by sEH inhibition. Treatment with an EET agonist also decreases adipocyte generation and increases HO-1, adiponectin, and glucose uptake by MSCs.[36,37] MSCs derived from HO-2 null mice demonstrate decreased HO-1 activity and increased adipogenesis.[38] An EET agonist increases HO-1, reduces adipogenesis, increases adiponectin, and decreases inflammatory cytokine production.[38]

***In vivo*, EETs can enhance insulin sensitivity not only through direct effects on insulin signaling, but also by increasing blood flow and insulin and glucose delivery.** In wild-type and obese insulin-resistant mice (*db/db*), administration of an sEH inhibitor increases muscle capillary blood volume and microvascular blood flow as measured by contrast-enhanced ultrasound; in *db/db* mice this is accompanied by a decrease in glucose.[22] Conversely, insulin-induced increases in muscle capillary blood volume and microvascular blood flow are prevented by administration of an epoxygenase inhibitor.[22]

EETs or sEH inhibition may enhance insulin secretion. Studies in islets provide conflicting data regarding the effect of EETs on insulin release.[39-41] Luo et al. reported that *Ephx2*-null mice and sEH inhibitor-treated mice demonstrate preserved glucose-stimulated insulin secretion and are protected against islet cell apoptosis following STZ treatment.[24,25] Insulin release in response to glucose or potassium channel inhibition is increased in isolated islets from *Ephx2*-null mice and mice treated with an sEH inhibitor.[24] EETs may also influence pancreatic islet cell number or size in models of T2DM. In HF-fed mice, for example, targeted deletion of *Ephx2* or selective sEH inhibition increases islet size and vascular density measured by staining for CD31.[19] Other investigators report that sEH inhibition increases the number of β cells without increasing islet size in high carbohydrate- and HF-fed rats.[20] ***We have not observed an effect of EPHX2 genotype on glucose-stimulated insulin***

secretion in humans, and for this reason focus this proposal on insulin sensitivity.

Soluble epoxide hydrolase inhibitors are under development in humans. Among the pharmacological inhibitors of soluble epoxide hydrolase tested in rodents, the first to be tested in humans was 1-(1-acetyl-piperidin-4-yl)-3-adamantan-1-yl-urea or AR9281.[17] A study of the effects of AR9281 in patients with hypertension and impaired glucose tolerance (NCT00847899) was completed, but not published. The sEH inhibitor GSK2256294 [(1R,3S)-N-(4-cyano-2-(trifluoromethyl)benzyl)-3-((4-methyl-6-(methylamino)-1,3,5-triazin-2-yl)amino)cyclohexanecarboxamide] has been studied safely in Phase I and II studies.[18] The tmax is 0.5 hrs, and $t_{1/2}$ is 48 hrs. With multiple dosing in overweight smokers, steady-state was achieved within seven days. GSK2256294 10 mg achieved more than 95% inhibition of sEH throughout the dosing interval.[18]

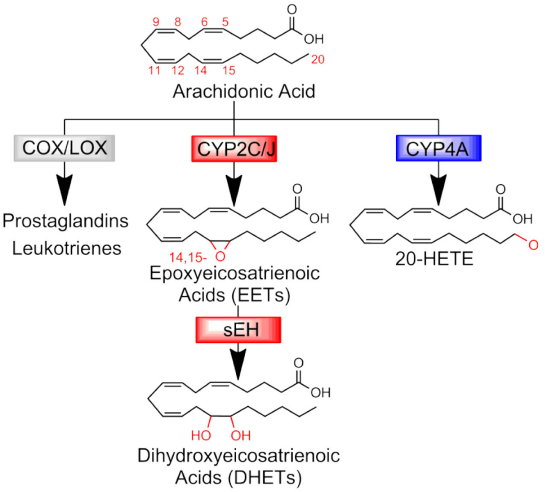
While sEH inhibition may have beneficial cardiovascular and renal effects in T2DM by improving endothelial function,[11,29] there could also be adverse consequences of long-term sEH inhibition. In pre-clinical models, EETs increase vascular endothelial growth factor (VEGF), increase proliferation and survival of endothelial cells, and promote angiogenesis, leading to metastasis of existing tumors.[42] The effect of sEH inhibition on angiogenesis and tumor growth is complex, however. While EETs promote angiogenesis, other fatty acid substrates of sEH, such as the epoxydocosapentaenoic acids (from omega-3 fatty acids) reduce angiogenesis.[43] In some models, dual cyclooxygenase and sEH inhibition decreases angiogenesis and tumor growth.[44] In addition, chronic inflammation contributes to the development of many cancers, and the anti-inflammatory effects of EETs may prevent these. For example, sEH expression is increased in colon cancer; *Ephx2*-null mice are protected against dextran sulfate sodium-induced colitis and colon cancer.[45] There was no effect of sEH inhibitor GSK2256294 on circulating VEGF in Phase II studies.[18] In this proof-of-concept study, we will treat patients with an sEH inhibitor for one week to avoid any risk of long-term effects.

