Role of CD36 in Nutrient Delivery and its Dysfunction in African Americans

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1.0 Background

CD36 is highly expressed in skeletal muscle, heart and adipose tissue of mice [1] both on tissue cells and in the endothelium of blood vessels.[2, 3] Endothelial cells (EC) of large arteries have low CD36 while expression is high in EC from medium arteries, arterioles and capillaries. RNAseq data in humans show that aorta CD36 expression is 1/3 that of coronary arteries. CD36 content of the capillary endothelium is highest in heart, muscle and adipose tissues compared to other tissues.[2, 4] CD36 has a regulatory role in Fatty acids (FA) uptake and oxidation by heart and muscle [5-7] and its expression in EC could contribute to this role. **Our hypothesis is that EC CD36 is involved in vascular maintenance and regulation of "nutritive blood flow".**

Preliminary data

Endothelial dysfunction in humans carrying the minor allele (G) of coding CD36 SNP rs3211938 (Figs. 1-4): We recently examined effect of treatment with sildenafil, which blocks cyclic GMP (cGMP) degradation on insulin sensitivity in pre-diabetic subjects.[8] In another trial (NCT01334554) we examined effect on endothelial function of a 4-week sildenafil treatment in obese African American (AA) women with metabolic syndrome (MetS). Genotyping for CD36 single nucleotide [illegible] (SNP) rs3211938 was done retrospectively at the end of the trial using genomic DNA extracted from blood.[9, 10] Samples had 100% concordance rate and included validated controls for each group (G/G, G/T, T/T). Endothelial function was assessed [illegible] artery flow-mediated dilation (FMD). Linear regression was used to examine differences between treatment groups and effect of genotype.

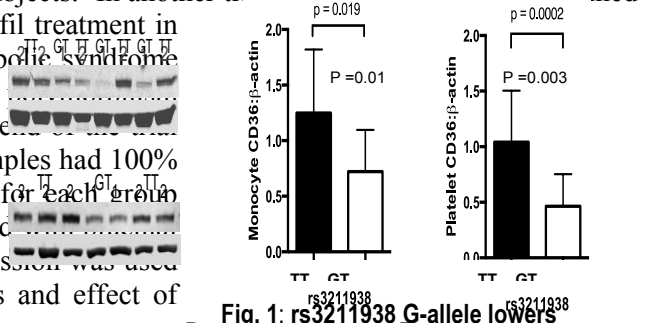


Fig. 1: **rs3211938 G-allele lowers CD36**: G carriers have 50% less CD36 on monocytes and platelets (also WB).

G-allele of coding SNP rs3211938 reduces CD36 **(Fig. 1):** The minor allele (G) of CD36 rs3211938 intro exon 10 and was reported to have a frequer and was ~13% in our recruited AA wome subjects with G-allele) was ~25% of th reduces CD36 protein by ~50% in monocyt

G-allele reduces cyclic GMP levels **(Fig. 2**) level in G-allele carriers impacts the NO-c were measured in blood. G-allele carrie (n=31) had 30% less cGMP suggesting low

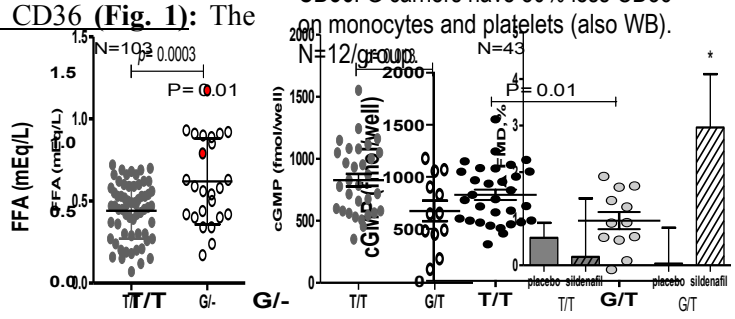


Fig. 2: **Rs3211938 G-allele lowers cGMP level.**

G-allele reduces endothelial function **(Fig. 3):** Endothelial dysfunction associates with less vasodilatory response.[12-14] We examined the impact of rs3211938 on FMD in GT and TT subjects matched for age and BMI and with no metabolic syndrome (MetS) according to ATP criteria[15] since MetS reduces endothelial function. FMD was 40% lower in GT (p=0.035) vs. TT indicating that the G-allele reduces the vasodilatory response to 5 min ischemia.

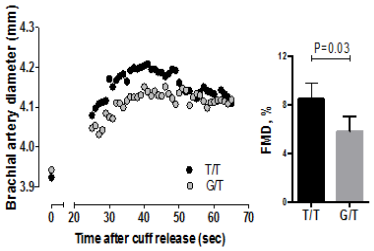
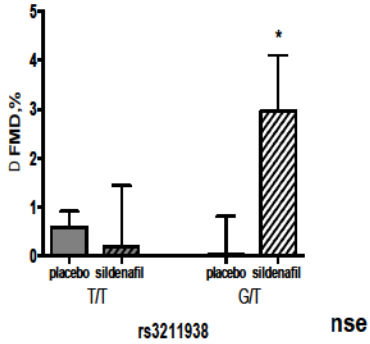


Fig. 3: **G-allele reduces endothelial function**. Left: Changes in brachial artery diameter before and after 5 min of ischemia. Right: Flow mediated dilation (FMD) is 44% lower in GT, n=8, than in T/T, n=9, p=0.03.

