RACIAL DIFFERENCES IN VAGAL CONTROL OF GLUCOSE HOMEOSTASIS

NCT02365285
June 5, 2017

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1.0 Specific Aims

Obesity has a greater detrimental impact on the health of African American women (AAW) than on any other racial or gender group. Nearly 80% of AAW are overweight or obese.[1] Reduced insulin sensitivity is more prevalent in AAW than in white women and men of both races.[4-8] This condition puts AAW at increased risk for the development of Type 2 diabetes mellitus (T2DM) and explains its higher prevalence among AAW (15% versus 7% in whites, respectively).[2] The exact mechanism underlying these pathophysiological differences remains unknown. We found that obese AAW have decreased parasympathetic (PNS) nerve activity compared to whites (**Figure 2A**) whereas, sympathetic (SNS) activity was not significantly increased in this group (**Figure 2B**). Reduced PNS activity has been reported in AA children even prior to the development of obesity and its co-morbidities.[9, 10] Recent animal studies showed that the PNS confers protection against oxidation[11, 12] generated in response to different stimuli (ricin poisoning,[12] myocardial infarction,[13, 14] fulminant hepatitis[15]). This effect is in part mediated through the α7-nicotinic acetylcholine receptor (α7nAChR).[15, 16] In our cohort of AAW, PNS activity was directly correlated with insulin sensitivity even after controlling for differences in age, blood pressure, and visceral adiposity (**Figure 3**). Equally important, the decrease in insulin sensitivity was associated with increased oxidative stress as measured by plasma F2 isoprostanes levels. Similar associations between insulin resistance and oxidative stress are found in animal models[17] and in humans,[18] including a single study done in AAW.[19] Notably, a 4-week administration of galantamine, a central acetylcholinesterase inhibitor and positive allosteric modulator of α7nAChR, increases PNS tone and improves insulin sensitivity in high-fat fed mice.[20] Taken together these findings lead us to hypothesize that the decreased PNS activity in obese AAW has deleterious effects on oxidative stress and insulin sensitivity (Figure 1). We will test this hypothesis by two specific aims.

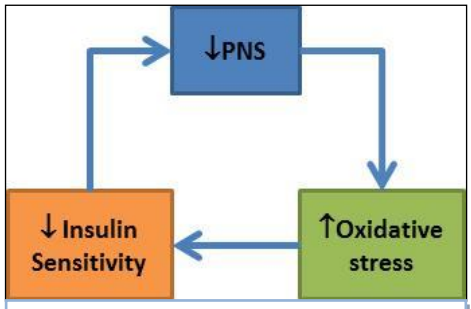

Figure 1,**Central hypothesis.** Decreased parasympathetic (PNS) activity in obese African American women, increases oxidative stress leading to reduced insulin sensitivity. Central restoration of PNS tone should result in decreased oxidative stress and improved insulin sensitivity

2.0 Background

Approximately 80% of AAW are overweight or obese, and this percentage may increase even more;[1] according to the National Health and Nutrition Examination Survey (NHANES) the prevalence of obesity in AAW increased by 4.3% in a 5-year period, whereas in whites remained the same. Multiple studies have reported that for the same body mass index (BMI), AAW had ~36-60% reduced insulin sensitivity compared to whites.[4-8] These racial differences are even present in early adolescence[21] and are not explained by differences in diet.[22] Reduced insulin sensitivity is a well-known risk factor for the development of T2DM and explains the higher prevalence of this condition among AAW (15% vs. 7% in whites).[2] T2DM is associated with a 4-year reduction in the life expectancy of AAW compared to whites.[3]

Table 1. Anthropometric, Body Composition and Metabolic Risk Factors in 229 Obese Women

	White Women (n = 123)	AA Women (n = 106)	P value
Age (y)	37 ± 8.3	40 ± 9.1	0.61
Height (cm)	165 ± 9.2	164 ± 7.9	0.19
Weight (kg)	96 ± 13.7	100 ± 16.4	0.06
Body Mass Index (kg/m^2)	35 ± 3.5	37 ± 4.8	0.001
Waist Circumference (cm)	105 ± 10.7	107 ± 11.4	0.13
Total cholesterol (mg/dL)	170 ± 30.4	173 ± 34.7	0.98
HDL-cholesterol (mg/dL)	47 ± 13.6	48 ± 13.3	0.47
LDL-cholesterol (mg/dL)	102 ± 26.6	109 ± 31.7	0.32
Triglycerides (mg/dL)	107 ± 61.7	83 ± 48.6	<0.001
hs-CRP (mg/L)	5 ± 5.8	7 ± 7.2	0.21
Glucose (mg/dL)	103 ± 42.9	98 ± 25.9	0.76
Insulin (mu/mL)	11 ± 8.6	14 ± 10.9	0.006
HOMA-IR	3 ± 3.9	4 ± 4.4	0.009
Lean Mass (kg)	49 ± 8.4	52 ± 8.3	0.003
Fat Mass (kg)	43 ± 7.8	45 ± 9.7	0.32
Body Fat (%)	47 ± 4.6	46 ± 4.5	0.16
VAT area (cm^3)	1646 ± 1007.5	1300 ± 661.7	0.006

VAT, visceral adiposity measured by Dual-Enery-X-Ray Absorptionmetry (Silver H., Shibao C. et al, manuscript in preparation)

Reduced insulin sensitivity is typically associated with increased visceral adiposity, elevated triglycerides, and reduced HDL-cholesterol;[23] AAW, however, have different clinical presentation. We recently studied 229 obese women (in collaboration with Dr. H. Silver) and found that AAW have reduced insulin sensitivity despite lower concentrations of triglycerides and 20% lower volume of visceral adiposity (**Table 1**). In addition, we have also shown that insulin sensitivity was inversely associated with F2-isoprostanes (**Table 2**). Similar observations have been previously reported by others,[24] including Gower et al[19] who found an inverse relationship between insulin sensitivity with protein carbonyls, a marker of oxidative stress, in AAW but not in white women.