2.0 Rationale and Specific Aims

The World Health Organization estimates that 422 million adults or 8.5% of the world's adult population was living with diabetes in 2014, compared to 108 million or 4.7% of the adult population in 1980.[1] Exercise, weight loss, and drugs like metformin can reduce the incidence of type 2 diabetes (T2DM), but lifestyle changes are difficult to maintain, and not all patients can take metformin.[2,3] Several exciting new classes of drugs have been developed for the treatment of diabetes,[4] yet diabetes remains uncontrolled in at least one-third of patients.[5] In addition, controlling glucose with anti-diabetic agents may not reduce macrovascular events.[6-9]

This proposal addresses the potential of small molecule inhibitors of soluble epoxide hydrolase (sEH) to improve insulin sensitivity in humans. Epoxyeicosatrienoic acids (EETs) are formed from arachidonic acid by the action of P450 epoxygenases (CYP2C and CYP2J) (**Figure 1**).[10] EETs act as potent vasodilators and have been identified as endothelium-derived hyperpolarizing factors.[11] In the kidney, EETs promote sodium excretion by inhibiting the translocation of the Na+/H+ exchanger (NHE3) in the proximal tubule.[12] EETs reduce inflammation by preventing NFκB activation.[13] It follows that increasing the actions of EETs in rodent models protects against hypertension, endothelial dysfunction, cardiovascular remodeling, and renal injury.[10,14,15] The effects of EETs are limited by hydrolysis by sEH to the less active dihydroxyepoxyeicosatrienoic acids (DHETs), and potential strategies to increase EETs include either increasing epoxygenase expression or reducing sEH activity.[16] Thus, specific pharmacological sEH inhibitors have been under development in clinical studies for the treatment of hypertension and lung disease.[17,18]

Figure 2: Epoxyeicosatrienoic acid (EET)/soluble epoxide hydrolase (sEH) pathway. sEH is encoded by *Ephx2* in mice and *EPHX2* in humans.



Studies in rodents point to an important role for EETs and sEH in the regulation of glucose homeostasis and insulin sensitivity (**Figure 2**). Specifically, inhibiting sEH or disrupting *Ephx2,* the gene encoding *sEH,* increases insulin sensitivity in rodent models of T2DM and insulin resistance.[19-21] These effects have been attributed to enhanced insulin signaling in liver, muscle, and adipose tissue, as well as to beneficial effects on muscle capillary blood volume and microvascular blood flow.[19-22] Administration of exogenous EETs replicates these effects.[23] Other studies have identified a beneficial effect of EETs or sEH inhibition on islet cell function or apoptosis.[19,20,24,25] Unfortunately, studies in rodents also suggest that tissue sEH activity increases with obesity.[26,27]

Our group has begun to elucidate the role of EETs and *EPHX2* in the regulation of glucose homeostasis in humans. We have found that circulating EETs correlate with insulin sensitivity and are significantly decreased in individuals with insulin resistance.[28] In addition, we have found that insulin sensitivity is increased in overweight carriers of a loss-of-function variant in *EPHX2* (rs751141 or Arg287Gln), but not in obese carriers of the variant.[28] The 287Gln allele is also associated with decreased vascular resistance.[29] We propose to study the mechanism of these effects and to test ***the overarching hypothesis that genetic or***

pharmacological factors that decrease sEH activity will improve insulin sensitivity by increasing blood flow and/or by increasing tissue insulin signaling. We will t**est the hypothesis that treatment with a specific sEH inhibitor decreases sEH activity and increases insulin-stimulated vasodilation and insulin sensitivity, via effects on tissue (muscle or adipose) insulin signaling and/or increased microvasculature**.

3.0 Animal Studies and Previous Human Studies

Circulating EET concentrations are decreased in obesity or insulin resistance. EET concentrations are decreased in obese patients with coronary artery disease (CAD) compared to non-obese patients with CAD, and body mass index (BMI) correlates inversely with plasma EETs.[46] We have found that plasma EETs are lower in individuals with metabolic syndrome than in those without metabolic syndrome (**Figure 3A**).[28] Similarly, EET concentrations correlate with insulin sensitivity calculated in two independent studies in subjects undergoing hyperglycemic clamps (**Figure 3B**) or frequently sampled intravenous glucose tolerance tests (**Figure 3C**). The relationship between plasma EET concentrations or sEH activity and EET concentrations or sEH activity in insulin-sensitive tissues is not known.

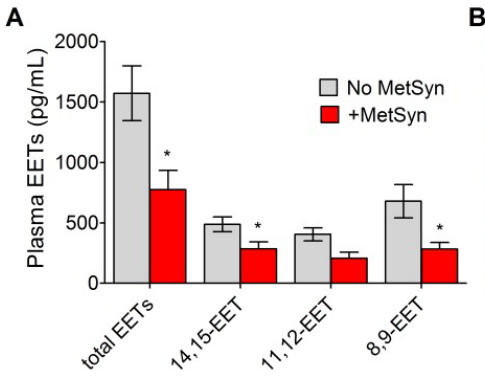


Figure 3: (A) Plasma epoxyeicosatrienoic ac
(N=28) metabolic syndrome. Relationship be
calculated in subjects undergoing hyperglyce
versus without metabolic syndrome

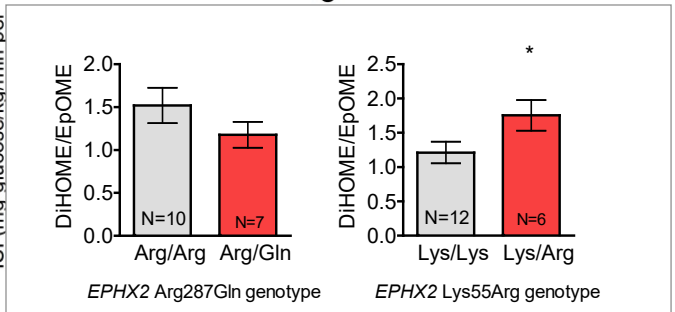


Figure 4: Plasma ratio of DiHOME to EpOME, as a marker of sEH activity, in a small number of subjects of known *EPHX2* loss-of-function 287Arg genotype (**Left**) or gain-of-function Lys55Arg genotype (**Right**). *p=0.05 versus Lys/Lys

