CLINICAL STUDY PROTOCOL

Interventional Drug or Biologic

Sympathetic activation in obesity

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Synopsis

Primary Objective

The primary objective of this study is to determine whether sympathetic nervous system (SNS) activity in adipose tissue (WAT and BAT), muscle and brain is altered in individuals with obesity in comparison to individuals with normal weight. Simultaneous multi-organ SNS activation will be obtained with a radiotracer for norepinephrine transporters (NET) for whole-body Positron Emission Tomography in combination with low dose computed tomography (PET/CT) imaging and microneurography (gold standard test for assessment of muscle SNS activity).

Secondary Objective (if applicable)

The secondary objectives of this study are: 1) to evaluate whether gender differences affect peripheral SNS in healthy normal weight and obese men and women in adipose tissue (WAT and BAT) and muscle (resting/fasting); 2) To investigate the relationship between peripheral and central SNS activity in obesity, by correlating SNS activity in peripheral tissues (WAT, BAT, and muscle) and brain; and 3) To investigate NET CNS and peripheral SNS activity before and after a high carbohydrate mixed meal in lean and obese men and women.

Study Duration

4 years

Study Design

Aim: ***To measure peripheral SNS in healthy normal weight and obese men and women in adipose tissue (WAT and BAT), deltoid skeletal muscle, and brain NET at fast and after a mixed meal test (MMT).***

The goal of this study is to measure in 20 individuals with normal weight (BMI 18-25 kg/m^2) and 20 individuals with obesity (BMI 30-50 kg/m^2) NET-binding in WAT and BAT to better understand the role of SNS in obesity. SNS activation of a) peripheral tissues: adipose tissue (BAT and WAT) and skeletal muscle; and b) CNS NET will be measure synchronously by using [^{11}C]MRB whole body PET/CT imaging in combination with the gold standard measurement of peripheral SNS activity (muscle sympathetic nervous activity (MSNA)).

Visit 1 - Screening procedures:

Potential subjects will complete pre-screening over the telephone to determine preliminary eligibility based on inclusion/exclusion criteria and basic socio-demographic information will be collected. Potentially qualifying participants will then be screened in person at the Yale New Haven Hospital (YNHH) Research Unit (HRU) or Church Street Research Unit (CSRU) with vital signs, EKG, a medical history and physical exam. Fasting blood will be collected for HbA1C, creatinine, liver enzymes (ALT, AST), TSH, hematocrit, and lipid panel. A urine pregnancy test will be administered.

Oral glucose tolerance test (OGTT) will be administered as follows: Following a 10-hour overnight fast, a nurse will insert an intravenous (IV) catheter. Subsequently, subjects will ingest 7.5 oz of Glucola, which contains 75 g of dextrose in orange flavored water. Blood samples will be taken at -

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15, 0, 10, 20, 30, 60, 90 and 120 minutes (after glucola ingestion) for the measurement of plasma glucose, insulin, and C-peptide concentrations, and calculation of measurements of insulin sensitivity index (Matsuda Index). Approximately 40 mL of blood will be drawn at this visit.

Body Composition, Percent Body Fat & Percent Body Water: will be assessed using bioelectrical impedance analysis Tanita® scale (FDA cleared); which is a special multi frequency seg-mental body composition analyzer that delivers a very mild electrical current that allows measurement of fat mass, percent body fat, fat free mass, total body water, and percent body water. This test will be performed at screening visit. For better accuracy, measurement of percent fat and percent body water will be obtained with whole body Dual X-Ray Absorptiometry (DXA) scan (Hologic®) (located in the HRU) on screening-OGTT visit. The scanner arm will move over the participant's body from feet to head. The machine uses a small amount of radiation (one tenth of the amount of radiation from a chest x-ray) to measure body fat, muscle and bone density.

Visit 2 – Whole body PET/CT Imaging Visit - FASTING:

Two weeks after the screening visit, participants who qualified for the study will be asked to come to the PET Center to undergo the whole body [^{11}C]MRB PET scan. Participants will be asked to maintain their regular exercise routine.

Human PET-CT Protocol: [^{11}C]MRB will be synthesized at the Yale PET center based on procedures described and previously performed [1,2]. An intravenous bolus dose of MRB (~20mCi) will be injected by an infusion pump. PET data will be acquired dynamically for 120 min using the Siemens mCT-X whole-body PET/CT scanner using continuous bed motion to image perform multiple PET bed acquisitions from the top of the head to the lower abdominal region. A CT scan will be performed for attenuation correction and to help delineate the BAT and other regions of interest (ROIs). Images will be reconstructed with an ordered subset expectation maximization algorithm using point spread function correction and time-of-flight information. To delineate fat (WAT, BAT) and skeletal muscle regions of interests (ROIs), CT images are first resliced to match the resolution and location of PET images. As performed in previous studies [2], a supraclavicular ROI is first manually drawn on the CT images. Within that area, the fat ROI is segmented using the CT images and an intensity window from -200 to -50 HU. Subsequently the fat ROI is segmented into WAT and BAT ROIs by using the ^{11}C-MRB PET images: Standardized Uptake Value (SUV) ≥ 1.25 for BAT, SUV < 1.25 for WAT. Brain ROIs for predetermined NET-rich areas of the brain, including the locus ceruleus, raphe, thalamus and hypothalamus were defined in template space and are applied to the PET images by using the participant's MR image as an intermediate step. For analysis of tracer uptake, mean SUV will be reported for segmentation of adipose tissue. Because the distribution volume ratio (DVR) is a more precise measurement of uptake for reversible tracers [3] such as ^{11}C-MRB, regional quantification of ^{11}C-MRB uptake as DVR (V_T/ V_{ND}), estimated via multilinear reference tissue model 2 (MRTM2) [4,5] (t*=30 min) using the occipital cortex, a region with low NET density [2,5,6] as the reference region. MRTM2 reduces the variability of MRTM (*4*) parameter estimates by fixing one of the three parameters, the parameter k_2', which is only related to the reference region time-activity curve, to a common value for all target regions. In this study, the parameter k_2' was fixed to 0.021 min^{-1}, based on previous brain studies and previous BAT PET studies [2]. In addition to DVR, MRTM2 can estimate R_1, the relative radioligand delivery, using a 1 tissue compartment model (1TC). For

the 1TC model, volume of distribution, $V_T = K_1/k_2$, where K_1 (mL/min^{-1}/mL^{-1}) is the rate constant of radiotracer from plasma to tissue of target organ and k_2 is the rate constant from tissue to plasma. Using a reference region with non-displaceable binding (e.g. occipital lobe) we can estimate similar rate constants: $V_{ND} = K'_1/k'_2$. Recalling that K_1 and K'_1 are the rate constants for transfer from plasma to the tissue and reference region, respectively, then we can estimate relative radioligand delivery as $R_1 = K_1/K'_1$.

Visit 3 – Whole body PET/CT Imaging Visit - High-Carbohydrate Mixed Meal Test (MMT)

High Carbohydrate Mixed meal test (MMT): Two weeks after visit 2, participants will come to the PET Center after a 10-hour overnight fast. A nurse will insert an intravenous (IV) catheter and subjects will ingest a liquid meal (65% CHO, 20% fat, and 15% protein, equal to 40% of daily energy expenditure, per ~16 ounces) to be prepared by the Metabolic Kitchen located at the HRU. Blood samples will be collected at −10, 0, 15, 30, 60, 90, and 120 min) for measurement of glucose, insulin and C-peptide concentrations [7]. Plasma samples will be drawn at baseline and throughout the MMT for measurement of additional hormones (such as leptin, ghrelin, thyroid hormones, and catecholamines) and free fatty acids (FFA) levels. Insulin and glucose will be used to calculate indexes of insulin sensitivity [7-9]. Hormones, glucose and FFA will be correlated with PET measurements of brown fat at baseline and during the MMT. Approximately 60 mL of blood will be drawn at this visit.

Participants will undergo whole body [^{11}C]MRB PET scan as described in visit 3. After the MMT, PET data will be acquired dynamically for 120 min using the Siemens mCT-X whole-body PET/CT scanner after an intravenous bolus dose of MRB (~20mCi).

The order of the PET scan visits 2 and 3 will be randomized.

Measurement of Energy Intake: Hunger and fullness will be determined immediately before, during and after the PET scans with Visual Analog Scales (VAS).

Visit 4 – Sympathetic Nervous System Activity (SNSA) measurement Visit - Fasting

Two weeks after visit 3, participants will be asked to return for a separated visit for measurements of SNS activity at rest and fasting. This visit will be performed at the Pierce Laboratory (Dr. Stachenfeld's laboratory). The participants will come in the morning (~9am) and will be asked to fast for 10-hour and to maintain their regular exercise routine. The following measurements will be obtained: MSNA, heart rate (HR), blood pressure (BP), electrocardiogram (EKG), and heart rate variability,. The proposed procedures will be performed at fasting (baseline). Participants will be asked to maintain their regular exercise routine.

Muscle sympathetic nerve activity (MSNA): Microneurography is the gold standard, most sensitive and reliable MSNA assessment in humans. The primary measure of sympathetic nervous system activity is peripheral nerve activity, as measured by MSNA.

Muscle sympathetic nerve activity will be quantified by identification and measurement of sympathetic bursts in the integrated neurogram and expressed as burst frequency (number of bursts per unit time). ECG surface electrodes are applied to the chest, an elastic band incorporating a strain gauge element is strapped around the thorax for recording respiratory movements, and a sensor cuff of a servopulse-plethysmography arterial pressure monitor (FINAPRES, Ohmeda) is placed on the middle phalanx of the long finger (held at heart level). The experiments are carried out in an environmentally controlled chamber at an ambient temperature of 27.0 ± 0.1 °C. The experiment will begin with a 60-minute control period. During such time the subjects rest in the reclined position.

Measurements of HR, BP, and EKG (beat-to-beat) will also be obtained during the procedure. An intravenous catheter will be inserted into a forearm vein for blood sampling. Following the instrumentation period, resting HR, pulse interval (PI), BP, and MSNA will be recorded for 10 minutes. Following these measurements, plasma will be taken for measurements of insulin, glucose, free fatty acids, norepinephrine, and epinephrine levels. Approximately 20 mL of blood will be drawn at this visit. After the first blood sample and the 10-min rest phase, subjects will begin a seven-minute period of rest with paced breathing (12 breaths per minute) while HR, BP, PI and MSNA are measured. The subjects will synchronize breathing to a metronome set to a cadence of ~12 breaths per minute (0.2 Hz).

Procedure: Microneurography Recording of multiunit postganglionic muscle sympathetic nerve activity (MSNA) will be made from the common peroneal nerve as it winds around the fibular head with the subject in the supine position with the subject's thigh comfortably supported. This procedure requires us to first trace the course of the nerve using small electrical stimuli applied to the surface of the skin over the nerve or use the ultrasound to visually assist. This allows the microneurographer to find and focus the recording electrode tip on a nerve fascicle associated with muscle sympathetic activity. The time required to focus the electrode on the nerve averages about 30 min and will not exceed 60 min. In optimal conditions the track of the nerve from superficial stimulation is accurate and the nerve remains in position during insertion. An uninsulated subcutaneous electrode will serve as the reference. Mechanoreceptor afferent activity can be recorded during light tapping on the muscle belly or passive muscle stretch. A suitable intrafascicular recording site for MSNA consists of regular bursts and synchronous with the cardiac cycle. The neural activity is amplified (2x104), filtered (0.5-5.0 kHz), rectified, and integrated. The amplified and filtered nerve signal is also led to an audio monitor and through a resistance capacitance circuit (time constant 100 ms). The integrated nerve signal is digitized at 400 Hz along with the ECG signal while respiratory and arterial pressure signals are also sampled at 400 Hz (MacLab 8s). Data for analysis is selected from periods of quiet breathing in which subjects were relaxed and not talking. Microneurography was approved under HIC protocols # 8079, #0512000875, #1609018353 and is currently used under # 2000020950.