African Americans have impaired parasympathetic function

Heart rate variability (HRV) provides a noninvasive and quantitative method of investigating cardiac autonomic modulation.[25] Spectral analysis techniques can distinguish between sympathetic and parasympathetic sources of HRV, as these rhythms occur at different frequencies. There is consensus that the relative power in the high frequency (HF_{rri}) band (>0.15 Hz) reflects PNS activity, whereas the ratio low frequency (LF_{rri}) to HF_{rri} reflects SNS modulation.[25] Using this technique, we found that obese AAW have reduced PNS activity compared to age and BMI-matched white women (**Figure 2A**). Similarly, Choi et al.[26] reported that young non-diabetic AA had low PNS activity compared to age-matched whites (log HF_{rri} =3.9±3.2 in AA vs. 5.3±4.7 ms^2 in whites, P<0.05), even in the absence of changes in SNS activity as measured by LF/HF ratio. Impaired vagal function in AA is not explained by the presence of hypertension (HTN) because Zion et al[9] reported low HF_{rri} even in normotensive young AA men without family history of HTN (ln HF_{rri}= 8.9 ± 1.1 vs. 9.7 ± 1.1 ms^2; P = 0.006). Reduced HF_{rri} was also reported in AA independently of differences in physical activity and psychological factors.[27] Low HF_{rri} has been also reported in young AA women[28] and AA children.[10]

Parasympathetic function is an important regulator of oxidative stress and inflammation

Recent studies showed that the PNS confers protection against oxidation and has also been implicated in the regulation of inflammation (cholinergic anti-inflammatory pathway).[29]In animal models stimulation of the PNS decreases oxidative stress generated in response to different stimuli (ricin poisoning[12], myocardial infarction,[13, 14]fulminant hepatitis[15]). This effect is in part mediated through the α7nAChR,[15, 16, 30]and also via vagal afferents.[29] Sub-diaphragmatic surgical vagotomy in rats exposed to endotoxin showed increased oxidative stress in the brain,[11] and direct electrical stimulation of the peripheral vagus nerve during lethal endotoxemia inhibited TNF synthesis in the liver.[29]These findings are important because increased oxidative stress and sub-clinical chronic inflammation have been implicated in the pathogenesis of insulin resistance.[17, 18]

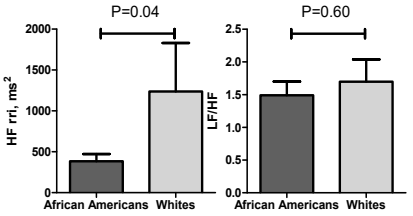

Figure 2. **A** PNS activity (HF_{rri}) is decreased in AAW compared to whites **B.** Sympathetic modulation (LF/HF) was similar between groups (mean±sem, N=28 AAW vs. 12 whites).

African Americans have increased oxidation in response to lipid infusion

Lopes et al[31] compared oxidative stress induced by standardized acute hyperlipidemia (4 h infusions of Intralipid®) in 30 subjects (15 AA and 15 whites, matched by age, gender and BMI). They found that hyperlipidemia-induced oxidation, as measured by plasma F2-isoprostanes, was at least 2-fold higher in AAs compared to whites. In this cohort, AAs were found to be more insulin resistant compared to whites as measured by fasting glucose, insulin levels and the HOMA index.

Table 2. Spearman correlations with insulin sensitivity in 41 AAW

Clinical Characteristics	r	P value
Age, years	0.24	0.88
Body mass index, Kg/m^2	-0.22	0.16
Waist circumference, cm	-0.21	0.18
Fat mass, Kg	-0.19	0.25
Fat free mass, Kg	-0.02	0.89
Percentage fat mass, %	-0.13	0.42
Visceral fat mass, Kg	-0.16	0.31
Systolic blood pressure, mm Hg	-0.15	0.36
Diastolic blood pressure, mm Hg	-0.09	0.59
Heart rate, bpm	0.13	0.43
Spectranalysis of BP & HR		
HF rri, ms*ms	0.32	0.04
LF to HF ratio (LF/HF)	-0.04	0.82
Marker of oxidative stress		
F2-Isoprostanes, ng/ml	-0.47	0.01

HF rri= absolute power in high frequency range.

Central acetylcholinesterase inhibition improves insulin sensitivity in an animal model of obesity

Satapathy SK et al[20] examined whether galantamine, a central acetylcholinesterase inhibitor and allosteric modulator of α7nAChR alleviates obesity-related inflammation and its metabolic effect. After 8 weeks on a high-fat diet, mice were treated with either galantamine (4 mg/kg intraperitoneally or saline) injections for 4 weeks. Galantamine effectively reduced fasting plasma glucose and insulin, improved insulin resistance and obesity-associated inflammation by reducing the levels of inflammatory cytokines. Differences in oxidative stress were not assessed in this study.