G-allele impacts response to sildenafil therapy **(Fig. 4):** AA women (n=46) with high insulin or 3 of 5 MetS traits participated in a trial that examined potential benefits of 4-week treatment with sildenafil vs. placebo in a double blind randomized study. We confirmed compliance by detecting sildenafil levels in subjects assigned the drug but not placebo. Forty women completed the study and 34 were genotyped for rs3211938 (6 withheld consent). We found a significant genotype/drug response interaction: Sildenafil improved FMD in G-

allele carriers but not in T/T controls, p=0.018.

In conclusion a genetic variant that reduces CD36 level that is prevalent in AA, diminishes endothelial function and impacts response to treatment with PDE5 inhibition. The primary goal of this project is to study the role of EC CD36 in vascular regulation. Our study will be the first to assess the impact of the CD36 gene on the risk of endothelial dysfunction and its role in mediating vascular effects of insulin and FAs. The work will provide fundamental information with health relevance considering the high prevalence of obesity and is particularly relevant in AA where obesity is about 1.5 fold higher than in whites; 68% of AA men and 82% of AA women being overweight or obese.



nse to sildenafil. AA women were given drug or placebo for 4 weeks. Endothelial function (% change in FMD) only improved in G-allele (GT)

Significance

Vascular stiffening and hypertension are major health care problems in Western societies and risk factors for stroke, myocardial infarction, and kidney and heart failure. One third of the adult population is hypertensive with another having "pre-hypertension" that commonly develops within few years into hypertension.[16-18] Vascular stiffening is also an early event in obesity-induced diabetes and cardiovascular disease and associates with worse long-term outcomes. African Americans (AA) are genetically predisposed to endothelial dysfunction, which contributes to the disproportionately higher burden of cardiovascular disease.[19-21] Compared with whites, healthy adults and adolescent AA have impaired microvascular vasodilatory function and greater large arterial stiffness.[19, 22]Identifying new targets and therapeutic approaches for preventing and treating endothelial dysfunction is of critical importance in the general population and especially in the AA subpopulation. Our study will be the first to assess impact of the CD36 gene on the risk of endothelial dysfunction and its role in mediating vascular effects of insulin and FAs. The work will provide fundamental information with health relevance considering the high prevalence of obesity and is particularly relevant in AA where obesity is about 1.5 fold higher than in whites; 68% of AA men and 82% of AA women being overweight or obese. [23]

2.0 Specific Aims

Our preliminary data show evidence of endothelial dysfunction in subjects carrying the G-allele of CD36 coding SNP rs3211938 that results in 50% reduction of CD36 levels in ~25% of AA. Subjects with endothelial dysfunction develop impairment of insulin's vascular actions and eventually reduced insulin sensitivity. Insulin induces microvascular recruitment via stimulation of NO-cGMP pathway, which facilitates nutrient flux, e.g., glucose to skeletal muscle. Elevated FAs impair insulin-stimulated microvascular recruitment and reduce insulin sensitivity. As our Vanderbilt colleague and collaborator (Dr. Wasserman) showed, chronic treatment with sildenafil increases vascularity and muscle glucose uptake in high fat fed mice. In humans, Drs. Shibao (PI) and Brown recently reported that a 3-month treatment with sildenafil improves insulin sensitivity in patients with impaired glucose tolerance.[8] More relevant to this project, we showed that endothelial dysfunction improves after 4-week treatment with sildenafil only in G-allele carriers (**Fig. 2**). **In this proposal, we will test the hypothesis that chronic treatment with sildenafil with and without the use of NO substrate, L-arginine, protects against FA induced impairment of endothelial function, improves insulin-stimulated microvascular recruitment, insulin sensitivity and glucose uptake in CD36 rs3211938 G-allele carriers.**

3.0 Inclusion/Exclusion Criteria

We will screen and genotype 120 subjects. We will study 40 subjects (30-G allele carriers and 10 non-carriers). We will conduct the study in AA population only. The characteristics of the subject population are detailed under the inclusion and exclusion criteria.

Inclusion criteria

- African American men and women.
- Age 18-50 years
- BMI 25-40 kg/m^2

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

- Diabetes type 1 or type 2, as defined by a FPG > 126 mg/dL or history of abnormal OGTT (two-hour plasma glucose > 200 mg/dL), or the use of anti-diabetic medication
- The use of nitrates or any disease that might require the use of nitrates
- Pulmonary hypertension
- Hypertension, defined as SBP/DBP >140/90 mm Hg or the use of anti- hypertensive agents
- Use of a PDE5 inhibitor for erectile dysfunction
- Use of an alpha blocker
- Pregnancy or breast-feeding. Women of child-bearing potential will be required to have undergone tubal ligation or to be using an oral contraceptive or barrier methods of birth control.
- The use of any potent CYP3A4 inhibitor.
- Cardiovascular disease such as myocardial infarction, 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
- Allergies to eggs, soy products and Intralipid® infusion
- History of serious neurologic disease such as cerebral hemorrhage, stroke, or transient ischemic attack
- History or presence of immunological or hematological disorders
- Impaired hepatic function (aspartate amino transaminase [AST] and/or alanine amino transaminase [ALT] > 3.0 x upper limit of normal range)
- Impaired renal failure (eGFR < 60 mL/min/1.73m^2)
- Hematocrit < 34%
- 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
- Treatment with chronic systemic glucocorticoid therapy (more than 7 consecutive days in 1 month)
- History of alcohol or drug abuse
- Treatment with any investigational drug in the one month preceding the study
- Mental conditions rendering a subject unable to understand the nature, scope and possible consequences of the study
- Inability to comply with the protocol, e.g., uncooperative attitude, inability to return for follow-up visits, unlikelihood of completing the study, and investigator discretion

4.0 Enrollment/Randomization

Recruitment and informed consent: We will recruit from the Nashville AA community for this planned study. The genotype frequency of the G allele in AA is ~ 25%, we conservatively estimate that we need to screen around 120 subjects to find 30 G allele carriers. As in previous studies, we will use radio public service announcements, word of mouth, social media, and distribution of ads to local municipal sites previously approved by the Vanderbilt Institutional Review Board. We will also use established Internet resources maintained by the Vanderbilt CTSA such as ResearchMatch and SubjectLocator. We may use a screening electronic survey to determine if the volunteer qualifies for the study. All subjects that we screen and genotype for this study will be offered the possibility to participate in a CD36 registry. We will use e-consent to consent the subjects.