Cardiovascular variables. Recorded with a 16-channel computerized data-acquisition system at a sampling Speed of 400 Hz (ADI Instruments PowerLab 9, Castle Hill, Australia). Beat-to-beat DBP, SBP and pulse interval (PI) are determined using peak detection algorithms (44). Brachial artery BP and HR are measured with an automated sphygmomanometer (Colin Medical Instruments Corp,

Komaki, Japan). Heart rate and pulse interval will be determined beat-to-beat from lead II of the EKG recording.

Visit 5 – Sympathetic Nervous System Activity (SNSA) measurement Visit – Mixed Meal Test (MMT)

Two weeks after visit 4, participants will be asked to return for a separated visit for measurements of SNS activity at rest and after intake of liquid meal. Participants will undergo similar procedures described on visit 4. However, MSNA will be obtained after MMT. The participants will come in the morning (~9am) and will be asked to fast for 10-hour and to maintain their regular exercise routine. A nurse will insert an intravenous (IV) catheter and subjects will ingest a liquid meal (65% CHO, 20% fat, and 15% protein, equal to 40% of daily energy expenditure, per ~16 ounces) to be prepared by the Metabolic Kitchen. Measurements of HR, BP, and EKG (beat-to-beat) will be obtained during the procedure. An intravenous catheter will be inserted into a forearm vein for blood sampling. Following the instrumentation period, resting HR, pulse interval (PI), BP, and MSNA will be recorded for 10 minutes. Following these measurements, plasma will be taken for measurements of insulin, glucose, free fatty acids, norepinephrine, and epinephrine levels. Approximately 40 mL of blood will be drawn at this visit.

Participants will be asked to maintain their regular exercise routine. The order of SNSA Fasting and MMT visits (visits 4 and 5) will be randomized. All study visits will be conducted within an 8-week period (including the two PET and SNSA scans).

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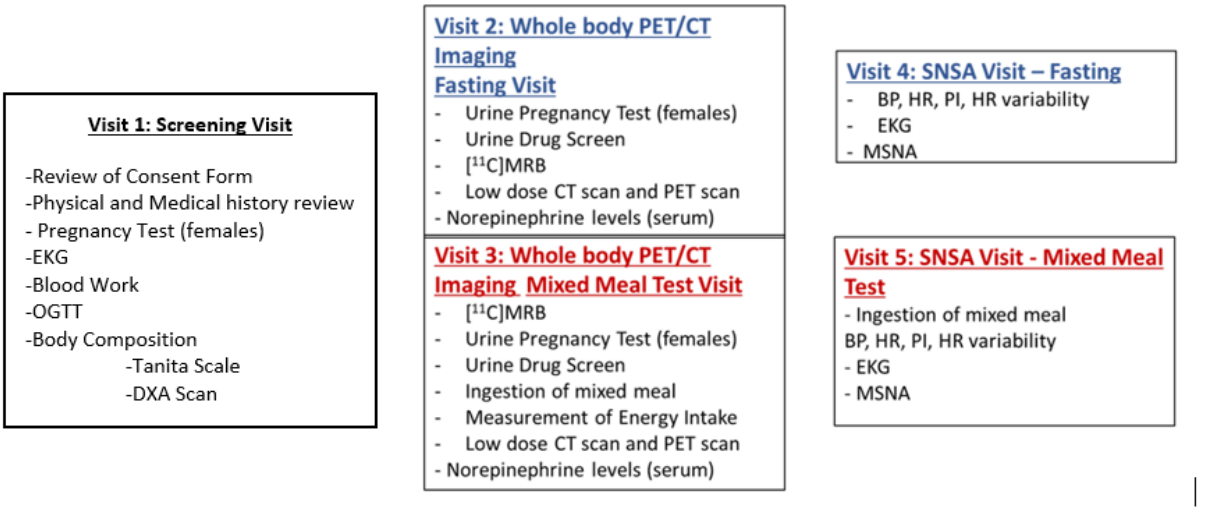
Schematic figure of the visits and procedures to be done. The order of the PET scans will be randomized.



Abbreviations: BP=blood pressure, EKG=electrocardiogram, HR=heart rate, MSNA=Muscle sympathetic nerve activity, SNSA=Sympathetic Nervous System Activity

PET and MSNA visits do not need to occur in sequence, i.e. PET scans can occur before or after the MSNA.

Number of Study Sites

1

Study Population

We plan to enroll 20 participants with normal BMI (10 women and 10 men) and 20 with obesity (10 women and 10 men).

Number of Participants

40

Primary Outcome Variables

Primary outcomes: Group differences in 1) [^{11}C]MRB-binding in brown adipose tissue (BAT), white adipose tissue (WAT), skeletal muscle, and norepinephrine-rich brain regions (hypothalamus and thalamus); and 2) microneurography (muscle sympathetic nerve activity (MSNA)). Correlations between NET in the brain and peripheral NET and MSNA.

HIC 2000030749

Secondary and Exploratory Outcome Variables (if applicable)

Secondary endpoints: Plasma hormone levels, heart rate, blood pressure, and electrocardiogram (beat-to-beat). Correlations of secondary endpoints with NET in the brain and peripheral NET and MSNA.

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Abbreviations

Abbreviation	Explanation

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HIC 2000030749

Glossary of Terms

Glossary	Explanation

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HIC 2000030749

Table of Contents

APPROVED BY THE YALE UNIVERSITY IRB 3/30/2022

HIC 2000030749

HIC 2000030749

HIC 2000030749

APPROVED BY THE YALE UNIVERSITY IRB 3/30/2022

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1 Introduction

1.1 Introductory Statement

This document is a protocol for a human research study. The purpose of this protocol is to ensure that this study is to be conducted according to ICH GCP guidelines, and according to CFR 21 Part 312, other applicable government regulations and Institutional research policies and procedures.

2 Background

2.1.1 Preclinical Experience

Obesity is one of the main drivers of insulin resistance, diabetes, and cardiovascular disease, and obesity-associated dysfunction of the sympathetic nervous system (SNS) may be implicated in the pathophysiology of these comorbidities [15-18]. The SNS is integral to modulating energy homeostasis via activation of target organs, such as adipose tissue, skeletal muscle, liver, pancreas, and adrenal glands [19]. However, this multi-organ SNS activation has been shown to be dysfunctional in obesity [20,21]. Central nervous system (CNS) noradrenergic neurons release norepinephrine (NE) in these target organs. NE recycling is a tightly regulated process modulated in large part by the norepinephrine transporter (NET) [22]. Due to its important role in regulating NE signaling, NET has been considered a marker of noradrenergic system activity [22].

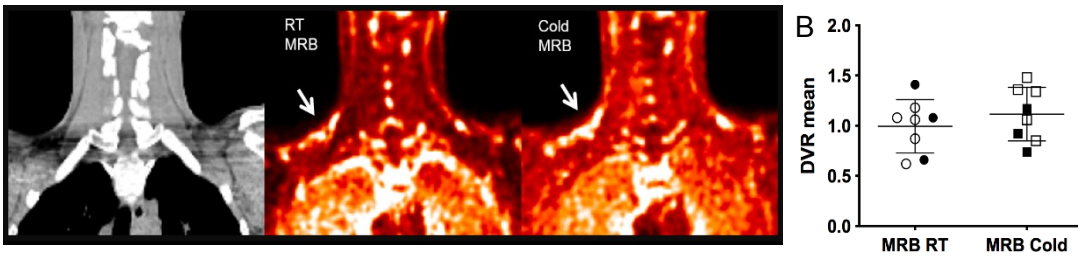
In humans, *in vivo* quantification of NET levels can be measured using a selective positron emission tomography (PET) radioligand for NET, (S,S)-^{11}C-O-methylreboxetine ([^{11}C]MRB) [23,24], and our group has shown that [^{11}C]MRB can measure NET in multiple tissues including brown (BAT) and white adipose tissue (WAT), skeletal muscle, and brain [12,25]. Furthermore, our preliminary data using [^{11}C]MRB scanning in healthy lean individuals as well as individuals with obesity suggest that obesity is associated with marked differences in NET availability in BAT [2] as well as the brain [26]. Use of PET allows us to assess NET binding in multiple tissues simultaneously. In these proposed studies, we will use NET-binding to investigate the role of SNS dysfunction in healthy men and women with obesity. Furthermore, because there is strong evidence that food induced SNS activity increment is blunted in individuals with obesity [15,27,28], we will also test the impact of obesity on response to a relevant stimulus (food). Our overall premise is that **coordinated multi-organ response (brown and white adipose tissue, muscle, liver, pancreas) is modulated by the SNS both in the fasting state and after a meal; however, this coordinated response becomes dysfunctional in obesity**. Therefore, to better understand the potential for dysregulated coordination of the SNS in obesity, we will synchronously measure SNS activation of a) peripheral tissues: adipose tissue (BAT and WAT) and skeletal muscle; and b) CNS by using *[^{11}C]MRB* whole body PET imaging in combination with muscle SNS activity (MSNA) during rest both in the fasting state and after a meal.

2.1.2 Clinical Experience

[^{11}C]MRB can be used to image NET-rich regions in the body at rest. Using a rodent model, [^{11}C]MRB PET imaging has been shown to appropriately 1) identify NET-rich regions in the body including BAT, heart, liver, pancreas, and kidney, 2) NET could be used as a highly specific target for BAT, and 3) that BAT can be detected in rodents under both room temperature and cold conditions [24]. Furthermore, the binding of [^{11}C]MRB in BAT was completely abolished by the pretreatment with a NET inhibitor, demonstrating the specific binding of the proposed ligand to BAT [24]. Building upon those rodent studies, the use of [^{11}C]MRB for imaging human BAT was validated under both cold and room temperature (RT) conditions at rest [12]. Ten healthy, Caucasian subjects (5M/5F: age 25±3, BMI 22±3 kg/m^2)

underwent [^{11}C]MRB PET-CT imaging for cervical/supraclavicular BAT under RT and cold-stimulated conditions (RPCM Cool vest; enthalpy 15°C) compared to ^{18}F-FDG PET-CT imaging. Uptake of [^{11}C]MRB, was quantified as the distribution volume ratio (DVR). As seen in Figure 1, BAT [^{11}C]MRB binding was similar under both RT and cold conditions (BAT DVR: RT 1.0±0.3 vs. cold 1.1±0.3, p=0.31). Importantly, BAT DVR [^{11}C]MRB at RT correlated positively with body temperature (r=0.92, p=0.004).