3. Preliminary Results

Parasympathetic activity is decreased in African American Women

We studied 28 obese AAW and 12 white women matched by age (42±10 vs. 37±9 years old), BMI (36±3 vs. 35±4 Kg/m^2), blood pressure (SBP 126±13 vs. 126±14 mm Hg) and heart rate (70±11 vs. 64±9 bpm). PNS activity was measured with spectral analysis of HRV. Our results showed that AAW have decreased PNS activity compared to white women (Figure 2A) whereas sympathetic activity was not increased in AAW compared to whites (Figure 2B). In a separate protocol, we studied 18 AAW and 13 white women matched by age (38±7 vs. 38±9 years old) and BMI (35±4 vs. 35±3 Kg/m^2), respectively. Racial differences in SNS activity were evaluated with 2 techniques. First, we determined the decrease in blood pressure during autonomic withdrawal with the ganglionic blocker trimethaphan.[32] At baseline, supine SBP was similar between groups, but the fall in SBP induced by autonomic withdrawal with trimethaphan was lower in AAW as compared to obese white women (-13±13 mm Hg vs. -24±15mm Hg, P=0.04). Second, in a subgroup of 8 subjects (4 AAW and 4 whites), matched by BMI, we measured direct muscle sympathetic nerve activity with microneurography technique. Sympathetic activity was lower in obese AAW women compared to whites (22±4 vs. 37±5 burst/min, p=0.06).

Insulin sensitivity is inversely associated with parasympathetic activity in African American Women

We studied 41 non-diabetic obese AAW (42±9 years old, BMI 39±5 Kg/m^2,% fat mass 54±6, SBP/DBP128±14/79±9 mm Hg). All subjects underwent measurements of insulin sensitivity using the frequently sampled intravenous glucose tolerance test analyzed using a modified minimal model (MINMOD) formula.[33] Parasympathetic and SNS activity were measured with spectral analysis of HRV according to established guidelines.[34, 35] We determined levels of F2-isoprostanes using GC/MS,[36] (Table 2). We found that ~ 50% of the subjects were insulin resistant (defined by insulin sensitivity index < 3 min-^{1}/uU per ml).[37] Insulin sensitivity was directly correlated with indices of PNS activity (HF_{rri}), (Figure 3). In a multiple linear regression analyses, this relationship remained significantly even after adjusting for differences in age, blood pressure and BMI (Ra^2=0.27, P<0.01). Measurement of sympathetic activity (LF/HF) was not associated with insulin sensitivity. Of note, levels of F2 isoprostanes, a marker of oxidative stress, were inversely associated with insulin sensitivity as well, (Table 2).

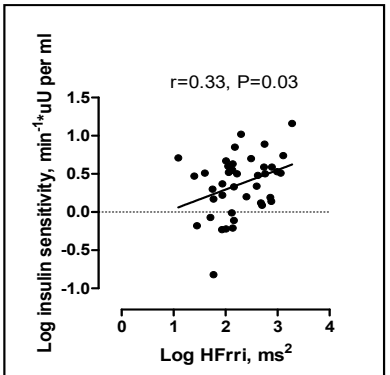

Figure 3. Association between insulin sensitivity and PNS activity (N=41 AAW)

4.0 Inclusion/Exclusion Criteria

Inclusion Criteria

- Female
- African American or white (race will be self-defined, but only subjects who report both parents of the same race will be included)
- 18-60 years old
- BMI 30-45 Kg/m2
- Not pregnant or breastfeeding

Exclusion Criteria

- Pregnant or breastfeeding
- Diabetes diagnosis (defined by the American Diabetes Association (ADA) criteria)[38]
- Cardiovascular disease such as myocardial infarction within 6 months prior to enrollment, presence of angina pectoris, significant arrhythmia, congestive heart failure (LV hypertrophy acceptable), deep vein thrombosis, pulmonary embolism, mitral valve stenosis, aortic stenosis, or hypertrophic cardiomyopathy.
- Arrhythmia (first-, second-, and third-degree AV block)
- Significant weight change >5% in the past 3 months
- Impaired hepatic function (AST and/or ALT >1.5X upper limit of normal range)
- Impaired renal function (eGFR <60ml/min)
- Users of strong inhibitors of CYP3A4 or CYP2D6

- Users of other acetylcholinesterase inhibitors such as pyridostigmine or bethanechol
- History of alcohol or drug abuse
- Mental conditions rendering the 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, and unlikelihood of completing the study
- Steroid use within 6 weeks prior to study entry
- 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
- Discretion of the investigator

5.0 Enrollment/Randomization

We will use established internet resources such as ResearchMatch, an IRB-approved volunteer database maintained by Vanderbilt University. We will also use newspaper and radio advertisements.

6.0 Study Procedures

6.1 Specific Aim 1 will test the hypothesis that acute central acetylcholinesterase inhibition will restore PNS activity and reduce oxidation in AAW compared to white women.