5.0 Study Procedures

In our preliminary data, **(Fig. 4)** we observed improvement in endothelial function after a 4-week treatment with sildenafil only in G-allele carriers with metabolic syndrome (MetS). It is well established that MetS is associated with endothelial dysfunction.

In the proposed study, we will recruit AA subjects G-allele carriers that have no evidence of MetS, but have evidence of endothelial dysfunction **(Fig. 3)**. We will assess the effect of the PDE5 inhibitor on insulin-stimulated microvascular recruitment and insulin sensitivity after 4 weeks on sildenafil treatment under conditions of elevated blood FA levels to produce insulin resistance. We will also explore the added effect of the NO substrate, L-arginine. The study is designed to obtain information on the potential of enhanced NO-cGMP pathway to rescue endothelial function, insulin-stimulated microvascular recruitment, and to mitigate the deleterious effects of elevated FA on insulin sensitivity in G-allele carriers.

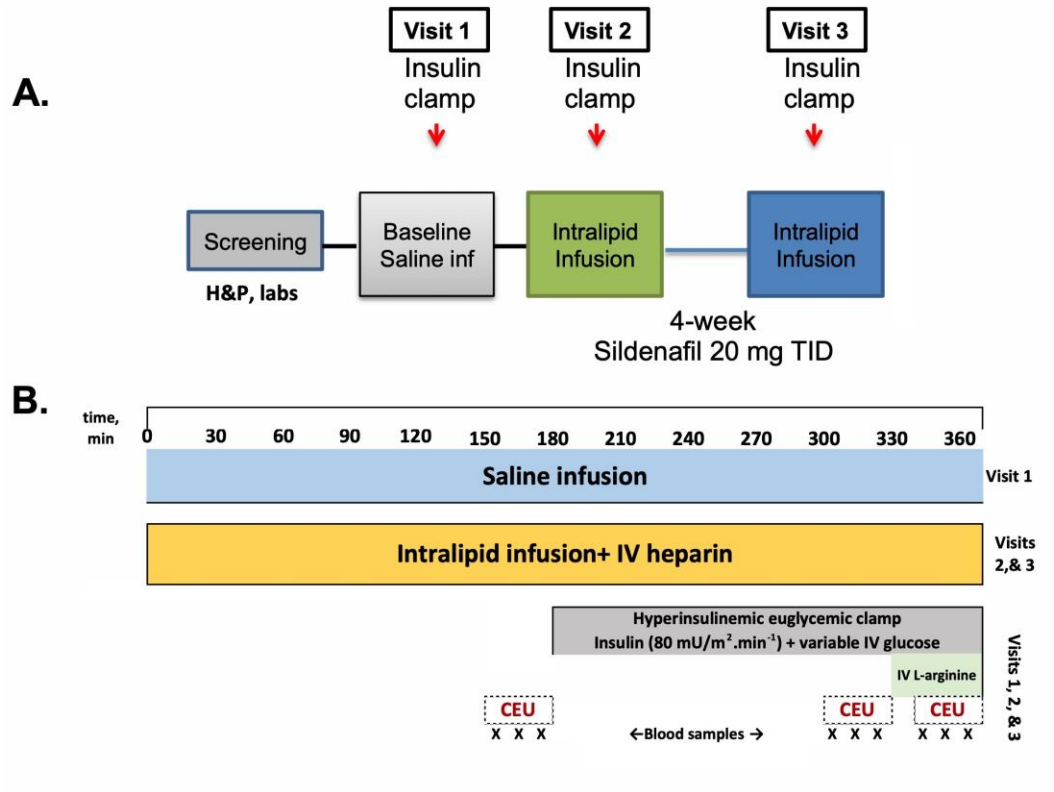


Figure 5 - Experimental Protocol: Panel A; study design, and panel B; infusion protocol. Each subject will undergo 3 insulin clamps.

Study Design:

We will invite subjects to participate in the following visits:

Pre-screening (this visit is optional, separate consent). We will collect a blood sample for DNA and other labs.

Screening visit: We will perform a history and physical, routine labs, including cell blood count, comprehensive metabolic panel, lipid profile, single ECG, and pregnancy test. Labs will be repeated if visit 1 is scheduled more than 3 months after the screening visit.

Visit 1: If the subjects meet inclusion criteria, this visit will occur after overnight fasting. They will receive a ~6-hour saline infusion in conjunction with approximately 3-hour "insulin clamp". L-arginine will be infused during the last 30 min of the clamp (see infusion protocol). Contrast enhanced ultrasonography (CEU) will be performed. Blood pressure (BP) and heart rate (HR) will be monitored throughout the study.

Visit 2 (will occur at least 2 weeks after visit 1)**:** This visit will occur in the CRC. Subjects are asked to fast overnight**.** In each study day, a catheter is placed into the antecubital vein for blood sampling. A second one is placed in the other antecubital vein for infusions (glucose, insulin, 20% Intralipid®, heparin and L-arginine, US contrast agent, (see infusion protocol). Blood pressure (BP) and heart rate (HR) will be monitored throughout the study. The Vanderbilt Investigational Pharmacy will be responsible for drug storage and dispensing logs.