Figure 1. A) Representative subject during scanning at RT and cold; B) [^{11}C]MRB Distribution Volume Ratio (DVR) in all subjects at RT and cold, closed marker = female



Obesity is associated with marked decrease in [^{11}C]MRB binding of human BAT. Recently, we have shown that there is a marked difference in [^{11}C]MRB binding of BAT amongst obese compared to lean women [2] (Figure 2). In this proof of concept study, 15 healthy, non-diabetic Caucasian women (9 lean, age 25.6 ± 1.7, BMI 21.8 ± 1.3 kg/m^2 and 6 obese age 30.8 ± 8.8 BMI 37.9 ± 6.6 kg/m^2) underwent PET-CT imaging of the neck/supraclavicular region using [^{11}C]MRB under RT conditions. We observed that [^{11}C]MRB binding was reduced in women with obesity in comparison to the lean group suggestive of decreased BAT. However, it remains to be determined whether NET in BAT is also reduced in men with obesity.

Figure 2. Decreased [^{11}C]MRB binding in BAT in women with obesity. A) Two representative subjects; B) All subjects.

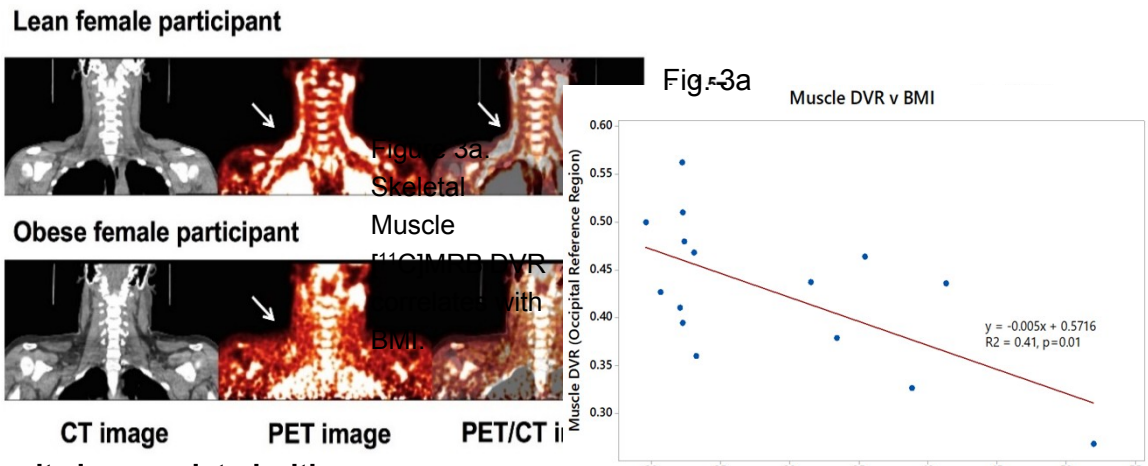


Fig. 3a

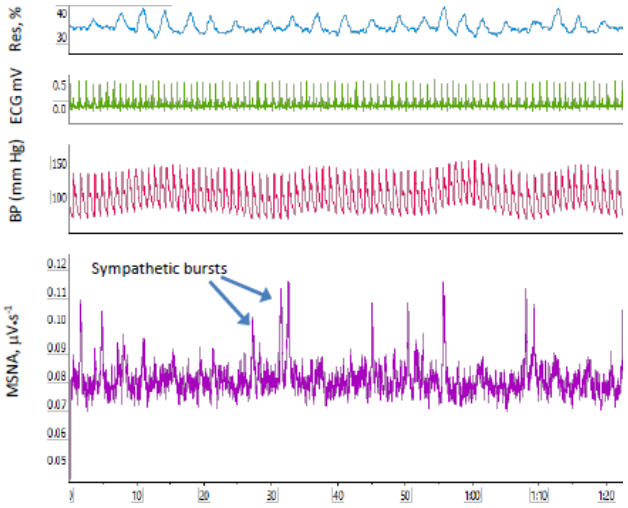
Figure 3a. Skeletal Muscle [^{11}C]MRB DVR correlates with BMI.

Obesity is associated with altered [^{11}C]MRB binding in skeletal muscle. Because SNS innervation of skeletal muscle has been shown to modulate skeletal muscle insulin resistance, we also performed preliminary analysis of 11CMRB binding

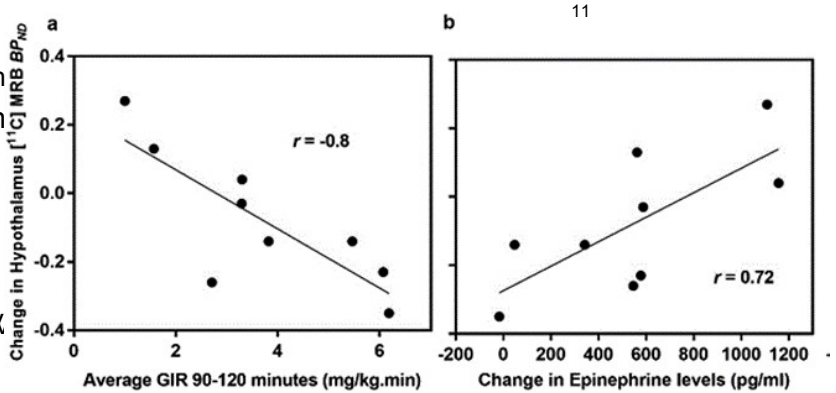
in muscle in these lean and obese women. In women, skeletal muscle (deltoid area) [^{11}C]MRB DVR correlated with BMI (Figure 3a), and was marginally lower (P=0.088) in women with obesity compared with women with normal BMI.

Based on these initial results, our goal is to expand our understanding of the importance of NET-binding in peripheral tissues (BAT and WAT) and skeletal muscle in obesity which may play a role in the pathogenesis of obesity-associated insulin resistance, type 2 diabetes and hypertension. We plan to perform whole body [^{11}C]MRB PET imaging to quantify NET-binding (an indicator of sympathetic activation of target organs) during rest in the fasted state in individuals with both normal BMI and with obesity. Furthermore, because in the past only women were included in our preliminary obesity study, we plan to determine whether [^{11}C]MRB binding in BAT will also be decreased in men with obesity [2].

Furthermore, to validate NET as an indicator of SNS activity, we plan to correlate the results from the whole body [^{11}C]MRB PET imaging scans with established measurements of SNS, such as heart rate variability, plasma catecholamine levels and MSNA [29]. Dr. Stachenfeld, one of the co-investigators in this project,is an expert in human studies of the autonomic nervous system. Her laboratory has used microneurography to measure MSNA in women with obesity [30]. The bottom tracing in Figure 4 shows sympathetic bursts as measured by microneurography in the peroneal nerve of an obese human while resting. The other tracings show beat to beat blood pressure, EKG and respiration. In our studies, we will examine changes in burst activity to determine changes in MSNA.



Human obesity is associated with altered brain NET. Central noradrenergic neuronal dysfunction has been observed in obesity [26,31-33]. Li et al [26] demonstrated that obese subjects have decreased NET-availability in the thalamus (a sensory relay region in the brain); although these findings were not confirmed in a European cohort [34]. Vettermann et al [35] showed that NET activity in the insula and hippocampus predicts successful weight loss. Furthermore, NET inhibitors used for the treatment of attention deficit disorder (ADHD) can promote weight loss [36,37]. In a prior study from our group [25], we observed that NET in the hypothalamus correlated with counterregulatory hormonal and metabolic response to hypoglycemia (a stimulus to induce stress response and increase hunger) F (Figure 6). However, it is h not clear whether NET- h rich regions in the CNS,

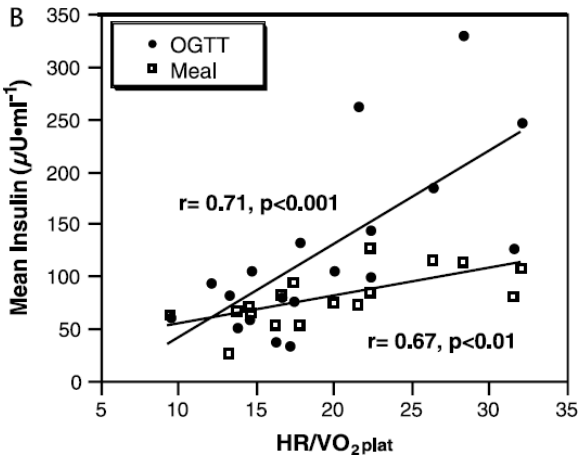


such as hypothalamus, thalamus, and brain stem (areas involved with glucose metabolism and autonomic function) are important in regulating SNS activation of target organs. We plan to investigate the relationship between CNS NET-availability and peripheral NET (measured by whole-body ([^{11}C]MRB PET imaging) and SNS activity (measured by MSNA) both in normal BMI and obese individuals. We propose that CNS NET-availability at rest in the fasting state in the hypothalamus and thalamus (brain regions involved with glucose and energy metabolism) will correlate with NET binding in BAT and skeletal muscle in individuals with normal BMI. In contrast, because of obesity-associated regionalized SNS dysfunction, CNS-NET availability will not correlate with peripheral NET-binding or MSNA in individuals with obesity.

High carbohydrate meal ingestion is a strong stimulus for activation of the SNS. Food intake (in particular carbohydrate-rich meals) is a strong stimulus for activation of SNS in both animals [38] and humans [39-42]. This has been demonstrated by plasma NE tracer kinetics [38,39] and MSNA [39,41-43]. In rodents, NE turnover in BAT, but not in the spleen or heart, increased in response to a meal [38]. Regionalized SNS activation was also observed in humans: increased in skeletal muscle and kidneys, but no or minimal change in heart and liver [39]. SNS play a major role in activating BAT after a meal [44], and a recent study using PET Imaging showed increased BAT activation after ingestion of a high carbohydrate-rich meal [45].

Food-stimulated SNS activation in obesity. Studies using MSNA have shown that obesity is associated with SNS hyperactivity in the fasting state [17,18]. However, food-stimulated increments in MSNA have been shown to be blunted in individuals with obesity [15,27]. Yeckel et al. [28] measured sympathetic activity during exercise with the exercise recovery index – calculated by a ratio of heart rate normalized for oxygen consumption (VO2). Sympathetic activity in response to exercise was compared with metabolic response in response to an OGTT and a high-carbohydrate meal challenge. The authors observed that insulin levels after the OGTT and test meal correlated with degree of sympathetic overactivity (Figure 9). Increased body fat (measured by densitometry) was associated with exercise-induced sympathetic overactivity. Therefore, based on the premise that obesity is associated with fasting SNS hyperactivation and diminished increment in SNS activation in response to a food challenge (high-carbohydrate mixed meal test), we plan to measure [^{11}C]MRB-binding in response to a meal in individuals with obesity in comparison to a group of individuals with normal BMI. In addition, to confirm the degree of SNS dysfunction in obesity, MSNA will be measured under similar circumstances (before and after a high-carbohydrate meal test).

Figure 9. Relationship between sympathetic activity (HR/V˙ O2 plateau) and insulin levels OGTT and high-carbohydrate meal



In our pilot study, sex differences were observed in response to a cold stimulus, with men showing a significantly increased [^{11}C]MRB-binding in response to cold [12]. Therefore, we will also investigate whether sex differences may play a role in [^{11}C]MRB-binding in response to a food stimulus. To build on our preliminary studies, we plan to quantify NET in peripheral tissues (adipose tissue (BAT and WAT) and skeletal muscle) and CNS at rest in fasting and post-prandial state, in both men and women with normal BMI and with obesity.