Our group and others have studied the relationship between functional polymorphisms in the gene encoding for sEH, *EPHX2*, and obesity, insulin sensitivity, or diabetes. The Arg287Gln variant encodes for an sEH enzyme with lower hydrolase activity, while Lys55Arg encodes for an sEH enzyme with increased hydrolase activity.[47] Both polymorphisms have lower phosphatase activity.[48] We have assessed the relationship between both variants and the ratio of DiHOME to EpOME in the plasma, as a marker of sEH activity. Like EETs, EpOMEs are substrates for hydrolysis by sEH to DiHOMEs. The ratio of DiHOME to EpOME is

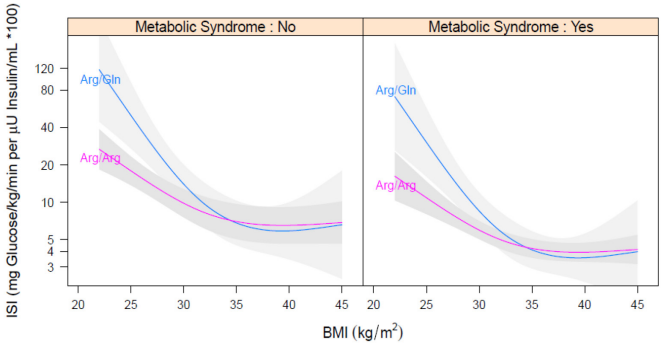
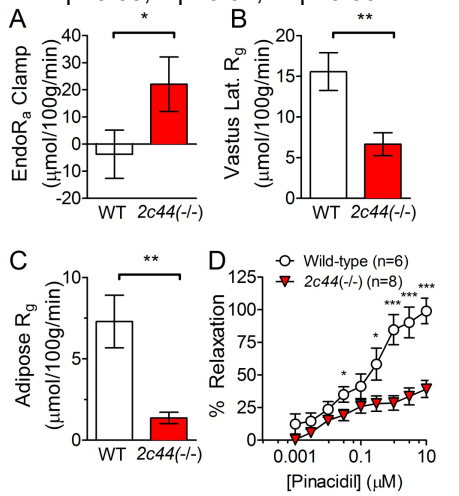


Figure 5: Effect of *EPHX2* Arg287Gln genotype on the relationship between body mass index (BMI) and insulin sensitivity index (ISI) calculated from hyperglycemic clamps.
p=0.02 for genotype, p=0.03 for genotype x BMI in all subjects.

used *in vivo* because the linoleic acid metabolites circulate at concentrations ten-fold higher than EETs and DHETs.[49] In **Figure 4**, preliminary data in individuals undergoing metabolic studies in whom we obtained DNA and measured plasma EpOME and DiHOME activity indicate that the loss-of-function 287Gln variant is associated with a non-significant (in this small number) 22% decrease in plasma sEH activity while the gain-of-function is associated with a significant 45% increase in activity compared to wild-type.

We have observed an association between the loss-of-function *EPHX2* 287Gln variant and increased insulin sensitivity calculated in individuals undergoing hyperglycemic clamps (Figure 5).[28] We found no relationship between *EPHX2* genotype and glucose-stimulated insulin secretion. Interestingly, the relationship between the *EPHX2* loss-of-function 287Gln variant and insulin sensitivity varied with BMI whether or not subjects had metabolic syndrome. These data suggest that decreasing sEH activity in patients at risk for T2DM could enhance insulin sensitivity. Nevertheless, questions remain to be addressed. Increased insulin sensitivity in *EPHX2* 287Gln carriers could result from enhanced insulin signaling in peripheral tissues, from increased capillary blood volume and microvascular blood flow or from enhanced endothelial function. We have found that basal forearm vascular resistance is decreased in *EPHX2* 287Gln carriers compared to Arg/Arg.[29]

Figure 6: (A) Endogenous glucose production, muscle **(B)** and adipose tissue **(C)** glucose uptake was impaired *in vivo* in *Cyp2c44(-/-)* mice assessed during hyperinsulinemic-euglycemic clamps. (**D**) Mesenteric artery relaxation in response to pinacidil was impaired in *Cyp2c44*(-/-) mice. *p<0.05, **p<0.01, ***p<0.001



Loss of epoxygenase *Cyp2c44* impairs muscle and adipose tissue insulin sensitivity *in vivo* in mice. In addition to EETs, other lipid mediators serve as substrates for hydrolysis by sEH. To determine whether EETs *per se* are involved in glucose homeostasis we assessed insulin sensitivity in mice lacking *Cyp2c44*, which demonstrate decreased EET production.[50] We found that hepatic glucose production (**Figure 6A**, EndoRa) was increased and insulin-stimulated glucose uptake in the gastrocnemius (not shown), vastus lateralis, and adipose tissues (**Figure 6B-C**) was significantly reduced in *Cyp2c44(-/-)* mice (manuscript in revision). This was accompanied by impaired vasodilation (**Figure 6D**), but not by changes in insulin-signaling in isolated muscles *ex vivo* (not shown). These data support a role for EETs in peripheral and hepatic insulin sensitivity and suggest that the effect of EETs may depend on enhanced endothelial function and glucose delivery to insulin-sensitive tissues.