Rationale. Obese AAW have decreased PNS activity compared to whites.[9, 10, 26] Continuous lipid infusion that causes cardiovascular autonomic imbalance (decrease in PNS activity and increase in SNS activity[39]) induces a greater increase in oxidative stress in AA compared to whites.[31] Considering that PNS protects against oxidation and that central acetylcholinesterase inhibitors has been shown to suppress oxidative stress in animal models.[40] We propose to test the hypothesis that the central acetylcholinesterase inhibitor galantamine will attenuate oxidation in response to lipid infusion in obese AAW compared to white women.

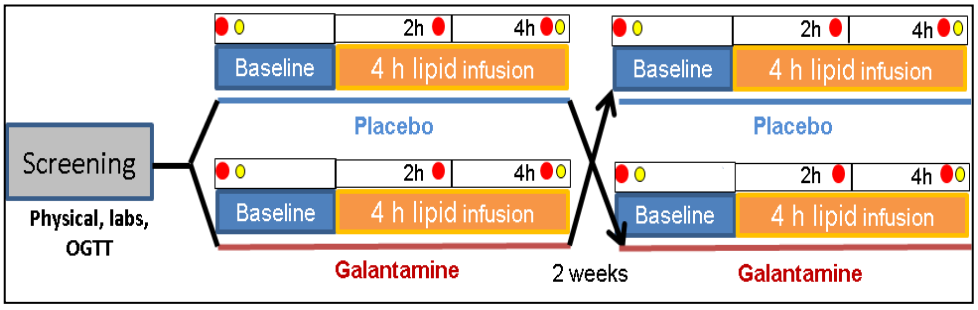

Figure 4. AIM 1 Study design.
red circle (blood collection for F2-isoprostanes, triglycerides, free fatty acids and inflammatory cytokines (TNF,IL-1β, IL-18, IL-6).
yellow circles (urine collection for F2-isoprostanes)

Study protocol. This will be a 2X2 cross-over design (**figure 4**).

We will enroll 24 subjects (12 obese AAW and 12 obese white women); subjects will be matched by age, BMI, and blood pressure. **Screening visit:** After informed consent is obtained, subjects will be invited to come to the clinical research center (CRC) for a screening visit (**screening visit 1**) that consist of a complete history, physical exam, lab work (fasting electrolytes, liver enzymes, creatinine, complete blood count, lipid panel, serum beta-hCG for women of child-bearing age), and ECG. In a separate visit (**screening visit 2**), subjects will return to the CRC to complete a 75g oral glucose tolerance test. For subjects who participated in specific aim 2, we will repeat screening visits 1 and 2 if more than 3 months have passed since the first screenings.

Study visits: Subjects who qualify will be admitted the day of the study in the CRC **(study visit 1)**. Blood samples will be obtained for DNA analysis.A 24-hr urine collection will be obtained for sodium, creatinine, and F2 isoprostanes measurements. After an overnight fast with the volunteer supine, an intravenous catheter will be placed in one arm for blood sampling and in the contralateral arm for lipid infusion. Body composition will also be determined with dual-energy X-ray absorptiometry (Lunar IDXA, GE Healthcare, CT, USA). We will obtain cardiovascular autonomic measurements with spectral analysis of HRV and blood pressure variability (BPV). The latter was previously validated by our group as a reliable measurement of SNS activity[35] (**standard**

techniques). The following parameters (PNS activity: HF_{rri}; SNS activity: $LF_{sbp,}$ ration LF/HF_{rri}) will be obtained. We will also collect measurements of microvascular circulation with contrast-enhanced ultrasound technique and flow-mediated dilation (**standard techniques**). At the end of this period, blood samples will be collected for non-esterified free fatty acids (NEFA), triglycerides, F2 isoprostanes and cytokines. Then subjects will be randomly assigned to drug order using a permuted-block randomization algorithm. The assignment to the treatment group will be blinded to the investigators and subjects. The Vanderbilt Investigational Pharmacy will be responsible for the storage, preparation, and labeling of all investigational agents [Intralipids®, heparin, and study drugs (16 mg p.o. galantamine and placebo)] and for maintaining accurate drug storage and dispensing logs. The blinded medication (single dose) will be administered in the morning, following the protocol of Lopes et al[31]. Intralipid® 20% (Baxter Healthcare Corp. Glendale, CA) will be infused at 0.8 mL/m^2/min for 4h. An initial heparin bolus of 1000 U will be followed by 200 U/h infusion during the 4-h period to activate endothelial lipoprotein lipase and accelerate hydrolysis of fatty acids from the glycerol backbone of the triglycerides. Blood samples will be obtained at baseline, 2h, and 4h for triglycerides, NEFA, F2 isoprostanes, and inflammatory cytokines (TNF, IL-1β, IL-18, IL-6). A urinary sample will be obtained at 4h for measurements of urine F2 isoprostanes. Cardiovascular autonomic measurements will be repeated at 2h and 4h. Microvascular circulation with contrast-enhanced ultrasound will be repeated at 4h. We will measure flow-mediated dilation at 2 and 4 hours. After the study is completed, we will allow a 2-week washout period before the subject is admitted to the CRC for a second study day **(study visit 2)** when all the procedures described above will be repeated, except for DEXA scan.