2.2 Background/prevalence of research topic

Obesity affects more than a third of the population of the United States [46] and carries an increased risk of developing type 2 diabetes, hypertension, heart disease, stroke, and certain types of cancers [47]. However, the exact mechanisms by which obesity contributes to these cardiometabolic disorders remains uncertain. Sympathetic nervous system (SNS) dysfunction has been described in individuals with obesity [15-18] and it has been proposed as one of the mechanisms involved in the development of obesity-related cardiometabolic complications [20,21]. It was initially proposed that obesity was associated with decreased SNS activity with a consequent decrease in energy expenditure and weight gain [48]. However, these findings were not confirmed by other reports. Some studies showed no change or even increased SNS activity (measured by plasma norepinephrine levels) [49]. Studies using microneurography, a direct measurement of SNS activation, showed that individuals with obesity may have sympathetic overactivity [17,18]. SNS activity may vary by tissue [21,50]. Specifically: SNS activation increases in kidneys and skeletal muscle and decreases or is unchanged in heart, and mostly unchanged in adrenal glands. Regionalized SNS hyperactivity in kidneys and skeletal muscle may lead to hemodynamic and metabolic changes in individuals with obesity. This results in an increased risk of developing hypertension and diabetes, and, in turn, cardiovascular morbidity and mortality. However, it is not exactly clear the interplay between obesity and regionalized SNS dysfunction. We propose that obesity is associated with dysfunctional coordination of SNS activation; and to prove that, we plan to measure synchronous SNS activity in various target organs in healthy individuals with normal body mass index (BMI) and with obesity.

SNS activation of target organs is mediated principally by the neurotransmitter norepinephrine (NE). Noradrenergic neurons release NE in the neuronal synapse of target organs and NE reuptake by pre-synaptic norepinephrine transporters (NET) are important for regulating NE levels in the synapse and neurotransmission [22]. NET availability is thus considered a marker of neuronal activation [22]. Our group has validated the use of [^{11}C]MRB which has high specificity for NET. In a rodent model, this tracer properly identified NET-rich regions in the body, such as brown adipose tissue (BAT), white adipose tissue (WAT), skeletal muscle, liver, heart, and brain [24]. In humans, [^{11}C]MRB has been used to quantify unstimulated BAT in men and women [51], and NET availability in BAT from healthy women with obesity was found to be reduced in comparison to healthy women with normal body mass index (BMI) [2]. In addition, preliminary unpublished data from our group showed that, in women, NET

availability in skeletal muscle (deltoid) inversely correlated with BMI. **[^{11}C]MRB PET imaging allow us to properly identify NET-rich regions in both in humans and animals and may be used as a novel technique to quantify SNS activation of target organs. Furthermore, we will investigate whether NET activity (as measured by [^{11}C]MRB PET imaging) correlates with muscle sympathetic nerve activity (MSNA), the gold-standard measurement for systemic SNS activation**. Altered coordinated SNS multi-organ activation in the fasting and post-prandial state in obesity may play a role in the pathophysiology of obesity-associated cardiometabolic disorders.

There is also compelling evidence that SNS activity is not only altered at baseline in obesity but also in response to stimuli. Studies have found that obesity is associated with altered SNS responses to various stimuli including oral glucose, food, hyperinsulinemic-euglycemic clamp [15,27,28,52]. Food-stimulated increments in MSNA have also been shown to be blunted in individuals with obesity [15,27]. Thus, we plan to simultaneously measure NET-binding in BAT, WAT, skeletal muscle, and brain in the fasted state and after a high carbohydrate meal challenge. Furthermore, we will evaluate the relationship between NET-rich regions in the brain involved in energy homeostasis (such as hypothalamus) and motor and sensory relay (thalamus) with peripheral NET-rich tissues, such as BAT and skeletal muscle

3 Rationale/Significance

3.1 Problem Statement

Sympathetic nervous system (SNS) dysfunction has been observed in obesity and may play a role in the pathophysiology of obesity-associated cardiometabolic disorders.

3.2 Purpose of Study/Potential Impact

This project will help further understand the impact of obesity in SNS activity and better understand the role of the SNS in the development of cardiometabolic disorders in patients with obesity.

3.2.1 Potential Risks

Risks Associated with Radiation:

Yale University Radioactive Investigational Drug Committee (Yale RIDC) will review the use of radiation in this research study, and no subjects will be scanned until RIDC approval is obtained. This research study involves exposure to radiation from [^{11}C]MRB PET scanning. This radiation exposure is not necessary for medical care and is for research purposes only.

For each individual PET scan, subjects will receive up to ≤ 20 mCi of, [^{11}C]MRB, plus transmission scans. This is equal to an effective dose of 0.428 rem per injection.

Although each organ will receive a different dose, the maximum amount of radiation exposure subjects will receive from participating in up to two PET scans is equal to an effective dose 0.856 rem, for a total of up to 40 mCi of [^{11}C]MRB in 2 injections. This calculated value is used to relate the dose received by each organ to a single value.

Subjects will also receive radiation exposure from low dose whole-body CT scans on the mCT, subjects will receive up to 1250 mrem (1.250 rem) from up to 2 low dose head CT scans (0.625 rem each).

The maximum amount of radiation an individual subject could receive in this study is from up to 2 injections of ≤ 20 mCi of [^{11}C]MRB, plus CT scans. This is equal to an ED of 2.106 rem.

The amount of radiation subjects will receive in this study is below the dose guidelines established by the RIDC guidelines for research subjects. This guideline sets an effective dose limit of 5 rem per year.

Adverse effects of the radiopharmaceuticals in this study have not been reported. However, the possibility exists for a rare reaction to any of the substances or procedures to which a subject is exposed.

Physical examination, venipuncture/IV placement, EKG, lab data collection and analysis: Subjects will receive a comprehensive medical history assessment, physical examination, EKG, and lab testing to ensure good physical health status and eligibility. These are routine medical procedures and should add no risks other than those normally associated with these procedures. Study doctors and/or nurse practitioners will assess the EKG and screening laboratory blood work.If any abnormal findings occur, they will provide appropriate medical advice. Potential participants who are excluded from the study due to medical reasons, or those who are in immediate need for medical or psychiatric attention, will be referred to the appropriate facilities at Yale New Haven Hospital so they can receive the clinical care needed to address their condition. Subjects will be exposed to the risk of venipuncture/IV placement that is routinely done in the HRU at the YNHH. Risks associated include: local bruising, hematoma,or infection. If this occurs, appropriate treatment will be provided immediately. On extremely rare occasions, a blood clot or infection might occur, and if this occurs, subjects will be treated at the HRU, which is fully staffed with nursing specialists and medical personnel. These risksare mitigated by the use of research HRU nurses who have extensive experience in venipuncture and IV catheterization. Transient vasovagal symptoms, e.g., nausea, sweating,and lightheadedness may also occur during IV catheter placement. Careful efforts to maintain confidentiality have been effective in previous research, and only patients' code numbers will be recorded on forms to protect confidentiality. Efforts to protect confidentiality are further detailed below.

Oral glucose tolerance test (OGTT): The OGTT is a commonly used outpatient test for diagnosis of type 2 diabetes. It is not associated with any specific adverse effects. Occasionally, some patients may experience mild nausea or GI discomfort following drinking the 75 gram glucose drink; however, this typically resolves after eating food.

Microneurography: The risk of microneurography is a peroneal paresthesia for 2-10 days following the experiment. The incidence of paresthesia is reported to be less than 10%, in less than 1% of all subjects paresthesia can persist for up to two months. Paresthesia resolves spontaneously. Microneurography can occasionally be painful, but this is very unusual.

Phlebotomy: The total amount of blood drawn is ~160 ml (including screening, OGTT). People who are in good health are not usually affected by this kind of blood loss. However, to be safe, subjects will be warned against donating blood for at least 6 weeks before and after completing this study. Additionally, subjects are screened for anemia at the first visit and females with a hematocrit less than 35% and males with hematocrit less than 40% will be excluded.

DXA scanning: DXA scanning for body composition is performed routinely by our group. The DEXA measurement poses no major risk to the subject. The amount of radiation the subject will be exposed to is small and is one tenth of the amount of radiation for a chest X-ray.

Medical treatment for injury: The consent form will specify that medical therapy will be provided for injuries sustained as a consequence of participation in this research. This will be provided at the HRU located at the YNHH or Church Street Research Unit (CSRU).

Minimizing Risks:

General: All investigators and research team members have taken the Human Investigations Training Course either on-line or in person at the Yale School of Medicine. Trained medical staff at the HRU will perform all clinical research procedures and all subjects will have health screens reviewed by a physician or nurse practitioner prior to their participation. In the event of an injury, medical therapy will be offered with the cost incurred by the subject's medical insurance carrier. Confidentiality of all study information is maintained by identifying subjects by code numbers, which are subsequently linked to their data files. No individuals, other than professionals directly involved in the study will be allowed to read data forms to ensure confidentiality. No subjects are identified by name in published data and only by code in data storage areas.

Informed consent: All subjects are consented by one of the study investigators or research staff, as part of determining their study eligibility. The purpose, nature, and potential risks and benefits of the study will be explained to each potential subject in detail during the screening interview. Prior to initiating the study, one of the study investigators or research staff will explain the planned study interventions, and each subject will be asked to read and sign a consent form approved by the Yale School of Medicine Human Investigation Committee (HIC). All potential subjects are given ample time to ask questions and only after this are they asked to provide informed consent in the research study. Subjects are given a copy of the signed consent for their personal records.

IV Insertion and Blood Drawing: Risks of bruising, clotting, and infection will be minimized by having venipuncture performed by trained, experienced personnel under sterile conditions. To avoid injury due to fainting, the antecubital vein catheter will be inserted when the subjects are recumbent. Females with a hematocrit less than 35% and males with a hematocrit less than 40% will be excluded. All subjects having donated blood within 6 weeks of the study will be

asked to postpone study participation (with a repeat blood count prior to future enrollment), and patients will be advised to refrain from blood donation for 6 weeks after each study.

Radiation. The Yale-New Haven Hospital Radiation Safety Committee (RSC) will review the use of radiation in this research study, and no subjects will be enrolled until RSC approval is obtained. This research study involves exposure to radiation from DXA scan and [^{11}C]MRB PET scanning. This radiation exposure is not necessary for medical care and is for research purposes only. The targeted amount of radiation an individual subject will receive in this study is from is from 2 injection(s) of ≤ 20 mCi of [^{11}C]MRB plus transmission scans and CT scans CT scans for attenuation correction and DXA scan. All scans will be done in the presence of medical supervision and trained nursing staff in an institution specifically designed to support imaging studies. In the event of serious medical complications, the Yale University PET scan facilities have immediate access to or consultation with specialized medical units at the Yale-New Haven Hospital. Preparation of radiopharmaceuticals and performance of PET scans will be by radiochemists and technologists of the Department of Diagnostic Radiology, Yale University School of Medicine. These professionals are qualified by training and experience in the safe use and handling of radiopharmaceuticals. Subjects will be asked about their previous radiation exposure, and those who have had research exposure within the past year will be excluded if their cumulative annual exposure (including the present study) exceeds FDA limits. No PET studies will be performed on pregnant or potentially pregnant women, asconfirmed by pregnancy testing during evaluation and on each scan day before initiation of any scan procedures. If subjects are breastfeeding they will not be able to participate in this research study.