4.0 Inclusion/Exclusion Criteria

Inclusion criteria

1. Men and women,
2. Age 21 to 60 years, and
3. Pre-diabetes as defined by
    a. Fasting plasma glucose 100-125 mg/dL, or
    b. Two-hour plasma glucose 140-199 mg/dL, or
    c. HbA1c 5.7-6.4%
4. BMI ≥ 30 kg/m^2, inclusive
5. For female subjects, the following conditions must be met:
    a. Postmenopausal status for at least one year, or
    b. Status-post surgical sterilization, or
    c. If of childbearing potential, utilization of adequate birth control and willingness to undergo serum β-hcg testing prior to drug treatment and on every study day.

Exclusion criteria

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

1. Diabetes type 1 or type 2, as defined by a fasting plasma glucose of 126 mg/dL or greater, a two-hour plasma glucose of 200 mg/dL or greater, a HbA1c >6.4%, or the use of anti-diabetic medication
2. Subjects who have participated in a weight-reduction program during the last six month or whose weight has increased or decreased more than two kg over the preceding six months
3. Resistant hypertension, defined as hypertension requiring the administration of more than three anti-hypertensive agents including a diuretic to achieve control
4. Use of spironolactone
5. Pregnancy or breast-feeding
6. Any significant history of smoking, as defined by smoking one pack-year over any cumulative time or smoking any amount within the last ten years
7. Any history of cancer including skin cancer, any history of a precancerous lesion, abnormal PSA, or lack of screening adherent to American Cancer Society Guidelines for the Early Detection of Cancer
8. Cardiovascular disease such as myocardial infarction within six months prior to enrollment, presence of angina pectoris, significant arrhythmia, congestive heart failure (left ventricular hypertrophy acceptable), deep-vein thrombosis, pulmonary embolism, second- or third-degree heart block, mitral valve stenosis, aortic stenosis, or hypertrophic cardiomyopathy
9. Abnormal corrected QT interval on screening ECG (QTc).
10. Treatment with anticoagulants
11. History of serious neurologic disease such as cerebral hemorrhage, stroke, or

transient ischemic attack

12. History or presence of immunological or hematological disorders
13. Diagnosis of asthma requiring regular inhaler use
14. Clinically significant gastrointestinal impairment that could interfere with drug absorption
15. Impaired hepatic function (aspartate amino transaminase [AST] and/or alanine amino transaminase [ALT] >3.0 x upper limit of normal range)
16. History of gastrointestinal bleed
17. Estimated glomerular filtration rate (eGFR)<60 mL/min/1.73 m^2 or with an albumin-to-creatinine ratio (UACR) $\geq$300µg/mg, where eGFR is determined by the four-variable Modification of Diet in Renal Disease (MDRD) equation, where serum creatinine is expressed in mg/dL and age in years: eGFR (mL/min/1.73m^2)=186 • $Scr^{-1.154}$ • $age^{-0.203}$ • (1.212 if black) • (0.742 if female)
18. Hematocrit <35%
19. 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
20. Treatment with chronic systemic glucocorticoid therapy
21. Treatment with lithium salts
22. History of alcohol or drug abuse
23. Treatment with any investigational drug in the month preceding the study
24. Mental conditions rendering a subject unable to understand the nature, scope, and possible consequences of the study
25. Inability to comply with the protocol, e.g., uncooperative attitude, inability to return for follow-up visits, and unlikelihood of completing the study

5.0 Enrollment/Randomization

Assignment to treatment order will be randomized and blinded to the investigators and subjects. Subjects will be randomly assigned to drug order using a permuted-block randomization algorithm. Dr. Yu, study biostatistician, will provide an allocation schedule. Vanderbilt Investigational Drug Services (IDS) 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 to Vanderbilt IDS. The pharmacist will confirm that consent was obtained and the subject met entry criteria, assign the subject a randomization number, and provide the investigator with labeled study drug as indicated in the protocol. An extra label containing the randomization number will be attached to each study drug. The investigator will affix this extra label to the subject's records. The Investigational Pharmacy will retain a secure set of sealed envelopes containing the treatment assignment. These will be opened in the event of a clinical scenario which necessitates unblinding, as determined by the PI and the DSMC (see Data and Safety Monitoring Plan). Subjects randomized who do not complete the whole protocol for any reason will be replaced.

6.0 Study Procedures

Rationale: Our preliminary studies indicate that the *EPHX2* loss-of-function variant (Arg287Gln) is associated with a 22% decrease in plasma sEH activity and enhanced insulin sensitivity. Studies in animal models suggest that sEH activity is increased in insulin-sensitive tissues in obesity, whereas epoxygenase activity is decreased.[26,27,32] A potent pharmacological inhibitor of sEH has the potential to decrease sEH activity in insulin-sensitive tissue even in obese subjects. In this Aim we will determine whether pharmacological sEH inhibition reduces sEH activity in insulin-sensitive tissues and whether pharmacological sEH inhibition enhances insulin sensitivity through increased insulin signaling or increased blood flow.