Visit 3 (will occur after ~ 4-week treatment with sildenafil 20 mg po three times a day).The last dose of sildenafil 20 mg po will be provided the morning of the study and prior to initiation of the "insulin clamp".

Drug infusion protocol (visits 1, 2 and 3, Fig. 5 B): In **visit 1**, subjects will receive i.v. infusion of saline (45ml/h) for 6 hours. During **visits 2 and 3**, subjects will receive i.v. 20% Intralipid® (45 ml/hr.) and heparin (200 units prime followed by 200 units/hr) for a total of 6 hours as previously described[26]. Heparin is given to activate endothelial lipoprotein lipase and to facilitate the conversion of circulating lipids to FA. The "insulin clamp" will start at minute 180 min after initiating the continuous infusion of Intralipid® + heparin and will last for 3 additional hours. During the "insulin clamp", plasma glucose will be clamped at the same basal levels (~90-95 mg/dl) using variable infusion of IV glucose. Blood samples for plasma FA and insulin will be collected at -10, 180, 300, 315, 330, 340, 350 and 360 min. Plasma FAs will be collected with 1 mg/L of tetrahydrolipstatin to prevent in vitro lipolysis[27] and measured using the NEFA-HR(2) Kit (Wako). We will measure microvascular recruitment (MBV) in the forearm brachioradialis muscle with contrast enhanced ultrasonography (CEU, **Fig. 6**), and measurements of brachial artery diameter and flow as previously described, see techniques. Insulin infusion (up to 80 $mU/m^2.min^{-1}$) will be started at time 180 min and continued for 3-hours, with simultaneous variable infusion of glucose. Ultrasound measurements will be carried out between 300-330 min to examine the effect of sildenafil or placebo. During the last 30 min, we will infuse i.v. L-arginine (10 mg/kg/min for 30 minutes) [24]. Then, we will repeat ultrasound measurements at the end of the L-arginine infusion to examine effect of L-arginine + sildenafil or L-arginine + intralipids alone on these endpoints.

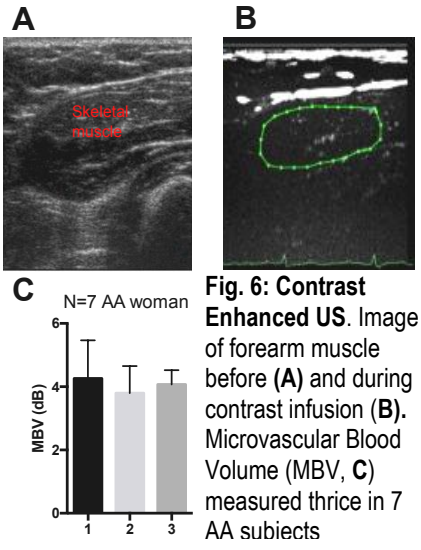


Fig. 6: Contrast Enhanced US. Image of forearm muscle before **(A)** and during contrast infusion (**B).** Microvascular Blood Volume (MBV, **C**) measured thrice in 7 AA subjects

Endpoints: The primary endpoint is the change in MBV (ΔMBV) during insulin infusion from baseline, an index of insulin-stimulated microvascular recruitment. We will compare ΔMBV in CD36 G-allele carriers with and without chronic sildenafil treatment (primary analysis) and with and without chronic treatment with sildenafil + L-arginine infusion. Secondary measurements are insulin sensitivity as measured by glucose infusion rate (M), insulin sensitivity index (M/I), and hemodynamics (BP and HR).

We hypothesize that chronic treatment with sildenafil will improve insulin-mediated increases in MBV in the CD36 G-allele carriers compared with placebo. We do not expect that sildenafil will affect insulin-mediated increases in MBV in non-carriers. Our preliminary data showed that 4-week treatment with sildenafil had no effect on flow-mediated dilation, a measurement of NO-dependent endothelial function in non-carriers. We expect that chronic treatment with sildenafil would improve insulin sensitivity as measured by the glucose infusion rate during the insulin clamp in G-allele carriers. Furthermore, we will determine if changes in insulin-stimulated microvascular recruitment can be potentiated with the addition of NO substrate such as L-arginine in G-allele carriers.

We do not anticipate difficulties in the conduct of the human studies. Dr. Shibao (PI) has experience infusing Intralipid® and heparin in humans (NCT02365285) and in assessing microvascular recruitment **(Fig.6)** and blood flow in skeletal muscle [30]. Dr. Abumrad NN has > 25-year experience in the conduct of the "insulin clamp" for evaluating insulin resistance and skeletal muscle uptake[31]. Further, we do not anticipate problems with recruitment of AA volunteers or with compliance as we previously reported [32]. Compliance will be monitored by measuring sildenafil concentration in plasma at the end of the study to prevent un-blinding.

Standard Techniques

Hyperinsulinemic euglycemic clamp ("insulin clamp"): The insulin infusion rate (up to 80 $mU/m^2.min^{-1}$) chosen will suppress hepatic glucose production and maximally stimulate peripheral glucose utilization allowing measurements of glucose utilization and insulin sensitivity [33].