Rationale for the use of galantamine. Galantamine hydrobromide (Razadyne®) is a competitive acetylcholinesterase inhibitor which increases the availability of acetylcholine. Okazaki et al[41] showed that central acetylcholinesterase inhibition increases PNS tone in an animal model as measured by the decrease in heart rate and HF_{rri}. Galantamine has been approved by the Food and Drug Administration (FDA) for the treatment of mild to moderately severe Alzheimer's dementia. We chose the dose of 16 mg p.o. based on pharmacokinetic studies in normal volunteers and patients with dementia of Alzheimer type.[42-45] This dose exhibits linear pharmacokinetics after oral administration; the oral bioavailability is about 90%,[42] the maximum concentration is achieved at 1 hour post-administration, and it has a short half-life of 7 hours. **The FDA has exempted our studies from the need of an Investigational New Drug Application (IND).**

Endpoints: The primary analyses will focus on the increase in plasma F2-isoprostanes levels with lipid infusion (∆ISO) during placebo versus galantamine. Secondary analyses will focus on the increase in urinary F2-isoprostanes, inflammatory cytokines, NEFA, triglycerides and cardiovascular measurements of PNS tone (HF_{rri}) and SNS tone ($LF_{sbp,}$ ration LF/HF_{rri}) with lipid infusion during placebo versus galantamine.

Sample size and power calculation: sample size was calculated based on the ability to detect a difference in ∆ISO measured in the same subjects during treatment with placebo versus galantamine. Lopes et al[31] reported that mean and standard deviation of ∆ISO was 5±2.9 in whites and 12±2.6 in AAs. We observed a standard deviation of 2.02 for F-2 isoprostanes (preliminary data). A sample size of 12 would provide 85% power to detect a 1.5 mmHg difference in whites (30% improvement in ∆ISO), with a standard deviation of differences of 1.55 (assuming a correlation of 0.7 between two repeated measurements in the same subject) using a paired t-test with a 0.05 two-sided significance level. The study has 99% power to detect a 30% improvement in ∆ISO in AA. For comparison modest caloric restriction in women improves F2-isoprostanes levels by 50%.[46]

Interpretation and alternative approach. Previous studies in animal models showed that the stimulation of the PNS decreases oxidative stress. However, this effect in humans is largely unexplored. If our hypothesis is true then restoration of PNS activity will greatly attenuate lipid-induced oxidation in AAW compared to white women. We also anticipate that restoration of PNS tone in AAW will improve sub-clinical inflammation as previously observed by Tracey KJ et al[29] in animal models. It is well established that lipid infusion induces activation of the SNS via increases in NEFA levels.[39] We have designed our study to measure both branches of the autonomic nervous system and will account for changes in SNS activity with our intervention, in the final analysis. Central acetylcholinesterase inhibitors can potentially induce bradycardia. This side effect has been reported to be increased mostly with donezepil (Aricept®) because of its long half life (~80 hours).[47] For comparison galantamine (our study drug) has a short half-life of 7 hours. The risk of bradycardia will be minimized by excluding subjects with AV blockade diagnosed during the screening visit. There is a possibility that acute

restoration of PNS activity may not be enough to suppress lipid-induced oxidation. In specific aim 2, we will determine if chronic restoration of PNS activity improves oxidation. Dr. Naji Abumrad (co-mentor) et al[48] reported that F2-isoprostanes levels are 10 times higher in adipose tissue compared to plasma and decreased within 1 week after gastric bypass surgery. In specific aim 2, we will obtain fat pad biopsies and will measure changes in oxidative stress in adipose tissue using this technique.

6.2 Specific Aim 2. We will test the hypothesis that chronic restoration of PNS activity with a central acetylcholinesterase inhibitor improves insulin sensitivity and reduces adipose tissue oxidation in obese AAW compared to white women. **Rationale** Our preliminary data showed that obese AAW have decreased PNS activity compared to whites; PNS activity was positively correlated with insulin sensitivity, and the decrease in insulin sensitivity was associated with increased oxidative stress as measured by plasma levels of F2-isoprostanes. Treatment with a central acetylcholinesterase inhibitor that increase PNS activity has been shown to improve insulin sensitivity and inflammation in an animal model of obesity,[49] but it is still unknown whether similar effects occur in humans. We will test the hypothesis that chronic restoration of PNS activity improves insulin sensitivity and reduces adipose tissue oxidation in obese AAW.

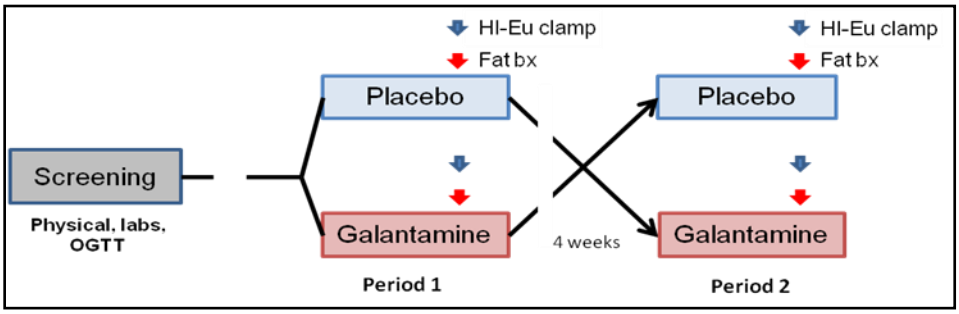

Figure 5. AIM 2 Study Design. OGTT, Oral Glucose Tolerance Test, HI-Eu clamp, hyperinsulinemic euglycemic clamp. Fat bx, fat biopsy is optional. Galantamine dose (8 mg/day), period 1 and period 2 will last 4 weeks. The wash-out period is 4 weeks.