Subjects:

We will study 34 obese (BMI ≥ 30 kg/m^2) and pre-diabetic subjects, age 21 through 50 years. We will study fifty percent black subjects, fifty percent white subjects, and fifty percent women. Subjects with any history of cancer including skin cancer, cardiovascular disease (other than hypertension), renal, pulmonary, endocrine (other than insulin resistance or hyperlipidemia), or hematologic disease will be excluded. Individuals with diabetes, resistant hypertension, or any history of smoking will be excluded. Pregnancy will be excluded. Detailed inclusion and exclusion criteria appear in **Human Subjects.**

Protocol:

Once informed consent has been obtained, subjects will report to the CRC after an overnight fast, and we will obtain a medical history and complete a physical examination. We will obtain screening laboratory and an ECG. Between screening and the first study day, subjects will return to the CRC in the fasting state to undergo measurement of waist and hip circumference, a 75-gram OGTT, and body composition measurement by Dual Energy X-ray Absorptiometry (DEXA, see Standard Techniques).Because this is a crossover study, hypertensive participants of this Aim will continue to take their anti-hypertensive medications, and the doses will be maintained constant throughout the study.

Subjects will then be randomized within one month of screening using a permuted-block algorithm to treatment with the sEH inhibitor GSK2256294 (10 mg/d) or matching placebo for one week in a double-blind, crossover design study. The Vanderbilt Investigational Drug Service will be responsible for storage, preparation, labeling, and dispensing of medication. GSK is supplying study drug and matching placebo. GSK2256294 has been studied safely in Phase I and II studies.[18] In multiple dosing studies in overweight smokers, steady state was achieved within seven days. The dose of GSK2256294 achieves >95% inhibition of sEH throughout the dosing interval. Subjects will also be provided a weight-maintenance diet containing 150 mmol/day sodium and 80 mmol/day potassium for one day prior to study. On the seventh day of drug treatment, subjects will report to the CRC in the morning after an overnight fast to undergo a hyperinsulinemic-euglycemic clamp. Urine will be collected overnight for 8 hours prior to the study day, and again after clamp completion. On the day of study, we will place IV catheters in both arms. We will obtain an adipose tissue biopsy (**Standard Techniques**) and optional muscle tissue biopsy if subject consents. We will administer paraminohippurate (PAH, 8 mg/kg) as a loading dose, followed by a 12 mg/min continuous infusion for measurement of renal blood flow. After the subject has been supine for 45 min (and PAH is at steady state), we will measure forearm blood flow (FBF), BP, and

heart rate in triplicate (**Standard Techniques**). We will draw blood for measurement ofGSK2256294 concentrations, PAH, plasma EETs, DHETs, EpOME, DiHOME, interleukin 6 (IL-6) and other inflammatory cytokines, adiponectin, VEGF, and potassium. Subjects will then undergo a hyperinsulinemic-euglycemic clamp (see **Standard Techniques**). We will infuse 6,6-dideuterated glucose to assess endogenous glucose appearance (EndoRa) under fasting conditions, and we will assess hepatic insulin sensitivity by the response to low (20mU/m^2/min) and high (80mU/m^2/min) insulin infusion rates (**Standard Techniques**). At the end of the clamp we will repeat FBF measurements, followed by adipose tissue and optional muscle biopsy. We have experience obtaining biopsies before and at the end of hyperinsulinemic clamps (**Preliminary Studies**). At the end of each study day, we will obtain safety laboratory including CBC with differential, complete metabolic profile, urinalysis, and ECG.

After completion of the study day, subjects will undergo a seven-week washout from study drug and then receive the opposite drug for one week, while eating the same standardized diet. On the seventh day of treatment they will report to the CRC after an overnight fast and repeat the study day protocol.

Standard Techniques

BP Measurements: During screening, washout, and active treatment outpatient BP will be measured with an aneroid sphygmomanometer (Welch Allyn, Skaneateles Falls, NY), using the appearance and complete disappearance of the Korotokoff sounds (K1 and K5) as systolic blood pressure (SBP) and diastolic blood pressure (DBP). The mean of three seated measurements will be used. During study days, BP will be measured every five min using an automated oscillometric recording device (Dinamap, Critikon, Carlsbad, CA).

Adipose harvest: Adipose tissue will be obtained from the periumbilical area using a Tulip™ Medical closed syringe system for lipoaspiration (see Letter of Support). Under aseptic conditions and local lidocaine anesthesia, a small incision is made in the skin. The GEMS Johnnie Snap lock is placed into a 60-cc syringe, the Tulip liposuction cannula with 60-cc syringe attached is inserted at an angle through the incision to below Scarpa's fascia, and suction is applied until the syringe activates the clicker lock. The needle is moved in and out at a rate of approximately 1 Hz without breaking suction with a twisting motion. The sampling continues until approximately 5 g of tissue is removed (approximately 5.5 cc at a specific gravity of 0.918 for human fat). The syringe containing the sample will be taken on ice to the lab where it will be separated into pieces for measurement of sEH activity, DiHOME, EpOME, EETs, DNA and RNA isolation, adipocyte size and number, cytokines, and adipokines.

PBMC harvest: PBMCs will be obtained through the peripheral IV into 3 EDTA tubes for total of 12 milliliters, and transported at room temperature to the lab for processing. Cells will then be passed through a Ficoll-Paque barrier, washed with PBS, and stored in DMSO.

Muscle biopsy: Biopsies will be obtained from the vastus lateralis under aseptic conditions and local lidocaine anesthesia by percutaneous needle biopsy using the modified Bergström technique. Briefly, after proper aseptic technique and local anesthesia, a 5 mm incision is made in the skin. The needle is inserted through the incision to the skeletal muscle. The inner trocar of the needle is retracted and suction applied to pull muscle into the outer trocar. The inner trocar is then closed to cut the muscle. This needle biopsy is then rotated (90°) and the procedure is repeated twice. After removing the needle, tissue is immediately

placed in ice-cold 0.9% NaCl, and the subcutaneous fat, if any, is separated from muscle. Muscle biopsies will be divided: a fresh sample will be taken on ice to the Eicosanoid Core for measurement of sEH activity, EETs, DHETs; two pieces will be frozen in liquid nitrogen and stored at -80ºC for DNA and RNA isolation and for Western blotting, one will be immersed in fixatives for electron microscopy.