Contrast-enhanced ultrasound (CEU): Images will be obtained using a linear-array transducer connected to an ultrasound (L9-3 mm transducer, iU22; Phillips). This equipment allows real-time imaging using low (0.08) and high (1.2) mechanical index as the contrast (microbubbles; Definity; Bristol-Myers Squibb) is infused (1.5 ml/min) for 10 min. At steady state (~4 min) the high mechanical index (1.2) used will destroy the microbubbles at the start of video recording. Switching to the low index (0.08) will let microbubbles resonate allowing real-time recording of vascular replenishment. Data are analyzed in our lab using QLAB software[34]. Our preliminary data in 7 AA **(Fig. 14)** show baseline MBV of 4.0±2.1 (3.8-4.3, SD 1.1 to 3.2).

Optional procedure

Harvesting of endothelial cells: A 3 J-shaped vascular guide wire will be advanced into antecubital vein and ECs will be retrieved from the wire tips by washing with cell dissociation buffer (Invitrogen) at 4 °C. Endothelial identity is verified by staining for endothelial antigens (CD31) and nuclear integrity by DAPI. We can safely recover ~800 EC/procedure. We will quantify protein levels of eNOS and p-eNOS by immunofluorescence. Two-control human umbilical vein EC slides will be included. Captured Images will be analyzed using Metamorph. EC protein expression/HUVEC will minimize the possible confound of differences in intensity of staining among different sessions (see letter of collaboration from Dr. A. Gamboa). For newly consented subjects, this procedure will be performed at the screening visit, if they provide verbal consent. Patients who are already enrolled and have completed the screening visit may return at any time to the clinic to have this procedure.

6.0 Risks

Blood Draws: Subjects may experience discomfort, bruising, and/or bleeding, or infection at the needle insertion site after a blood draw. Rarely, some people faint. Frequent blood sampling may cause anemia. We will exclude subjects with hematocrit <34%.

12-hour fast: Participants may experience hunger during a 12-hr fast, however this is a standard requirement by clinicians for accurate blood testing.

Ultrasound: The ultrasound test is a safe procedure that uses low-power sound waves. There are no direct risks from the ultrasound test.

PDE-5 inhibitors:
Sildenafil can cause severe hypotension when taken together with nitrates or sGC stimulator. We have included the use of this medication class as an exclusion criterion. We will monitor BP and HR throughout the study. Subjects with BP<90/50 mmHg and symptoms of hypotension at any pressure will be discontinued.

PDE5- inhibitors may cause prolonged erection (4 hours) or priapism (painful erections > 6 hours). In addition, PDE-5 inhibitors have been reported to cause headaches, dyspepsia, back pain, flushing, nasal congestion, and myalgia or limb pain. In prior studies by our group, headaches were more common among subjects randomized to a PDE-5 inhibitor than placebo.

Reports of changes in color vision (an effect of PDE-5 inhibition) are observed with sildenafil citrate.

An association of PDE-5 inhibitor use with acute hearing loss and non-arteritic ischemic optic neuropathy (rare) has been reported. The causal relationship between PDE-5 inhibitor use and hearing loss or AION is not clear, but subjects will be advised of a potential link.

We have reported edema as an unexpected, related AE in the first subject that we studied.

Hyperinsulinemic clamp:
There is a risk of hypoglycemia during the hyperinsulinemic euglycemic clamps. Glucose concentrations will be monitored every 5 minutes during the insulin clamp. We will infuse variable rate of 20% glucose to prevent hypoglycemia. Dr. Abumrad has more than 25 years' experience using this technique to assess for insulin sensitivity in humans. A CRC nurse and a physician will be present at all times during the insulin clamps.

Intravenous Cather Insertion:
Inserting a plastic catheter into the vein may cause a brief amount of pain and possibly a small bruise at the site, but will allow us to draw blood without repeated sticks throughout the study. It is also possible to develop an infection at the location of the catheter. It is uncomfortable to have the intravenous catheter in place for any length of time because it causes discomfort when the patient moves their arm.

Ultrasound contrast agent:
There is small risk of arrhythmias, chest pain, hypotension, and dyspnea with use of ultrasound contrast agents (microbubbles; Definity; Bristol-Myers Squibb). Subjects will be informed about this risk.

Intralipid®:
In some people, headache, dizziness, flushing, drowsiness, nausea, vomiting, or sweating may occur after taking Intralipid®. Other serious side effects include signs of infection (fever, persistent sore throat), pain/swelling/redness at injection site, pain/swelling/redness of arms/legs, bluish skin, sudden weight gain, shortness of breath and back/chest pain.

We will instruct the subjects to immediately call their doctor or the PI, Dr. Shibao (or designee) if any of these rare but very serious side effects occur: mental/mood changes, bone pain, muscle weakness, yellowing skin/eyes, dark urine, easy bruising/bleeding, severe stomach/abdominal pain, trouble breathing, or any symptoms of a serious allergic reaction, including: rash, itching/swelling (especially of the face/tongue/throat), severe dizziness, trouble breathing.

Heparin:
In some people, heparin can cause abdominal or stomach pain or swelling, back pain or backaches, bleeding from the gums when brushing teeth, blood in the urine, constipation, coughing up blood, dizziness, headaches, heavy bleeding or oozing from cuts or wounds, joint pain, stiffness, or swelling, unexpected or unusually heavy menstrual bleeding, unexplained bruising or purplish areas on the skin, unexplained nosebleeds, or vomiting of blood or material that looks like coffee grounds. These side effects are uncommon.

Rarely, heparin may cause blood under the skin (blood blister) at the place of injection, chest pain, chills or fever, fast or irregular breathing, irritation, pain, redness, or ulcers at the place of injection, itching and burning feeling, especially on the bottom of the feet, nausea or vomiting, numbness or tingling in the hands or feet, pain, coldness, or blue color of the skin on the arms or legs, peeling of the skin, puffiness or swelling of the eyelids or around the eyes, shortness of breath, skin color change, especially near the place of injection or in the fingers, toes, arms, or legs, skin rash, hives, or itching, tearing of the eyes, tightness in the chest, or trouble with breathing.