Study protocol. This is a 2X2 cross-over design (**Figure 5**). We will enroll 28 subjects (14 obese AAW and 14 obese white women). Subjects will be matched by age, BMI, and blood pressure. After informed consent is obtained, subjects will complete **screening visits 1 and 2** as previously described in section 6.1. For subjects who participated in specific aim 1, we will only repeat screening visits 1 and 2 if more than 3 months have passed. Subjects who will be screened for the first time will have the body composition assessment with DEXA scan during the study days only. Subjects who meet inclusion criteria will report to the CRC in the morning in the fasting state to undergo measurements of insulin, glucose levels, and serum beta hCG (only women of child-bearing potential). Subjects will be randomized to drug order using a permuted-block randomization algorithm. The investigational pharmacist will provide a 4-week supply of study drug (**period 1, Figure 5**). The dosage of galantamine will be slowly escalated to increase tolerability starting with 4 mg p.o. twice a day (bid) for 4 weeks (8 mg/day). Each subject will be provided with the study medication, a medication diary, and verbal and written instructions. Follow up telephone calls will be placed one, three, and seven days following randomization and then weekly thereafter. Subjects will be instructed on how to keep their diet constant. After subjects complete the 4-week treatment period, they will be admitted the day of the study to the CRC. They will be requested to bring their medication diary and pill bottles. A pill count will be performed. We will use structured case report forms to assess compliance, concurrent medications, medical conditions, and adverse events.

The study will start after a 12-hour, overnight fast. Subjects will receive the morning dose of the study drug. We will obtain blood samples for measurements of plasma F2-isoprostanes, inflammatory cytokines, and pancreatic polypeptide (marker of PNS activity). We will obtain blood samples for serum beta hCG (only women of child-bearing potential). We will obtain a urine sample for F2-isoprostanes. We will also obtain baseline blood pressure and cardiovascular autonomic measurements (PNS activity assessment: HF_{rri},; SNS assessment: $LF_{sbp,}$ ratio LF/HF_{rri}). Subsequently, the subject will undergo a one-step hyperinsulinemic-euglycemic clamp (**standard techniques**). Subjects will undergo a fat-pad biopsy for measurement of F2-isoprostanes in adipose tissue (patient can opt-out from fat biopsy) and body composition with dual-energy X-ray absorptiometry (Lunar IDXA, GE Healthcare, CT, USA, **standard techniques**). After completion of **period 1**, subjects will stop the medication,

wash out drug for 4 weeks, and then begin the next treatment period (**period 2**) in which all the procedures described above will be repeated.

Endpoints: The primary analyses will focus on insulin sensitivity as measured by glucose infusion rate (M), insulin sensitivity index (M/I),during hyperinsulinemic-euglycemic clamps. Secondary endpoints will focus on measurements of F2 isoprostanes in plasma and adipose tissue (if fat pad biopsy is obtained), fasting insulin, fasting glucose, systolic and diastolic blood pressure, cardiovascular measurements of PNS tone (HFrri) and SNS tone ($LF_{sbp,}$ ratio LF/HF_{rri}), pancreatic polypeptide, and inflammatory cytokines.

Sample size and power calculation: Sample size was calculated based on the ability to detect a difference in glucose infusion rate measured in the same subjects during treatment with placebo versus galantamine. We observed a standard deviation of 11.4 in glucose infusion rate from a previous study.[7] A sample size of 14 in each racial group would provide 85% power to detect a 10 μmol/Kg FFM/min difference in glucose infusion rate (30% improvement) with a standard deviation of differences of 11.4 (assuming a conservative correlation of 0.5 between two repeated measurements in the same subject) using a paired t-test with a 0.05 two-sided significance level.

Interpretation and alternative approaches. If our hypothesis is correct then restoring PNS tone with the central acetylcholinesterase inhibitor, galantamine, will produce a greater improvement in insulin sensitivity and adipose tissue oxidation in obese AAW compared to white women. Furthermore, we will also determine if restoration of PNS tone will improve obesity related inflammation. The effect of galantamine on insulin sensitivity could be explained by mechanism other than a reduction in adipose tissue oxidation. The PNS is known to regulate hepatic glucose production in dogs,[50] and galantamine decreases hepatic fat deposit in high-fat fed mice.[49] To determine changes in insulin sensitivity, we chose to perform a one-step hyperinsulinemic clamp technique. The effect of galantamine on insulin sensitivity could be explained by mechanism other than restoration of PNS tone. In a study by Satapathy SK et al[20], galantamine also induces weight loss in high-fat fed mice. We designed our study to control for this confounding effect; we will advise our subjects not to change their diet while in the study. Patient's weight will be collected in each study visit, and we will adjust for changes in weight, in the final analysis.