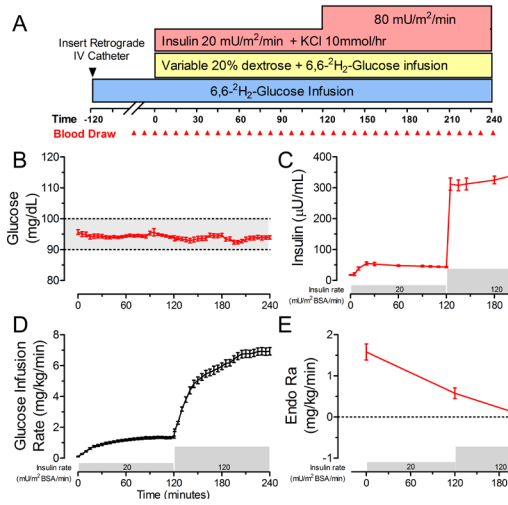


Figure 8: (A) Hyperinsulinemic-euglycemic clamp protocol and results demonstrating **(B)** achieved plasma glucose, **(C)** glucose infusion rate, **(D)** plasma insulin, and **(E)** endogenous glucose production (EndoRa). Values are mean±SEM, n=67 for B-D; n=10 for E.

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.

FBF measurements: We will measure FBF before and at the end of hyperinsulinemia using venous occlusion mercury-in-Sialastic strain-gauge plethysmography.[29,61,62] The wrist is supported in a sling to raise the forearm to above the level of the atrium, and the strain gauge is placed at the widest part of the forearm. The strain gauge is connected to a plethysmograph (model EX-5; D. E. Hokanson, Inc., Bellevue, WA), calibrated to measure the percentage change in volume and connected to a chart recorder. For each measurement, a cuff placed around the upper arm is inflated to 40 mm Hg with a rapid cuff inflator (model E-10; D. E. Hokanson, Inc., Bellevue, WA) to occlude venous outflow from the extremity. The hand is excluded from the measurement of blood flow by inflation of a pediatric sphygmomanometer cuff around the wrist to 200 mm Hg before and during measurements of FBF. Flow measurements are recorded for ~7 of 15 s, and the slope is derived from the first three or four pulses; five to seven readings are obtained for each mean value.

Hyperinsulinemic-euglycemic clamp: We will place a catheter in the antecubital vein for insulin and glucose infusion, and a catheter in a hand vein for glucose sampling. Subjects will rest at least 30 min before baseline measurements. We will infuse insulin in the arm contralateral to sampling. We have chosen insulin infusion rates to suppress hepatic glucose production partially during the low-dose infusion (20mU/m^2/min), completely during the high-dose infusion (80mU/m^2/min), and to stimulate peripheral glucose utilization maximally in insulin-resistant subjects during the high-dose infusion.[63-66] We will give a priming insulin dose at the time of each insulin infusion change. We will measure plasma glucose every 2.5 to 5 min and give an exogenous infusion of 20% glucose to control the rate of fall of glucose and maintain the plasma glucose at a target of 90-95 mg/dL.[67,68] We will administer potassium chloride (KCl) 40 mEq orally prior to hyperinsulinemia to maintain potassium levels. Heart rate and ECG will be monitored continuously during all glucose clamp studies. Participants will be instructed to empty their bladder prior to insulin infusion, at the end of the low dose

insulin infusion, and at the end of the high dose insulin infusion.

EndoRa and utilization (R_d) will be determined by stable isotope enrichment.[69] Total rates of appearance (R_a) and R_d will be calculated based on equations of Steele et al.[70] as modified by Debodo.[71] During hyperinsulinemic clamps, R_a and R_d, calculated using this approach, may be underestimated,[72] particularly during states of high glucose flux.[73] To avoid calculation errors due to glucose contamination, previously reported with ^{3}H-glucose tracers, we will use 6,6-dideuterated glucose as the tracer and analyze enrichment using MS. To minimize the effect of rapidly changing enrichment,[74] we will assess glucose turnover only when steady-state conditions exist, at the end of a 120-min tracer equilibration period and during the last 30 min of the clamp. (We define steady state as a period when the coefficient of variation of 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 the last 30 min of the clamp. To control for inter-individual variations in plasma insulin, the insulin sensitivity index will be calculated by dividing M by average steady-state insulin concentration. Tracer will be administered as a primed continuous infusion (3.6 mg/kg bolus followed by 0.06 mg/kg/min constant infusion) and will be added to the exogenous glucose infusion[73] to maintain plasma glucose enrichment throughout the study.

Laboratory Analyses

Clinical assays will be run in Vanderbilt CLIA-approved laboratories. Blood for research assays will be centrifuged for 20 min at 0^{o}C, and plasma/serum will be divided into aliquots, labeled, logged, and stored separately at -80^{o}C until sampling. Standard quality measures will be applied to all analyses including (but not limited to) tracking of inter-assay variability and inclusion of standards and internal controls.