Microneurography. The likelihood of the occurrence of symptoms following microneurography will be minimized by the following certain procedures. First, few problems or symptoms are reported in laboratories whose microneurographers have received training from individuals with a documented record of success and safety. Dr. Stone or Dr. Stachenfeld will perform all microneurography studies. Dr. Stone is a highly qualified microneurographer in the laboratory with over two years' experience, and Dr. Stachenfeld has performed microneurography in her laboratory for 10 years, and this will reduce the risk of occurrence of symptoms following the experiment. The time spent searching for a nerve fascicle within the peroneal nerve will be limited to < 60 min. No nerve site will be sampled more than once. The recording electrodes are inspected under a dissecting microscope to ensure electrode tip integrity (those electrodes found with damaged tips are not used) and each recording electrode will be used only once for intraneural recording. Placement of the electrodes and positioning in the common peroneal nerve are performed under sterile conditions. Metal microelectrodes are steam sterilized at Yale New Haven Hospital before use. The site of electrode placement is cleaned with alcohol and only investigators wearing sterile gloves handle the sterile microelectrodes. Following these simple guidelines, the risk of occurrence of symptoms following microneurography will be small. Microneurography studies are conducted at the Pierce Laboratory.

3.2.2 Potential Benefits

The proposed studies do not have direct, short-term subject benefits. Assessment of risks vs. benefits requires some consideration of the potential benefits to society from the studies

proposed. Obesity affects more than a third of the population of the United States and carries an increased risk of cardiometabolic disorders. Given the considerable potential benefit to society, we believe the risk-benefit ratio for participation in the proposed studies is favorable.

4 Study Objectives

4.1 Hypothesis

Our hypothesis is that coordinated multi-organ response (brown and white adipose tissue, muscle, is modulated by the SNS both in the fasting state and after a meal; however, this coordinated response becomes dysfunctional in obesity. Dysfunctional brain regulation of SNS activation to target organs may explain the increased risk of cardiometabolic disorders in obesity.

4.2 Primary Objective

The primary objective of this study is to determine whether sympathetic nervous system (SNS) activity in adipose tissue (WAT and BAT), muscle and brain is altered in individuals with obesity in comparison to individuals with normal weight. Simultaneous multi-organ SNS activation will be obtained with a radiotracer for norepinephrine transporters (NET) for whole-body Positron Emission Tomography (PET) imaging and microneurography (gold standard test for assessment of muscle SNS activity).

4.3 Secondary Objectives (if applicable)

The secondary objectives of this study are: 1) to evaluate whether gender differences affect peripheral SNS in healthy normal weight and obese men and women in adipose tissue (WAT and BAT) and muscle (resting/fasting); 2) To investigate the relationship between peripheral and central SNS activity in obesity, by correlating SNS activity in peripheral tissues (WAT, BAT, and muscle) and brain; and 3) To investigate NET CNS and peripheral SNS activity before and after a high carbohydrate mixed meal in lean and obese men and women.

4.4 Exploratory Objectives (if applicable)

N/A

5 Study Design

5.1 General Design Description

Aim: To measure peripheral SNS in healthy normal weight and obese men and women in adipose tissue (WAT and BAT), deltoid skeletal muscle, and brain NET at fast and after a mixed meal test (MMT).

The goal of this study is to measure in 20 individuals with normal weight (BMI 18-25 kg/m2) and 20 individuals with obesity (BMI 30-50 kg/m2) NET-binding in WAT and BAT to better understand the role of SNS in obesity. SNS activation of a) peripheral tissues: adipose tissue (BAT and WAT) and skeletal muscle; and b) CNS NET will be measure synchronously by using [11C]MRB whole body

PET imaging in combination with the gold standard measurement of peripheral SNS activity (muscle sympathetic nervous activity (MSNA)). PET and MSNA visits do not need to occur in sequence, i.e. PET scans can occur before or after the MSNA.

Visit 1 - Screening procedures:

Potential subjects will complete pre-screening over the telephone for approximately 10minutes to determine preliminary eligibility based on inclusion/exclusion criteria and basic socio-demographic information will be collected. Potentially qualifying participants will then be screened in person at the Yale New Haven Hospital (YNHH) Research Unit (HRU) or Church Street Research Unit (CSRU) with vital signs, EKG, and a medical history and physical exam. A urine pregnancy test will be administered for females.

Oral glucose tolerance test (OGTT) will be administered as follows: Following a 10-hour overnight fast, a nurse will insert an intravenous (IV) catheter. Subsequently, subjects will ingest 7.5 oz of Glucola, which contains 75 g of dextrose in orange flavored water. Blood samples will be taken at -15, 0, 10, 20, 30, 60, 90 and 120 minutes (after glucola ingestion) for the measurement of plasma glucose, insulin, and C-peptide concentrations, and calculation of measurements of insulin sensitivity index (Matsuda Index). Fasting blood obtained from the catheter will be collected for HbA1C, creatinine, liver enzymes (ALT, AST), TSH, hematocrit, and lipid panel. Approximately 40 mL of blood will be drawn at this visit.

Body Composition, Percent Body Fat & Percent Body Water: will be assessed using bioelectrical impedance analysis Tanita® scale (FDA cleared); which is a special multi frequency seg-mental body composition analyzer that delivers a very mild electrical current through the feet that allows measurement of fat mass, percent body fat, fat free mass, total body water, and percent body water. This test will be performed at screening visit. For better accuracy, measurement of percent fat and percent body water will be obtained with whole body Dual X-Ray Absorptiometry (DXA) scan (Hologic®) (located in the HRU) on screening-OGTT visit. The scanner arm will move over the participant's body from feet to head. The machine uses a small amount of radiation (one tenth of the amount of radiation from a chest x-ray) to measure body fat, muscle and bone density.

Subjects who fail any of the screening procedures will be informed and excluded from participating in the study, however they will be allowed to be re-screened at a later time,

Visit 2 – Whole body PET Imaging Visit - FASTING:

Two weeks after the screening visit, participants who qualified for the study will be asked to come to the PET Center to undergo the whole body [11C]MRB PET scan. Participants will be asked to maintain their regular exercise routine.

Human PET-CT Protocol: [11C]MRB will be synthesized at the Yale PET center based on procedures described and previously performed 1,2. An intravenous bolus dose of MRB (~120mCi) will be injected by an infusion pump. PET data will be acquired dynamically for 120 min using the Siemens mCT-X whole-body PET/CT scanner using continuous bed motion to image perform multiple PET bed acquisitions from the top of the head to the lower abdominal region. A CT scan will be performed for attenuation correction and to help delineatethe BAT and other regions of interest (ROIs). Images will be reconstructed with an ordered subset expectation maximization algorithm using point spread function correction and time-of-flight information. To delineate fat (WAT, BAT) and skeletal muscle regions of interests (ROIs),

CT images are first resliced to match the resolution and location of PET images. As performed in previous studies 2, a supraclavicular ROI is first manually drawn on the CT images. Within that area, the fat ROI is segmented using the CT images and an intensity window from -200 to -50 HU. Subsequently the fat ROI is segmented into WAT and BAT ROIs by using the 11C-MRB PET images: Standardized Uptake Value (SUV) ≥ 1.25 for BAT, SUV < 1.25 for WAT.

Brain ROIs for predetermined NET-rich areas of the brain, including the locus ceruleus, raphe, thalamus and hypothalamus were defined in template space and are applied to the PET images by using the participant's MR image as an intermediate step. For analysis of tracer uptake, mean SUV will be reported for segmentation of adipose tissue. Because the distribution volume ratio (DVR) is a more precise measurement of uptake for reversible tracers [3] such as ^{11}C-MRB, regional quantification of ^{11}C-MRB uptake as DVR (V_T/ V_{ND}), estimated via multilinear reference tissue model 2 (MRTM2) [4,5] (t*=30 min) using the occipital cortex, a region with low NET density [2,5,6] as the reference region. MRTM2 reduces the variability of MRTM (*4*) parameter estimates by fixing one of the three parameters, the parameter k_2', which is only related to the reference region time-activity curve, to a common value for all target regions. In this study, the parameter k_2' was fixed to 0.021 min^{-1}, based on previous brain studies and previous BAT PET studies [2]. In addition to DVR, MRTM2 can estimate R_1, the relative radioligand delivery, using a 1 tissue compartment model (1TC). For the 1TC model, volume of distribution, $V_T = K_1/k_2$, where K_1 (mL/min^{-1}/mL^{-1}) is the rate constant of radiotracer from plasma to tissue of target organ and k_2 is the rate constant from tissue to plasma. Using a reference region with non-displaceable binding (e.g. occipital lobe) we can estimate similar rate constants: $V_{ND} = K'_1/k'_2$. Recalling that K_1 and K'_1 are the rate constants for transfer from plasma to the tissue and reference region, respectively, then we can estimate relative radioligand delivery as $R_1 = K_1/K'_1$.

The maximum number of attempts for each failed/incomplete imaging and test is 2 times.

Visit 3 – Whole body PET Imaging Visit - High-Carbohydrate Mixed Meal Test (MMT)

High Carbohydrate Mixed meal test (MMT): Two weeks after visit 2**,** participants will come to the PET Center after a 10-hour overnight fast. A nurse will insert an intravenous (IV) catheter and subjects will ingest a liquid meal (65% CHO, 20% fat, and 15% protein, equal to 40% of daily energy expenditure, per ~16 ounces) to be prepared by the Metabolic Kitchen located at the HRU. Blood samples will be collected at −10, 0, 15, 30, 60, 90, and 120 min) for measurement of glucose, insulin and C-peptide concentrations [7]. Plasma samples will be drawn at baseline and throughout the MMT for measurement of additional hormones (such as leptin, ghrelin, thyroid hormones, and catecholamines) and free fatty acids (FFA) levels. Insulin and glucose will be used to calculate indexes of insulin sensitivity [7-9]. Hormones, glucose and FFA will be correlated with PET measurements of brown fat at baseline and during the MMT. Approximately 60 mL of blood will be drawn at this visit.

Participants will undergo whole body [^{11}C]MRB PET scan as described in visit 3. After the MMT, PET data will be acquired dynamically for 120 min using the Siemens mCT-X whole-body PET/CT scanner after an intravenous bolus dose of MRB (~20mCi).

The order of the PET scan visits 2 and 3 will be randomized.

PET and MSNA visits do not need to occur in sequence, i.e. PET scans can occur before or after the MSNA.

Measurement of Energy Intake: Hunger and fullness will be determined immediately before, during and after the PET scans with Visual Analog Scales (VAS).

Visit 4 – Sympathetic Nervous System Activity (SNSA) measurement Visit - Fasting

Two weeks after visit 3, participants will be asked to return for a separated visit for measurements of SNS activity at rest and fasting. This visit will be performed at the Pierce Laboratory (Dr. Stachenfeld's laboratory). The participants will come in the morning (~9am) and will be asked to fast for 10-hour and to maintain their regular exercise routine. The following measurements will be obtained: MSNA, heart rate (HR), blood pressure (BP), electrocardiogram (EKG), and heart rate variability. The proposed procedures will be performed at fasting (baseline). Participants will be asked to maintain their regular exercise routine.