Statistical Considerations

Data analysis plan: 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 randomization groups to describe the study sample. These basic data analyses will be conducted for **both study aims.** We are using a 2x2 cross-over design in both study aims. Although we have designed the study to avoid carryover effect, we will test for carryover effect using the T-test approach described in Section 2.3 (page 21) of Jones and Kenwood.[51] If there is evidence for a carryover effect, we will only use data collected before the crossover to estimate the treatment effects. This would result in a loss of power; however it would ensure valid estimates. Most likely, there will be no carryover effect due to the carefully chosen washout. We will use mixed-effect models with a random subject effect and treatment (galantamine versus placebo), race (AA versus whites), and their interaction as fixed effects. In Aim 1, the time effect (baseline, 2 h, and 4 h into the lipid infusion) will also be included as a fixed effect in the model. We will use an autoregressive model of order 1 [AR(1)] or other plausible covariance structures for the error covariance. In addition to evaluating these fixed effects using regression analysis, we will calculate within-subject mean differences and 95% confidence intervals for galantamine versus placebo comparison within each racial group and test for treatment effect using paired t-test or sign rank test as appropriate.

Standard Techniques

Hyperinsulinemic-euglycemic clamps. Insulin infusion rates were chosen to suppress hepatic glucose production and maximally stimulating peripheral glucose utilization during the infusion (80 mu/m^2/min) in insulin-resistant subjects.[52] A priming insulin dose will be given at the time of the insulin infusion change. Plasma glucose will be measured every 2.5-5 minutes, and an exogenous infusion of 20% glucose will be adjusted to maintain the plasma glucose at a target glycemia of 90 mg/dl by a modification of the glucose clamp technique.[53] Potassium will be administered orally as needed to achieve potassium levels above 4.0 mmol/l. Heart rate and ECG will be recorded continuously.

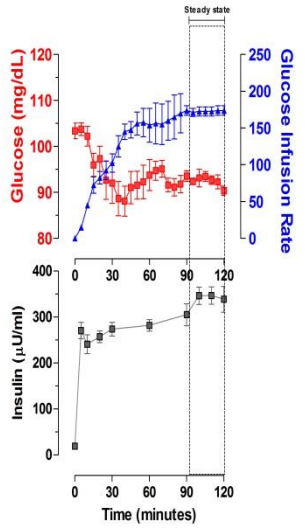

Figure 6. Hyperinsulinemic euglycemic clamp (high insulin dose 120 mU/m^2/min). Upper panel is the glucose level (in red) and glucose infusion rate (blue). Lower panel is the plasma insulin levels. Time points 90-> 120 min

Spectral analysis of heart rate and blood pressure. Blood pressure fluctuates with a 10-second periodicity, and these fluctuations are commonly termed "Mayer" waves. This low-frequency variability (LFSBP) is thought to reflect sympathetic regulation of vasomotor tone. LFSBP correlates with direct measurements of sympathetic traffic using microneurography. For spectral analysis, beat-to-beat data are recorded using the WINDAQ data acquisition system (DI220, DATAQ, Acron, OH, 14 Bit, 1000Hz) and processed off line using a custom-written software in PV-Wave language (PV-wave, Visual Numerics Inc., Houston, TX). Detected beat-to-beat values of R-R intervals and blood pressure values are interpolated and low-pass filtered (cutoff 2 Hz). Data segments of interest are used for spectral analysis. Linear trends are removed, and power spectral density is estimated with the FFT-based Welch algorithm. The power in the frequency range of low frequencies (LF: 0.04 to <0.15 Hz) and high frequencies (HF: 0.15 to < 0.40 Hz) were calculated according to the Task Force recommendations.[34]

Assays: Plasma glucose will be measured with a YSI glucose analyzer (YSI Life Sciences, Yellow Springs, OH). Plasma insulin concentrations will be determined by radioimmunoassay (RIA; Millipore, St. Charles, MO).

Lipid Analysis: Total cholesterol, triglycerides, and HDL will be determined using automated techniques (UniCel® DxC 800 Synchron® Clinical System, Beckman Coulter). NEFA will be determined using a commercially available enzymatic assay (Wako Chemicals, Dallas, TX). F2 isoprostanes will be measured by gas chromatography and negative-ion chemical ionization mass spectrometry.[36] Adipose tissue from the abdominal subcutaneous tissue will be obtained with a needle biopsy. F2 isoprostanes in adipose tissue will be analyzed as previously reported.[48]

Contrast-enhanced ultrasound. Imagings will be obtained using a linear-array transducer connected to an ultrasound system (L9-3 transducer, iU22; Phillips Ultrasound, Santa Ana, CA). This equipment has the capability to obtain real-time imaging using low (0.08) and high mechanical index (1.2) while the contrast (microbubbles; Definity; Bristol-Myers Squibb,Princeton, NJ)) are infused at a constant rate that produce moderate opacification in fat and muscle tissue. We will dilute one vial (2 ml) in 20 cc of normal saline. The medication will be administered intravenously (approximately, 1.2-1.5 ml/min) in 24 hours.
We will use one vial per day per subject. The high mechanical index (1.2) will be used to destroy the microbubbles at the beginning of each recording whereas the low mechanical index (0.08) will allow the microbubbles to resonate without destruction, thereby allowing recording of the replenishment of the microbubbles in the vasculature. The first seconds (0.5) of recording after destruction of the microbubbles correspond to filling of rapid-filling vessels (arterioles, veins, venules) which will be subtracted leaving only measurements of microvascular blood velocity. Images will be analyzed in our laboratory using Q-Lab; Philips software.

Flow-mediated dilation. Patient will be studied in a quiet, temperature-controlled room (22-23C). After 15-min of rest in a supine position, the right brachial artery will be imaged with a high ultrasound resolution machine.