EPHX2 genotyping: We will extract genomic DNA from whole blood using the AutoPure LS extraction system (Qiagen, Valencia, CA). *EPHX2* rs751141 (Arg287Gln) and rs41507953 (Lys55Arg) will be genotyped using TaqMan assays (Applied Biosystems, Foster City, CA). SDS v2.4 (Applied Biosystems) will be used to create cluster plots and identify sample-associated fluorescent markers for determination of genotype.

sEH activity and EETs: We will measure sEH activity in two ways. In tissue extracts, we will measure the rate of conversion of an added concentration of 14,15-EET (50 μM) to 14,15-DHET,[75] as well as the ratio of DiHOMEs to EpOMEs. The former is a true measure of enzyme activity, and we will compare the DiHOME/EpOME ratio to this gold standard. In plasma we will measure the molar ratio of DiHOME to EpOME. Plasma EETs, DHETs, EpOME, and DiHOMEs will be quantified using ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS).[28] Deuterated internal standards (Cayman Chemical, Ann Arbor, MI) will be added to each sample. For lipid extraction, three mL 0.15M KCl will be added to one mL plasma and extracted with eight mL of an acidified chloroform/methanol mixture at a two:one ratio. The organic phase will be collected and saponified (0.4M KOH in 80% methanol). EETs, EpOMEs, DHETs, and DiHOMEs will be separated by SiO_2 chromatography. SiO_2-purified EETs and EpOMEs will be hydrated to DHETs and DiHOMEs, respectively, in 9.5M acetic acid. EETs, EpOMEs, DHETs, and DiHOMEs will be analyzed by UPLC/MS/MS. The UPLC is equipped with an Acquity BEH C18 column 1.0 x 100 mm, 1.7 μm (Waters). To separate analytes, we will perform gradient elution starting with a 70% mobile phase A (15 mM ammonium acetate, pH 8.5) proceeding to a 60% mobile phase B (acetonitrile) over five min. Following gradient elution, columns will be washed for one minute with mobile phase B. The UPLC effluent will then be subjected to negative ion electrospray ionization using a triple quadrupole mass spectrometer, TSQ Quantum Vantage (Thermo Scientific, Hudson, NH), equipped with an electrospray source operating in negative ion-mode. Analytes and internal standards will be monitored using selected reaction monitoring.

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). Samples for C-peptide will be drawn into heparinized tubes containing 250 KIU Trasylol (Aprotinin) per mL of whole blood. C-peptide will be measured using RIA (Millipore).

Western blot analysis of pAKT/AKT, CD31, VEGF: Adipose and muscle samples will be homogenized in the presence of proteases and phosphatase inhibitors. For adipose tissue, homogenate will be centrifuged for 10 min at 10,000 g; the stromal vascular fraction from the pellet at will be recovered and the adipocyte fraction (top of the tube) will be used for Western blots. Homogenates will be denatured after mixing with Laemmli buffer and heated at 95°C for 5 min. Samples (30 µg of protein) will be resolved electrophoretically in 4-20% acrylamide gel and transferred to polyvinylidene difluoride membranes (Immobilon-FL, Millipore, Billerica, MA). Membranes will be blocked with Odyssey® blocking buffer and incubated overnight with antibodies: rabbit monoclonal antibody phospho-AKT, total AKT, (Cell Signaling, Danvers, MA), mouse monoclonal against CD31 (ThermoFisher Scientific, Rockland, IL), or mouse monoclonal against VEGF (Novus Biologicals, Littleton, CO). Membranes will then be incubated for one hour with Alexa Fluor 680-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) or IRDye® 800CW donkey anti-mouse (LI-COR Biosciences, Lincoln, NE). Membranes used for phospho-AKT will be stripped and re-probed for total AKT to obtain the ratio of pAKT/AKT expression. Odyssey® Infrared Imaging System (LI-COR Biosciences) will be used to detect fluorescence intensity. Band densities will be analyzed using NIH Image J software. Coomassie blue staining will be used as the loading control for other analyses.

Evaluation of PBMC immune cell populations: We will use flow cytometry to assess activated, memory, exhausted, and senescent CD4 and CD8 populations using surface markers CD38, HLA-DR, CD69, CD57, PD1, and CD45RO (as examples), iNKT cells using CD1d tetramer, as well as M1 (CD14+CD16+ with either CD86 or CD36) and M2 (CD163, CD301, and CD206) macrophage polarization.

7.0 Risks

Potential Risks

1. Frequent blood draws can lead to anemia.
2. Withholding BP medications may result in elevation in BP. We will monitor BP every one to three days after stopping or decreasing these medications. If the BP is too high (see **Protection against risk**) or there are symptoms of high BP, the study will be stopped and prior medications will be restarted.
3. Putting a catheter into a vein may cause bleeding, bruising, or infection (uncommon). We will use careful and sterile technique to minimize these side effects.
4. Adipose and muscle tissue biopsies may cause bleeding, bruising, pain, or infection (uncommon). Only trained investigators will complete these procedures. We will use sterile technique to minimize the risk of infection, and local anesthesia (lidocaine) will be used to minimize pain. We will monitor subjects for one hour after the biopsies.
5. Infusion of glucose may cause hyperglycemia. Symptoms of high blood sugar are hunger, thirst, frequent urination, dry mouth, and dry skin. Blood will be monitored during and for 2 – 3 hours after clamp completion to ensure blood sugar remains in 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.
6. Infusion of insulin 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, and numbness or tingling of the fingertips or lips, and trembling. Glucose (dextrose 20%) will be infused during periods of insulin administration, and glucose will be monitored every five min to maintain normal plasma glucose. After study completion, insulin will be stopped, a meal will be given, and intravenous glucose will be tapered. Blood glucose will be monitored for 2-3 hours after the study until it stabilizes within the normal range.
7. Eating a controlled diet for one day may be inconvenient.
8. GSK2256294 is an investigational drug. To date there are no safety concerns based on Phase I and II trials in humans. There has been skin irritation at the site of ECG lead placement during GSK2256294. There may be risks that we do not know about at this time. There is a theoretical risk that inhibiting sEH and increasing EETs could promote angiogenesis in pre-existing cancer.