Muscle sympathetic nerve activity (MSNA): Microneurography is the gold standard, most sensitive and reliable MSNA assessment in humans. The primary measure of sympathetic nervous system activity is peripheral nerve activity, as measured by MSNA.

Muscle sympathetic nerve activity will be quantified by identification and measurement of sympathetic bursts in the integrated neurogram and expressed as burst frequency (number of bursts per unit time). ECG surface electrodes are applied to the chest, an elastic band incorporating a strain gauge element is strapped around the thorax for recording respiratory movements, and a sensor cuff of a servopulse-plethysmography arterial pressure monitor (FINAPRES, Ohmeda) is placed on the middle phalanx of the long finger (held at heart level). The experiments are carried out in an environmentally controlled chamber at an ambient temperature of 27.0 ± 0.1 °C. The experiment will begin with a 60-minute control period. During such time the subjects rest in the reclined position.

Measurements of HR, BP, and EKG (beat-to-beat) will also be obtained during the procedure. An intravenous catheter will be inserted into a forearm vein for blood sampling. Following the instrumentation period, resting HR, pulse interval (PI), BP, and MSNA will be recorded for 10 minutes. Following these measurements, plasma will be taken for measurements of insulin, glucose, free fatty acids, norepinephrine, and epinephrine levels. Approximately 20 mL of blood will be drawn at this visit. After the first blood sample and the 10-min rest phase, subjects will begin a seven-minute period of rest with paced breathing (12 breaths per minute) while HR, BP, PI and MSNA are measured. The subjects will synchronize breathing to a metronome set to a cadence of ~12 breaths per minute (0.2 Hz).

Procedure: Microneurography Recording of multiunit postganglionic muscle sympathetic nerve activity (MSNA) will be made from the common peroneal nerve as it winds around the fibular head with the subject in the supine position with the subject's thigh comfortably

supported. This procedure requires us to first trace the course of the nerve using small electrical stimuli applied to the surface of the skin over the nerve or use the ultrasound to visually assist. This allows the microneurographer to find and focus the recording electrode tip on a nerve fascicle associated with muscle sympathetic activity. The time required to focus the electrode on the nerve averages about 30 min and will not exceed 60 min. In optimal conditions the track of the nerve from superficial stimulation is accurate and the nerve remains in position during insertion. An uninsulated subcutaneous electrode will serve as the reference. Mechanoreceptor afferent activity can be recorded during light tapping on the muscle belly or passive muscle stretch. A suitable intrafascicular recording site for MSNA consists of regular bursts and synchronous with the cardiac cycle. The neural activity is amplified (2x104), filtered (0.5-5.0 kHz), rectified, and integrated. The amplified and filtered nerve signal is also led to an audio monitor and through a resistance capacitance circuit (time constant 100 ms). The integrated nerve signal is digitized at 400 Hz along with the ECG signal while respiratory and arterial pressure signals are also sampled at 400 Hz (MacLab 8s). Data for analysis is selected from periods of quiet breathing in which subjects were relaxed and not talking. Microneurography was approved under HIC protocols # 8079, #0512000875, #1609018353 and is currently used under # 2000020950.

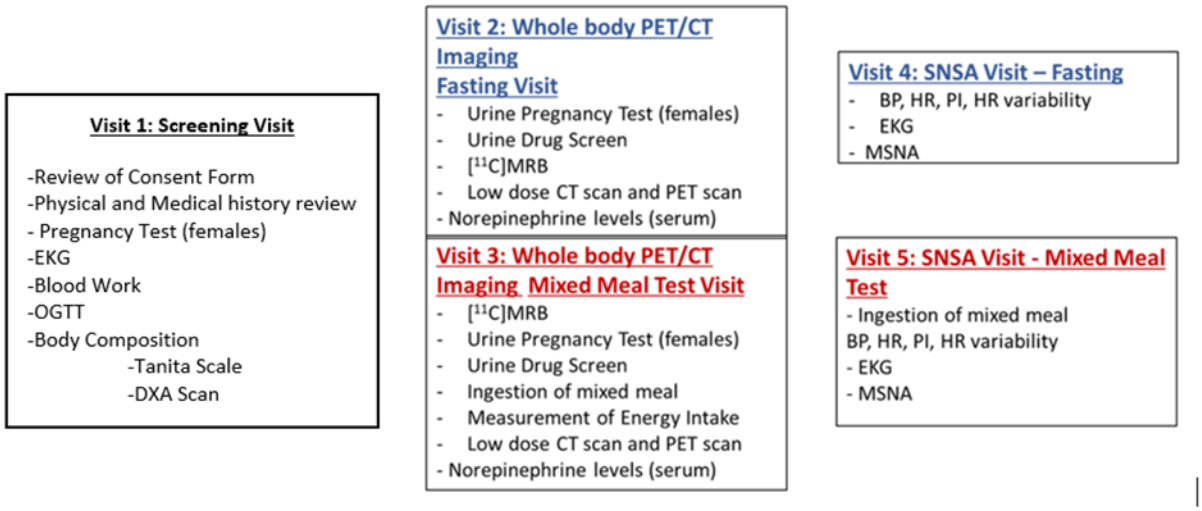
Cardiovascular variables. Recorded with a 16-channel computerized data-acquisition system at a sampling Speed of 400 Hz (ADI Instruments PowerLab 9, Castle Hill, Australia). Beat-to-beat DBP, SBP and pulse interval (PI) are determined using peak detection algorithms (44). Brachial artery BP and HR are measured with an automated sphygmomanometer (Colin Medical Instruments Corp, Komaki, Japan). Heart rate and pulse interval will be determined beat-to-beat from lead II of the EKG recording.

Visit 5 – Sympathetic Nervous System Activity (SNSA) measurement Visit – Mixed Meal Test (MMT)

Two weeks after visit 4, participants will be asked to return for a separated visit for measurements of SNS activity at rest and after intake of liquid meal. Participants will undergo similar procedures described on visit 4. However, MSNA will be obtained after MMT. The participants will come in the morning (~9am) and will be asked to fast for 10-hour and to maintain their regular exercise routine. A nurse will insert an intravenous (IV) catheter and subjects will ingest a liquid meal (65% CHO, 20% fat, and 15% protein, equal to 40% of daily energy expenditure, per ~16 ounces) to be prepared by the Metabolic Kitchen. Measurements of HR, BP, and EKG (beat-to-beat) will be obtained during the procedure. An intravenous catheter will be inserted into a forearm vein for blood sampling. Following the instrumentation period, resting HR, pulse interval (PI), BP, and MSNA will be recorded for 10 minutes. Following these measurements, plasma will be taken for measurements of insulin, glucose, free fatty acids, norepinephrine, and epinephrine levels. Approximately 40 mL of blood will be drawn at this visit.

Participants will be asked to maintain their regular exercise routine. The order of SNSA Fasting and MMT visits (visits 4 and 5) will be randomized. All study visits will be conducted within an 8-week period (including the two PET and SNSA scans).

Schematic figure of the visits and procedures to be done. The order of the PET scans will be randomized.



Abbreviations: BP=blood pressure, EKG=electrocardiogram, HR=heart rate, MSNA=Muscle sympathetic nerve activity, SNSA=Sympathetic Nervous System Activity

Method of Assignment/Randomization- The order of the PET scan visits 2 (PET-CT Fasting) and 3 (PET/CT-MMT) will be randomly assigned (with a 50% chance of being assigned to each visit). SNSA visits 4 (fasting) and 5 (MMT) will be also be also be randomized (with a 50% chance of being assigned to each visit).

PET and MSNA visits do not need to occur in sequence, i.e. PET scans can occur before or after the MSNA.

5.1.1 Study Date Range and Duration

07/01/2021-06/30/2025

5.1.2 Number of Study Sites

1

5.2 Outcome Variables

Group differences in 1) [^{11}C]MRB-binding in brown adipose tissue (BAT), white adipose tissue (WAT), skeletal muscle, and norepinephrine-rich brain regions (hypothalamus and thalamus);

and 2) microneurography (muscle sympathetic nerve activity (MSNA)). Correlations between NET in the brain and peripheral NET and MSNA.

5.2.1 Primary Outcome Variables

The primary objective of this study is to determine whether sympathetic nervous system (SNS) activity in adipose tissue (WAT and BAT), muscle and brain is altered in individuals with obesity in comparison to individuals with normal weight. Simultaneous multi-organ SNS activation will be obtained with a radiotracer for norepinephrine transporters (NET) for whole-body Positron Emission Tomography (PET) imaging and microneurography (gold standard test for assessment of muscle SNS activity).

Primary outcome: SNS activity measured by [^{11}C]MRB binding in adipose tissue, muscle and brain; and sympathetic burst in muscle (measured with microneurography).

5.2.2 Secondary Outcome Variables (if applicable)

The secondary objectives of this study are: 1) to evaluate whether gender differences affect peripheral SNS in healthy normal weight and obese men and women in adipose tissue (WAT and BAT) and muscle (resting/fasting); 2) To investigate the relationship between peripheral and central SNS activity in obesity, by correlating SNS activity in peripheral tissues (WAT, BAT, and muscle) and brain; and 3) To investigate NET CNS and peripheral SNS activity before and after a high carbohydrate mixed meal in lean and obese men and women.

5.2.3 Exploratory Outcome Variables (if applicable)

N/A

5.3 Study Population

The subjects we plan to enroll are in good medical health as evidenced by the medical history review during screening and lean (BMI 18.5-24.9 kg/m2) or non-diabetic obese (BMI 30-50 kg/m2).

5.3.1 Number of Participants

We plan to enroll 20 participants with normal BMI (10 women and 10 men) and 20 with obesity (10 women and 10 men).

5.3.2 Eligibility Criteria/Vulnerable Populations

Inclusion Criteria:

HIC 2000030749

In order to be eligible to participate in this study, an individual must meet all of the following criteria:

1. Provision of signed and dated informed consent form
2. Stated willingness to comply with all study procedures and availability for the duration of the study
3. Age 18–45 years
4. In good general health as evidenced by medical history and lean (BMI 18.5-24.9 kg/m_2) or non-diabetic obese (BMI 30-50 kg/m^2) with a fasting plasma glucose (FPG) <100 mg/dL and a hemoglobin A1c <5.7%.