The artery will be scanned over a longitudinal section 3-5 cm above the elbow. The focus zone will be set to the depth of the anterior vessel wall. Depth and gain settings will be optimized to identify the lumen vessel wall interface. The diameter of the right brachial artery will be measured continuously at rest, during reactive hyperemia and after 10 minutes recovery period. A pneumatic tourniquet will be placed around the forearm distal to the target artery and will be inflated to a pressure 50 mm Hg above patient's systolic blood pressure for 5 min. Reactive hyperemia will be induced by sudden cuff deflation. The brachial artery will be continuously imaged for 30 s prior to and 180 s after cuff release.

7.0 Risks

Potential risks and adequacy of protection against risks

- Central acetylcholinesterase inhibitors can potentially induce bradycardia. However, this risk has been reported to be increased only with donezepil (Aricept®) because of its long half-life (~80 hours).[47] For comparison galantamine (our study drug) has a half-life of 7 hours. We will exclude subjects with AV blockade diagnosed during the screening visit by ECG to minimize the risk of bradycardia.
- Gastrointestinal symptoms, particularly nausea, are the most common side effects associated with the use of central acetylcholinesterase inhibitors. This adverse event (AE) could be minimized with a dose-escalation schedule. For specific aim 2 (chronic study), we are planning to initiate galantamine with the lower available dose 4 mg p.o. twice a day for 2 weeks followed by 8 mg p.o. twice a day (16mg/day, target dose).
- The metabolism of galantamine is primarily through the cytochrome P450 system, specifically the CYP2D6 and CYP3A4 isoenzymes. We will exclude subjects who have impaired hepatic function and/or who are currently using strong inhibitors of CYP3A4 and CYP2D6 (e.g., ketoconazole and paroxetine, respectively).

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

Any 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 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 adverse event will be classified as serious if it a) results in death, b) is life-threatening, c) requires inpatient hospitalization or prolongation of existing hospitalization, d) results in persistent or significant disability or incapacity, e) is a congenital anomaly or birth defect. Serious adverse events will be reported to Dr. Satish R. Raj, the Data and Safety Monitoring Officer (DSMO), and the IRB within 10 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: The PI will be responsible for preparing an administrative progress report every 12 months describing the progress of the study and safety data. The reports will be reviewed internally by her mentors and also by Dr. Satish Raj, the Data and Safety Monitoring Officer (DSMO). All studies will be registered in ClinicalTrials.gov prior to enrolling subjects.

9.0 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 life threatening by the physician, even if not addressed on the potential risk section (Section 7.0)

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. Member of the research team will have access to the 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).

Appendix A:

Title: Racial Differences in Vagal Control of Glucose Homeostasis
Study Procedure Calendar
Specific Aim 1

Schedule of Activities	**Screening 1**	**Screening 2**	**Study Visit 1**	**Study Visit 2**
Informed consent	X			
Medical history	X			
Physical examination	X			
Lab analysis (CBC, CMP, lipid profile)	X			
Pregnancy test (serum β-hCG)	X		X	
Pregancy test (urine β-hCG)				X
ECG without interpretation	X			
75 gram OGTT		X		
Body composition (DEXA scan)			X	
24-hr urine collection (sodium, creatinine)			X	X
Baseline urine F2 isoprostanes			X	X
4 hr urine F2 isoprostanes			X	X
Study drug (placebo or galantamine)			X	X
Contrast-enhanced ultrasound with microbubbles/flow-mediated dilation			X	X
Spectranalysis of BP & HR variability			X	X
Lipid infusion (intralipids)			X	X
Baseline blood (NEFA, F2 isoprostanes, triglycerides, FFA, cytokines)			X	X
2h blood (NEFA, F2 isoprostanes, triglycerides, FFA, cytokines)			X	X
4h blood (NEFA, F2 isoprostanes, triglycerides, FFA, cytokines)			X	X
Medication reconciliation			X	X
Evaluation of adverse events (AE)			X	X
Heparin bolus/infusion			X	X

Appendix B:

Title: Racial Differences in Vagal Control of Glucose Homeostasis
Study Procedure Calendar
Specific Aim 2

Schedule of Activities	Screening 1	Screening 2	Phone 1 visit	Phone 3 visit	Phone 7 visit	Phone weekly visit	Study day 1	wash out	Short visit	Phone 1 visit	Phone 3 visit	Phone 7 visit	Phone weekly visit	Study day 2
Informed consent	X													
Medical history	X													
Physical Examination	X													
Lab analysis (CBC, CMP, lipid profile)	X													
Pregnancy test (serum β-hCG)	X						X							X
ECG without interpretation	X													
75 gram OGTT		X												
Body composition (DEXA scan)							X							X
4-wk drug supply administered (8 mg/day for 2 wks, 16 mg/day for 2 wks)		X							X					
Medication diary, verbal/written instructions			X	X	X	X	X			X	X	X	X	X
Evaluation of adverse events (AEs)			X	X	X	X	X			X	X	X	X	X
one-step hyperinsulinemic-euglycemic clamp							X							X
Blood collection (F2 isoprostanes, cytokines, pancreatic polypeptide)							X							X
Urine collection (F2 isoprostanes)							X							X
Spectranalysis of BP & HR variability							X							X
Fat-pad biopsy (F2 isoprostanes measurement in adipose tissue (optional)							X							X
Medication reconciliation							X							X

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