Protections against risk

1. A nurse or physician will be present at all times during the study days.

2. Medication withdrawal (hypertensive subjects): Anti-hypertensive medications will be discontinued three weeks prior to the study day to avoid confounding effects on sEH activity or EETs. We have excluded individuals with resistant hypertension to minimize risk. Whenever appropriate, medications will be tapered before they are discontinued. During the washout period, BP will be measured every one to three days. If at any time the seated systolic BP is >170 mmHg or the seated diastolic BP >110 mm Hg or if a hypertensive subject develops symptoms of high BP 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 five percent of enrolled subjects may be excluded by these BP criteria. Excluded subjects will be replaced.

3. Subjects with a history of a hematocrit less than 35% will be excluded from study.

4. Only trained investigators will complete adipose and muscle tissue biopsies. We will use sterile technique to minimize the risk of infection, and local anesthesia (lidocaine) will be used to minimize pain. We will monitor subjects for one hour after the biopsies.

5. We will monitor glucose concentrations frequently during and immediately after hyperinsulinemic-euglycemic clamps. Should hypoglycemia occur, we will administer glucose.

6. We have limited the duration of GSK2256294, and we are excluding participants with a history of cancer or precancerous lesions. We are requiring that subjects be current with American Cancer Society Guidelines for the Early Detection of Cancer and also have PSA screening. We are excluding smokers.

7. We will monitor subjects actively for new adverse events using a standard questionnaire.

Should any adverse effects of GSK2256294 become known during the study, we will notify the IRB, we will revise the protocol and consent form as appropriate, and we will advise and re-consent study participants

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

All protocols will be reviewed and approved by the Vanderbilt Institutional Review Board (IRB) before any subject is enrolled. The Principal Investigators will closely oversee the protocol in conjunction with the dedicated research nurse and Co-investigators.

A DSMC will provide objective review of treatment results as they relate to human safety and data quality. The committee will be comprised of Naji Abumrad, M.D., The John L. Sawyers Professor of Surgery; Alvin C. Powers, M.D., Director of the Vanderbilt Diabetes Center, Professor of Medicine and Joe C. Davis Professor of Biomedical Science; and Dr. Yu Shyr, Harold L. Moses Chair in Cancer Research, Director of the Vanderbilt Center for Quantitative Sciences, and Director of the Vanderbilt Technologies for Advanced Genomics Analysis and Research Design (VANGARD). Dr. Abumrad will chair the committee. An important aspect of the membership of the DSMC is that all three members hold a primary appointment outside the Department of Medicine (Abumrad and Shyr) or lead a Research Center that reports directly to the Dean (Powers).

The DSMC will meet at least twice, prior to the initiation of study procedures to review the protocol and after the first five subjects have been enrolled to receive a safety and progress report. These reports will provide information regarding recruiting, safety reporting, and data quality. No early stopping is planned. The committee will assess safety data including hypertension or hypotension, hypoglycemia, common adverse events, hospitalizations, and other serious adverse events. Interim data will be provided to the committee by Drs. Brown and Yu. The randomization will be blinded and presented on a coded basis unless the Committee votes to receive unblinded data. The Committee will have the authority to modify the protocols or to terminate the study if it deems such actions to be warranted. The DSMC will provide summary reports to the investigators. Dr. Luther will convey such reports to the NIH (or FDA) as appropriate.

The DSMC will also receive quarterly reports of enrollment, protocol adherence, data quality, and adverse events via e-mail. The DSMC will review all serious adverse events. Any serious adverse event will be reported to the DSMC, IRB, NIH, and FDA within 7 days of the PI's notification of the event. 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, or e) is a congenital anomaly or birth defect. The DSMC may choose to become unblinded; however, it is expected that such unblinding would not occur without reasonable concern related either to patient safety or to data validity.

Non-serious, unexpected adverse events will be reported to the IRB at the time of annual continuing review. If required, appropriate changes will be made to the consent form. Adverse events 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 Not related, Unlikely, Possible, Probable, or Definite. Any protocol deviation will be reported at the time of annual continuing review. 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, 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.

9.0 Study Withdrawal/Discontinuation

If at any time during the study, a subject develops any symptoms related to study participation that subject will be withdrawn from the study. If, in the opinion of the investigator, a subject is non-compliant, that subject will be withdrawn from the study. Subjects who are withdrawn will be followed until symptoms have resolved.

10.0 Privacy/Confidentiality Issues

A unique identification case number will be used to protect the confidentiality of the study participants. Only case numbers will be included in spreadsheets used for the statistical analysis.

We will use the web-based Vanderbilt Research Electronic Data Capture (REDCap) system to design electronic data collection forms. These forms 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 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 predoctoral student in the laboratory.

11.0 Follow-up and Record Retention

All records will be retained for 7 years following publication of the data. After that time, records may be archived for an additional 5 years and then shredded.