Exclusion Criteria:

An individual who meets any of the following criteria will be excluded from participation in this study:

1. Hypertension
2. Cardiac or pulmonary disease
3. Known history of Type 1 or Type 2 diabetes
4. Hepatic disease, swallowing and gastrointestinal disorders, including but not limited to diverticulitis and inflammatory bowel disease
5. Disorders or impairment of the gag reflux
6. Subjects with previous gastrointestinal surgery.
7. Subjects with a pacemaker or other implanted electro medical device
8. Current use of anti-obesity medications, supplements and/or anti-hyperglycemic medications
9. Neurological injury or illness, and psychiatric medications
10. Women who are pregnant or lactating
11. Subjects who suffer from claustrophobia
12. Subjects who have received a diagnostic or therapeutic radiopharmaceutical within 7 days prior to participation in this study
13. Subjects who work with radiation or have participated in other research studies involving ionizing radiation within one year of the PET scans that would cause the subject to exceed the yearly dose limits.
14. Subjects with history of IV drug use which would prevent venous access for PET tracer injection
15. Severe motor problems that prevent the subject from lying still for PET and MR imaging
16. Subjects who complain of chronic pain
17. Blood donation within 8 weeks of the study
18. Non-English speaking subjects
19. Subjects with poor venous access
20. Subjects with an allergy or intolerance to the carbohydrate meal or IV contrast.
21. Subjects with a history of bleeding disorders

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22. Subjects on anticoagulation medications.
23. Subjects who are Transgender

6 Methods

6.1 Treatment

6.1.1 Identity of Investigational Product

[^{11}C]MRB will be prepared at the Yale PET Center under the supervision of Drs. Henry Huang and Nabeel Nabulsi in accordance with local Chemistry Manufacturing & Control (CMC) procedures and quality specifications described in our local Drug Master File (DMF), which has been approved by the Yale University Radioactive Drug Research Committee (YU RDRC).

6.1.2 Dosage, Administration, Schedule

N/A

6.1.3 Method of Assignment/Randomization

Method of Assignment/Randomization- The order of the PET scan visits 2 (PET-CT Fasting) and 3 (PET/CT-MMT) will be randomly assigned (with a 50% chance of being assigned to each visit). SNSA visits 4 (fasting) and 5 (MMT) will be also be also be randomized (with a 50% chance of being assigned to each visit).

Blinding and Procedures for Unblinding

N/A

6.1.4 Packaging/Labelling

N/A

6.1.5 Storage Conditions

N/A

6.1.6 Concomitant therapy

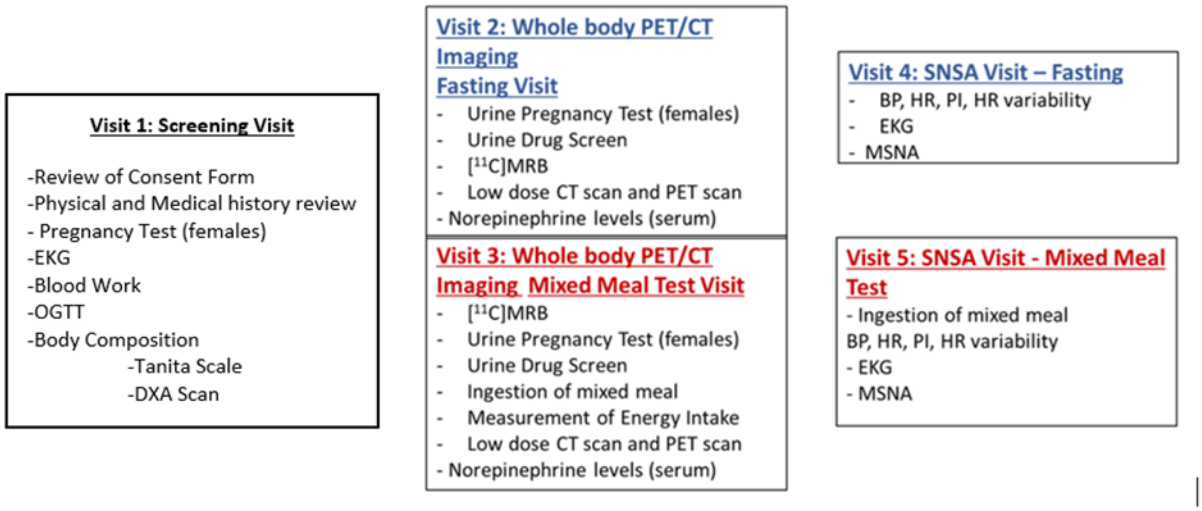
There are no restrictions.

6.1.7 Restrictions

There are no restrictions.

6.2 Assessments

Assessments are detailed in the figure below:



Abbreviations: BP=blood pressure, EKG=electrocardiogram, HR=heart rate, MSNA=Muscle sympathetic nerve activity, SNSA=Sympathetic Nervous System Activity

Please note: The order of visits 2 and 3 are will be randomly assigned with a 50% chance of being assigned to each visit

6.2.1 Efficacy

See 5.1 General Design Description

6.2.2 Safety and Pregnancy-related policy

Risks Associated with Radiation:

No PET studies will be performed on pregnant or potentially pregnant women, as confirmed by pregnancy testing during evaluation and on each scan day before initiation of any scan procedures. If subjects are breastfeeding, they will not be able to participate in this research study.

6.2.3 Adverse Events Definition and Reporting

1. Personnel responsible for the safety review and its frequency:

The principal investigator, Dr. Belfort De Aguiar will be responsible for monitoring the data, assuring protocol compliance, and conducting the safety reviews at the specified frequency, which must be conducted at a minimum of every 6 months (including when reapproval of the protocol is sought). During the review process, the principal investigator will evaluate whether the study should continue unchanged, require modification/amendment, or close to enrollment. Either the principal investigator, the HIC, or the RSC have the authority to stop or suspend the study or require modifications.

HIC 2000030749

2. The risks associated with the current study are deemed greater than minimal for the following reasons: (choose those that apply)

The total amount of radiation in this study is from up to 3 low dose CT scans - used to optimize the PET scan – and up to 3 injections of radioactive material (20 mCi), [11C]MRB and from a low dose CT scans used to help obtain the PET images and the DXA scan .

The average yearly background radiation in the United States is about 0.3300 mrem. The amount of additional radiation from participating in this study is about 2.307 rem. This is equal to about 7.69 years worth of natural radiation.

The amount of radiation subjects will receive in this study is below the dose guidelines established by the RIDC guidelines for research purposes. This guideline sets an effective dose limit of 5 rem per year.

Adverse effects of the radiopharmaceuticals in this study have not been reported. However, the possibility exists for a rare reaction to any of the substances or procedures to which a subject is exposed.

3. Attribution of Adverse Events:

Adverse events will be monitored for each subject participating in the study and attributed to the study procedures / design by the principal investigator Dr. Belfort De Aguiar, according to the following categories:

a.) Definite: Adverse event is clearly related to investigational procedures(s)/agent(s).

b.) Probable: Adverse event is likely related to investigational procedures(s)/agent(s).

c.) Possible: Adverse event may be related to investigational procedures(s)/agent(s).

d.) Unlikely: Adverse event is likely not to be related to the investigational procedures(s)/agent(s).

e.) Unrelated: Adverse event is clearly not related to investigational procedures(s)/agent(s).

4. Plan for Grading Adverse Events:

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The following scale will be used in grading the severity of adverse events noted during the study:

1. Mild adverse event
2. Moderate adverse event
3. Severe

Plan for Determining Seriousness of Adverse Events:

Serious Adverse Events:

In addition to grading the adverse event, the PI will determine whether the adverse event meets the criteria for a Serious Adverse Event (SAE). An adverse event is considered serious if it results in any of the following outcomes:

1. Death.
2. A life-threatening experience in-patient hospitalization or prolongation of existing hospitalization.
3. A persistent or significant disability or incapacity.
4. A congenital anomaly or birth defect; OR
5. Any other adverse event that, based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition.

An adverse event may be graded as severe but still not meet the criteria for a Serious Adverse Event. Similarly, an adverse event may be graded as moderate but still meet the criteria for an SAE. It is important for the PI to consider the grade of the event as well as its "seriousness" when determining whether reporting to the IRB is necessary.

6. Plan for reporting UPIRSOs (including Adverse Events) to the IRB

The principal investigator will report the following types of events to the IRB:

Any incident, experience or outcome that meets ALL 3 of the following criteria:

1. Is unexpected (in terms of nature, specificity, severity, or frequency) given (a) the research procedures described in the protocol-related documents, such as the IRB-approved protocol and informed consent document and (b) the characteristics of the subject population being studied; AND

2. Is related or possibly related to participation in the research (possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); AND

3. Suggests that the research places subjects or others at greater risk of harm (including physical, psychological, economic, legal, or social harm) than was previously known or recognized.

Unanticipated Problems Involving Risks to Subjects or Others (UPIRSOs) may be medical or non-medical in nature, and include – but are not limited to – serious, unexpected, and related adverse events and unanticipated adverse device effects. Please note that adverse events are reportable to the IRB as UPIRSOs only if they meet all 3 criteria listed above.

These UPIRSOs/SAEs will be reported to the IRB in accordance with IRB Policy 710, using the appropriate forms found on the website. All related events involving risk but not meeting the prompt reporting requirements described in IRB Policy 710 should be reported to the IRB in summary form at the time of continuing review. If appropriate, such summary may be a simple brief statement that events have occurred at the expected frequency and level of severity as previously documented. In lieu of a summary of external events, a current DSMB report can be submitted for research studies that are subject to oversight by a DSMB (or other monitoring entity that is monitoring the study on behalf of an industry sponsor).

7. Plan for reporting adverse events to co-investigators on the study, as appropriate the protocol's research monitor(s), e.g., industrial sponsor, Yale Cancer Center Data and Safety Monitoring Committee (DSMC), Protocol Review Committee (PRC), DSMBs, study sponsors, funding and regulatory agencies, and regulatory and decision-making bodies.

For the current study, the following individuals, funding, and/or regulatory agencies will be notified:

- All Co-Investigators listed on the protocol.
- National Institute of Health

1 Other Data Safety Monitoring Board (DSMB) or Committee (DSMC): The Yale PET Center's internal Data Safety Monitoring Board, composed of the Directors of the PET Center, also meets to review any adverse events on a monthly basis.

1 Yale New-Haven Radiation Safety Committee (if applicable)

Yale University Radiation Safety Committee (if applicable)

The principal investigator Dr. Belfort De Aguiar will conduct a review of all adverse events upon completion of every study subject. The principal investigator will evaluate the frequency

and severity of the adverse events and determine if modifications to the protocol or consent form are required.

Research staff will call subject 1-3 days after the PET scan to check in and address any adverse events that may have arisen post PET scan.

The study will be stopped if either of the following occur:

1) If 1 or more subjects experiences a serious adverse event (SAE), which is considered by the investigator to be possibly or probably related to the radiotracer.

2) If 2 or more subjects experience AEs of severe intensity, which are considered by the investigator to be possibly or probably related to the radiotracer and of clinical concern.

After review of the safety data, SAEs and AEs confirmed to have been related to the radiotracer will be reported to the FDA and the appropriate regulatory committees at Yale. The study will be placed on hold until a determination is made as to whether the study may continue as written, if modifications are needed, or if the study will be discontinued.

Definitions

Adverse event (AE) means any untoward medical occurrence associated with the use of an intervention in humans, whether or not considered intervention-related (21 CFR 312.32 (a)).

An AE or suspected adverse reaction is considered "serious" (SAE) if, in the view of either the investigator or sponsor, it results in any of the following outcomes:

- death,
- a life-threatening adverse event,
- inpatient hospitalization or prolongation of existing hospitalization,
- a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions,
- a congenital anomaly/birth defect, or
- An important medical event that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

Severity

Adverse events will be graded according to [name grading scale, e.g. CTCAE v5.0]. For AEs not included in the protocol defined grading system, the following guidelines will be used to describe severity.