L-arginine:
Due to the hypertonicity of the solution, the medication needs to be administered via IV infusion. Extravasation has resulted in burn-like reactions and skin necrosis requiring surgical intervention. Excessive rates of infusion (e.g. < 30 minutes) may result in local irritation, flushing, nausea, or vomiting. Other side effects associated with this medication are headaches, numbness.

Breach of Confidentiality:
In the event of a security breach, confidential information may be stolen. We have strong security procedures in place to minimize the possibility of a breach. Although we cannot provide a 100% guarantee that the participant's data will be safe, our procedures minimize the chance that a breach would take place.

There is no benefit of the proposed research to the subjects per se. AA are genetically predisposed to endothelial dysfunction. Our study will determine if a genetic variation in the CD36 receptor associated with a ~50% decreased expression is associated with a reduction in insulin vascular actions and/or negative metabolic outcome such as reduced insulin sensitivity and glucose uptake in the setting of elevated FA. Furthermore, our study will determine if these abnormalities could be prevented with the use of sildenafil citrate.

7.0 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 PI will be responsible for ensuring both data integrity and for ensuring that study participants are safely cared for and that all adverse events (AEs) are noted, followed, and reported to the IRB. Any untoward medical event will be classified as an adverse event, regardless of its causal relationship with the study. An AE 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. Serious AEs

will be reported to Dr. Talat A. Ikizler, Catherine McLaughlin Hakim Professor of Medicine and Nephrology at Vanderbilt University, the Data and Safety Monitoring Officer (DSMO), and the IRB within 7 days of the PI's notification of the event. Non-serious, unexpected adverse events will be reported to the IRB at the time of the annual continuing review.

Data and Safety Monitoring Plan: Data and Safety Monitoring Plan: The protocol will be reviewed and approved by the Vanderbilt IRB before any subject is enrolled. The investigators will closely oversee the protocol in conjunction with the dedicated research fellow. A Data and Safety Monitoring Officer (DSMO) will provide objective review of treatment results as they relate to human safety and data quality. The officer will be Dr. Talat A. Ikizler. The DSMO will meet with the investigators prior to the initiation of the study to review the protocol and after one-third of the subjects have been enrolled in the study, to receive reports of the progress of the study. These reports will provide information regarding recruitment, safety reporting and data quality. No early stopping is planned. The DSMO will assess safety data including hypoglycemia, hypotension, common adverse events and other serious AEs. Interim data will be provided to the committee by Dr. Yu and the PIs (Drs. Shibao and Abumrad NN).

The DMSO will have the authority to modify the protocol or to terminate the study if it deems such actions to be warranted. The DSMO will provide summary reports to the IRB and the investigators.

The DSMO will review all serious AEs. Any serious AE will be reported to the DSMO, IRB, and NIH (and FDA if appropriate) as soon as possible, but not more than seven days from the investigators' awareness of the event. Any untoward medical event will be classified as an AE, regardless of its causal relationship with the study. An AE 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

Non-serious, unexpected AEs will be reported to the IRB at the time of annual review, unless we observe a pattern of such events, in which case we expect the DSMO will recommend earlier notification at the time of quarterly review. If warranted, appropriate changes will be made to the consent form. 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 Not related, Unlikely, Possible, Probable, or Definite. Any instance of non-compliance with the protocol will be reported at the time of annual review. Summary Reports will be submitted to the IRB at least annually and will contain a) The number of AEs 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 instances of non-compliance with the protocol and how each was handled.

8.0 Study Withdrawal/Discontinuation

Criteria for study withdrawal/discontinuation

- Drug-related toxicity
- Requirement for prohibited concomitant medications (see exclusion criteria)
- Pregnancy
- Request by subject to terminate treatment
- Clinical reasons believed to be life threatening by the physician, even if not addressed on the potential risk section (Section 7.0)

9.0 Statistical Considerations

Data Analysis Plan: We have chosen a sample size based on the power to detect differences in our primary endpoint, insulin-stimulated MBV as measured by CEU. Our preliminary data showed baseline MBV measure with a mean in the range of 3.8 to 4.3 and an SD in the range of 1.1 to 3.2 (average mean±SD: 4.0±2.1, Fig.6). In our previous study (preliminary data, **Fig. 4**), we did not observe any improvement in endothelial function after a 4-week treatment with sildenafil in non-carriers whereas in G-allele carriers endothelial function as measured by FMD improved by 40% (mean±SD: from 4.8±2.2 to 8.0±2.2). We assume sildenafil will have no effect on MBV among the non-carriers and sildenafil will increase CEU conservatively by about 30% among the G-allele carriers. A paired t-test will have 84% power to detect a 30% increase of CEU to 5.3 post-sildenafil treatment compared to placebo (4.0±2.1, mean±SD), assuming a within-subject correlation coefficient of 0.6 with a sample size of 24. The type I error rate is 0.05 and the study is on G carriers. We conservatively estimated 20% of dropout rate, and therefore we will enroll 30 subjects in this protocol. For insulin sensitivity (secondary endpoint), as previously shown[25] Intralipid® + heparin infusion decreased glucose infusion rate from 5.6±2.3 to 4.5±1.7mg/kg/min. Our study has 81% power to detect a similar difference using an SD for the within-subject difference of 1.8 (calculated using the higher SD of 2.3 assuming a within-subject correlation coefficient of 0.7).

Data Analyses: Standard graphing and screening techniques will be used to detect outliers and to ensure data accuracy. We will assess continuous outcomes for normality. If normality is violated, we will apply data transformation or consider non-parametric analysis methods. Summary statistics for both continuous and categorical variables will be provided by groups (intralipids, intralipids+sildenafil to describe the study sample. Our analysis will use mixed effect models with a random subject effect and treatment (intralipids vs. intralipids+sildenafil), genotype (G-allele carriers vs. non-carriers), and their interaction as fixed effects.

10.0 Privacy/Confidentiality Issues

All data will be collected specifically for the proposed research project. 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. PHI and access to the key for the ID numbers will only be viewable by members of the research team. Members of the research team will have access to the patient's medical record during the screening visit and throughout the study until the patient completes her participation in the study or meets any of the criteria for study withdrawal/discontinuation.

11.0 Follow-up and Record Retention

Research records will be maintained for at least three (3) years from the date the research is closed with the Vanderbilt University IRB. All research records will be accessible for inspection and copying by authorized representatives of the IRB, federal regulatory agency representatives, and the department or agency supporting the research.

All Health Insurance Portability and Accountability Act (HIPAA)-related documentation will be maintained for at least six (6) years from the date of the last use or disclosure of the Protected Health Information (PHI).

REFERENCES

1. Abumrad NA, el-Maghrabi MR, Amri EZ, Lopez E,Grimaldi PA. Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is

induced during preadipocyte differentiation. Homology with human CD36. *The Journal of biological chemistry*. 1993;268:17665-8.
2. Greenwalt DE, Scheck SH,Rhinehart-Jones T. Heart CD36 expression is increased in murine models of diabetes and in mice fed a high fat diet. *The Journal of clinical investigation*. 1995;96:1382-8.
3. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM,Stern DM. Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood*. 1998;91:3527-61.
4. Vistisen B, Roepstorff K, Roepstorff C, Bonen A, van Deurs B,Kiens B. Sarcolemmal FAT/CD36 in human skeletal muscle colocalizes with caveolin-3 and is more abundant in type 1 than in type 2 fibers. *Journal of lipid research*. 2004;45:603-9.
5. Bonen A, Dyck DJ, Ibrahimi A,Abumrad NA. Muscle contractile activity increases fatty acid metabolism and transport and FAT/CD36. *The American journal of physiology*. 1999;276:E642-9.
6. Ibrahimi A, Bonen A, Blinn WD, Hajri T, Li X, Zhong K, Cameron R,Abumrad NA. Muscle-specific overexpression of FAT/CD36 enhances fatty acid oxidation by contracting muscle, reduces plasma triglycerides and fatty acids, and increases plasma glucose and insulin. *The Journal of biological chemistry*. 1999;274:26761-6.
7. McFarlan JT, Yoshida Y, Jain SS, Han XX, Snook LA, Lally J, Smith BK, Glatz JF, Luiken JJ, Sayer RA, Tupling AR, Chabowski A, Holloway GP,Bonen A. In vivo, fatty acid translocase (CD36) critically regulates skeletal muscle fuel selection, exercise performance, and training-induced adaptation of fatty acid oxidation. *The Journal of biological chemistry*. 2012;287:23502-16.
8. Ramirez CE, Hui N, Yu C, Gamboa JL, Luther JM, Brown NJ,Shibao CA. Treatment with Sildenafil Improves Insulin Sensitivity in Prediabetes: A Randomized, Controlled Trial. *The Journal of clinical endocrinology and metabolism*. 2015:jc20153415.
9. Love-Gregory L, Sherva R, Schappe T, Qi JS, McCrea J, Klein S, Connelly MA,Abumrad NA. Common CD36 SNPs reduce protein expression and may contribute to a protective atherogenic profile. *Human molecular genetics*. 2011;20:193-201.
10. Love-Gregory L, Sherva R, Sun L, Wasson J, Schappe T, Doria A, Rao DC, Hunt SC, Klein S, Neuman RJ, Permutt MA,Abumrad NA. Variants in the CD36 gene associate with the metabolic syndrome and high-density lipoprotein cholesterol. *Human molecular genetics*. 2008;17:1695-704.
11. Ellis J, Lange EM, Li J, Dupuis J, Baumert J, Walston JD, Keating BJ, Durda P, Fox ER, Palmer CD, Meng YA, Young T, Farlow DN, Schnabel RB, Marzi CS, et al. Large multiethnic Candidate Gene Study for C-reactive protein levels: identification of a novel association at CD36 in African Americans. *Human genetics*. 2014;133:985-95.
12. Barrett EJ,Rattigan S. Muscle perfusion: its measurement and role in metabolic regulation. *Diabetes*. 2012;61:2661-8.
13. Gruber HJ, Mayer C, Mangge H, Fauler G, Grandits N,Wilders-Truschnig M. Obesity reduces the bioavailability of nitric oxide in juveniles. *Int J Obes (Lond)*. 2008;32:826-31.
14. Kim JA, Montagnani M, Koh KK,Quon MJ. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. *Circulation*. 2006;113:1888-904.

15. Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. The Expert Panel. *Arch Intern Med*. 1988;148:36-69.
16. Julius S, Nesbitt SD, Egan BM, Weber MA, Michelson EL, Kaciroti N, Black HR, Grimm RH, Jr., Messerli FH, Oparil S, Schork MA,Trial of Preventing Hypertension Study I. Feasibility of treating prehypertension with an angiotensin-receptor blocker. *The New England journal of medicine*. 2006;354:1685-97.
17. Narayan KM, Ali MK,Koplan JP. Global noncommunicable diseases--where worlds meet. *The New England journal of medicine*. 2010;363:1196-8.
18. Murray CJ, Atkinson C, Bhalla K, Birbeck G, Burstein R, Chou D, Dellavalle R, Danaei G, Ezzati M, Fahimi A, Flaxman D, Foreman, Gabriel S, Gakidou E, Kassebaum N, et al. The state of US health, 1990-2010: burden of diseases, injuries, and risk factors. *Jama*. 2013;310:591-608.
19. Hoffman RP. Metabolic syndrome racial differences in adolescents. *Current diabetes reviews*. 2009;5:259-65.
20. Kalinowski L, Dobrucki IT,Malinski T. Race-specific differences in endothelial function: predisposition of African Americans to vascular diseases. *Circulation*. 2004;109:2511-7.
21. Morris AA, Patel RS, Binongo JN, Poole J, Al Mheid I, Ahmed Y, Stoyanova N, Vaccarino V, Din-Dzietham R, Gibbons GH,Quyyumi A. Racial differences in arterial stiffness and microcirculatory function between Black and White Americans. *J Am Heart Assoc*. 2013;2:e002154.
22. Duck MM,Hoffman RP. Impaired endothelial function in healthy African-American adolescents compared with Caucasians. *The Journal of pediatrics*. 2007;150:400-6.
23. An R. Prevalence and Trends of Adult Obesity in the US, 1999-2012. *ISRN obesity*. 2014;2014:185132.
24. Creager MA, Gallagher SJ, Girerd XJ, Coleman SM, Dzau VJ,Cooke JP. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J Clin Invest*. 1992;90:1248-53.
25. Roden M, Stingl H, Chandramouli V, Schumann WC, Hofer A, Landau BR, Nowotny P, Waldhausl W,Shulman GI. Effects of free fatty acid elevation on postabsorptive endogenous glucose production and gluconeogenesis in humans. *Diabetes*. 2000;49:701-7.
26. Chai W, Liu J, Jahn LA, Fowler DE, Barrett EJ,Liu Z. Salsalate attenuates free fatty acid-induced microvascular and metabolic insulin resistance in humans. *Diabetes Care*. 2011;34:1634-8.
27. Krebs M, Stingl H, Nowotny P, Weghuber D, Bischof M, Waldhausl W,Roden M. Prevention of in vitro lipolysis by tetrahydrolipstatin. *Clin Chem*. 2000;46:950-4.
28. Colombo PC, Ashton AW, Celaj S, Talreja A, Banchs JE, Dubois NB, Marinaccio M, Malla S, Lachmann J, Ware JA,Le Jemtel TH. Biopsy coupled to quantitative immunofluorescence: a new method to study the human vascular endothelium. *J Appl Physiol (1985)*. 2002;92:1331-8.
29. Silver AE, Christou DD, Donato AJ, Beske SD, Moreau KL, Magerko KA,Seals DR. Protein expression in vascular endothelial cells obtained from human peripheral arteries and veins. *J Vasc Res*. 2010;47:1-8.
30. Marinos A, Celedonio JE, Ramirez CE, Gottlieb J, Gamboa A, Hui N, Yu C, Michael Stein C, Biaggioni I,Shibao CA. Time-Course Analysis of Flow Mediated Dilation for the

Evaluation of Endothelial Function After a High-Fat Meal in African Americans. *J Am Heart Assoc*. 2015;4.
31. DeFronzo RA, Tobin JD,Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *The American journal of physiology*. 1979;237:E214-23.
32. Johnson DA, Joosten YA, Wilkins CH,Shibao CA. Case Study: Community Engagement and Clinical Trial Success: Outreach to African American Women. *Clin Transl Sci*. 2015;8:388-90.
33. Fabbrini E, Tamboli RA, Magkos F, Marks-Shulman PA, Eckhauser AW, Richards WO, Klein S,Abumrad NN. Surgical removal of omental fat does not improve insulin sensitivity and cardiovascular risk factors in obese adults. *Gastroenterology*. 2010;139:448-55.
34. Sjoberg KA, Rattigan S, Hiscock N, Richter EA,Kiens B. A new method to study changes in microvascular blood volume in muscle and adipose tissue: real-time imaging in humans and rat. *Am J Physiol Heart Circ Physiol*. 2011;301:H450-8.

Addendum

SILDENAFIL 20 MG BLINDED CAPSULES
#0 SIZE (PURPLE OPAQUE)
IRB # 160955

Ingredients: Sildenafil 20 mg tablets # 100
(Teva, NDC 00093-5517-98)
Microcrystalline Cellulose, NF to cover
(PCCA Part 30-1130)
Capsule #1 Purple Locking (Gelatin) #100
(PCCA Part 30-3015)

Yield **100 capsules**

Starting Materials: Capsule machine
Size "0" purple caps

Compounding:

1. Don appropriate attire for compounding.
2. Place one sildenafil 20 mg tablet in each of 100 empty capsule bottoms.
3. Cover tablet with microcrystalline cellulose, NF making sure capsule is completely full.
4. Replace tops, clean capsules.
5. Complete record in IDS log book.
6. Package and label appropriately.

References: USP/NF Chapter 795 Pharmaceutical Compounding

Expiration Date: 6 months

Storage: Room temperature

Sample Labels:

Vanderbilt Investigational Drug Service
Blinded Sildenafil 20 mg caps #100
Dr. Cyndya Shibao
Lot# IDSXXXX Exp: XX/XX/XXXX
IRB # 160955 XX/XX