- Mild – Events require minimal or no treatment and do not interfere with the participant's daily activities.
- Moderate – Events result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.
- Severe – Events interrupt a participant's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually potentially life-threatening or incapacitating. Of note, the term "severe" does not necessarily equate to "serious".]

Relationship to Investigational Product

All AEs must have their relationship to study intervention assessed by the clinician who examines and evaluates the participant based on temporal relationship and his/her clinical judgment. The degree of certainty about causality will be graded using the categories below. In a clinical trial, the study product must always be suspect.

- Definitely Related – There is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out. The clinical event, including an abnormal laboratory test result, occurs in a plausible time relationship to study intervention administration and cannot be explained by concurrent disease or other drugs or chemicals. The response to withdrawal of the study intervention (dechallenge) should be clinically plausible. The event must be pharmacologically or phenomenologically definitive, with use of a satisfactory rechallenge procedure if necessary.
- Probably Related – There is evidence to suggest a causal relationship, and the influence of other factors is unlikely. The clinical event, including an abnormal laboratory test result, occurs within a reasonable time after administration of the study intervention, is unlikely to be attributed to concurrent disease or other drugs or chemicals, and follows a clinically reasonable response on withdrawal (dechallenge). Rechallenge information is not required to fulfill this definition.
- Potentially Related – There is some evidence to suggest a causal relationship (e.g., the event occurred within a reasonable time after administration of the trial medication). However, other factors may have contributed to the event (e.g., the participant's clinical condition, other concomitant events). Although an AE may rate only as "possibly related" soon after discovery, it can be flagged as requiring more information and later be upgraded to "probably related" or "definitely related", as appropriate.

- Unlikely to be related – A clinical event, including an abnormal laboratory test result, whose temporal relationship to study intervention administration makes a causal relationship improbable (e.g., the event did not occur within a reasonable time after administration of the study intervention) and in which other drugs or chemicals or underlying disease provides plausible explanations (e.g., the participant's clinical condition, other concomitant treatments).
- Not Related – The AE is completely independent of study intervention administration, and/or evidence exists that the event is definitely related to another etiology. There must be an alternative, definitive etiology documented by the clinician.

Expectedness

The Principal Investigator will be responsible for determining whether an AE is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the study intervention.

6.2.4 Pharmacokinetics (if applicable)

N/A

6.2.5 Biomarkers (if applicable)

N/A

6.3 Study Procedures

Visit 1 - Screening procedures:

Potential subjects will complete pre-screening over the telephone for approximately 10 minutes to determine preliminary eligibility based on inclusion/exclusion criteria and basic socio-demographic information will be collected. Potentially qualifying participants will then be screened in person at the Yale New Haven Hospital (YNHH) Research Unit (HRU) with vital signs, EKG, and a medical history and physical exam. Fasting blood will be collected for HbA1C,creatinine, liver enzymes (ALT, AST), TSH, hematocrit, and lipid panel. A urine pregnancy test will be administered.

Oral glucose tolerance test (OGTT) will be administered as follows: Following a 10-hour overnight fast, a nurse will insert an intravenous (IV) catheter. Subsequently, subjects will ingest 7.5 oz of Glucola, which contains 75 g of dextrose in orange flavored water. Blood samples will be taken at -15, 0, 10, 20, 30, 60, 90 and 120 minutes (after glucola ingestion) for the measurement of plasma glucose, insulin, and C-peptide concentrations, and calculation of measurements of insulin sensitivity index (Matsuda Index). Approximately 40 mL of blood will be drawn at this visit.

Body Composition, Percent Body Fat & Percent Body Water: will be assessed using bioelectrical impedance analysis Tanita® scale (FDA cleared); which is a special multi frequency seg-mental body composition analyzer that delivers a very mild electrical current

that allows measurement of fat mass, percent body fat, fat free mass, total body water, and percent body water. This test will be performed at screening visit. For better accuracy, measurement of percent fat and percent body water will be obtained with whole body Dual X-Ray Absorptiometry (DXA) scan (Hologic®) (located in the HRU) on screening-OGTT visit. The scanner arm will move over the participant's body from feet to head. The machine uses a small amount of radiation (one tenth of the amount of radiation from a chest x-ray) to measure body fat, muscle and bone density.

Visit 2 – Whole body PET/CT Imaging Visit - FASTING:

After 2 weeks from visit 1, -participants who qualified for the study will be asked to come to the PET Center to undergo the whole body [11C]MRB PET scan. Participants will be asked to maintain their regular exercise routine.

Human PET-CT Protocol: [11C]MRB will be synthesized at the Yale PET center based on procedures described and previously performed 1,2. An intravenous bolus dose of MRB (~120mCi) will be injected by an infusion pump. PET data will be acquired dynamically for 120 min using the Siemens mCT-X whole-body PET/CT scanner using continuous bed motion to image perform multiple PET bed acquisitions from the top of the head to the lower abdominal region. A CT scan will be performed for attenuation correction and to help delineate the BAT and other regions of interest (ROIs). Images will be reconstructed with an ordered subset expectation maximization algorithm using point spread function correction and time-of-flight information. To delineate fat (WAT, BAT) and skeletal muscle regions of interests (ROIs), CT images are first resliced to match the resolution and location of PET images. As performed in previous studies 2, a supraclavicular ROI is first manually drawn on the CT images. Within that area, the fat ROI is segmented using the CT images and an intensity window from -200 to -50 HU. Subsequently the fat ROI is segmented into WAT and BAT ROIs by using the 11C-MRB PET images: Standardized Uptake Value (SUV) ≥ 1.25 for BAT, SUV < 1.25 for WAT.

Brain ROIs for predetermined NET-rich areas of the brain, including the locus ceruleus, raphe, thalamus and hypothalamus were defined in template space and are applied to the PET images by using the participant's MR image as an intermediate step. For analysis of tracer uptake, mean SUV will be reported for segmentation of adipose tissue. Because the distribution volume ratio (DVR) is a more precise measurement of uptake for reversible tracers 3 such as 11C-MRB, regional quantification of 11C-MRB uptake as DVR (VT/ VND), estimated via multilinear reference tissue model 2 (MRTM2) 4,5 (t*=30 min) using the occipital cortex, a region with low NET density 2,5,6 as the reference region. MRTM2 reduces the variability of MRTM (4) parameter estimates by fixing one of the three parameters, the parameter k2', which is only related to the reference region time-activity curve, to a common value for all target regions. In this study, the parameter k2' was fixed to 0.021 min-1, based on previous brain studies and previous BAT PET studies 2. In addition to

DVR, MRTM2 can estimate R1, the relative radioligand delivery, using a 1 tissue compartment model (1TC). For the 1TC model, volume of distribution, VT = K1/k2, where K1 (mL/min−1/mL−1) is the rate constant of radiotracer from plasma to tissue of target organ and k2 is the rate constant from tissue to plasma. Using a reference region with non-displaceable binding (e.g. occipital lobe) we can estimate similar rate constants: VND = K'1/k'2. Recalling that K1 and K'1 are the rate constants for transfer from plasma to the tissue and reference region, respectively, then we can estimate relative radioligand delivery as R1 = K1/K'1.

Visit 3 – Whole body PET/CT Imaging Visit - High-Carbohydrate Mixed Meal Test (MMT)

High Carbohydrate Mixed meal test (MMT):

After 2 weeks from visit 2, participants will come to the PET Center after a 10-hour overnight fast. A nurse will insert an intravenous (IV) catheter and subjects will ingest a liquid meal (65% CHO, 20% fat, and 15% protein, equal to 40% of daily energy expenditure, per ~16 ounces) to be prepared by the Metabolic Kitchen located at the HRU. Blood samples will be collected at −10, 0, 15, 30, 60, 90, and 120 min) for measurement of glucose, insulin and C-peptide concentrations 7. Plasma samples will be drawn at baseline and throughout the MMT for measurement of additional hormones (such as leptin, ghrelin, thyroid hormones, and catecholamines) and free fatty acids (FFA) levels. Insulin and glucose will be used to calculate indexes of insulin sensitivity 7-9. Hormones, glucose and FFA will be correlated with PET measurements of brown fat at baseline and during the MMT. Approximately 60 mL of blood will be drawn at this visit.

Participants will undergo whole body [11C]MRB PET scan as described in visit 3. After the MMT, PET data will be acquired dynamically for 120 min using the Siemens mCT-X whole-body PET/CT scanner after an intravenous bolus dose of MRB (~20mCi).

The order of the PET scan visits 2 and 3 will be randomized.

Measurement of Energy Intake: Hunger and fullness will be determined immediately before, during and after the PET scans with Visual Analog Scales (VAS).

Visit 4 – Sympathetic Nervous System Activity (SNSA) measurement Visit - Fasting

After 2 weeks from visit 3, participants will be asked to return for a separated visit for measurements of SNS activity at rest and fasting. This visit will be performed at the Pierce Laboratory (Dr. Stachenfeld's laboratory). The participants will come in the morning (~9am) and will be asked to fast for 10-hour and to maintain their regular exercise routine. The following measurements will be obtained: MSNA, heart rate (HR), blood pressure (BP), electrocardiogram (EKG), and heart rate variability,. The proposed procedures will be performed at fasting (baseline). Participants will be asked to maintain their regular exercise routine.

Muscle sympathetic nerve activity (MSNA): Microneurography is the gold standard, most sensitive and reliable MSNA assessment in humans. The primary measure of sympathetic nervous system activity is peripheral nerve activity, as measured by MSNA.

Muscle sympathetic nerve activity will be quantified by identification and measurement of sympathetic bursts in the integrated neurogram and expressed as burst frequency (number of bursts per unit time). ECG surface electrodes are applied to the chest, an elastic band incorporating a strain gauge element is strapped around the thorax for recording respiratory movements, and a sensor cuff of a servopulse-plethysmography arterial pressure monitor (FINAPRES, Ohmeda) is placed on the middle phalanx of the long finger (held at heart level). The experiments are carried out in an environmentally controlled chamber at an ambient temperature of 27.0 ± 0.1 °C. The experiment will begin with a 60-minute control period. During such time the subjects rest in the reclined position.

Measurements of HR, BP, and EKG (beat-to-beat) will also be obtained during the procedure. An intravenous catheter will be inserted into a forearm vein for blood sampling. Following the instrumentation period, resting HR, pulse interval (PI), BP, and MSNA will be recorded for 10 minutes. Following these measurements, plasma will be taken for measurements of insulin, glucose, free fatty acids, norepinephrine, and epinephrine levels. Approximately 20 mL of blood will be drawn at this visit. After the first blood sample and the 10-min rest phase, subjects will begin a seven-minute period of rest with paced breathing (12 breaths per minute) while HR, BP, PI and MSNA are measured. The subjects will synchronize breathing to a metronome set to a cadence of ~12 breaths per minute (0.2 Hz).

Procedure: Microneurography Recording of multiunit postganglionic muscle sympathetic nerve activity (MSNA) will be made from the common peroneal nerve as it winds around the fibular head with the subject in the supine position with the subject’s thigh comfortably supported. This procedure requires us to first trace the course of the nerve using small electrical stimuli applied to the surface of the skin over the nerve or use the ultrasound to visually assist. This allows the microneurographer to find and focus the recording electrode tip on a nerve fascicle associated with muscle sympathetic activity. The time required to focus the electrode on the nerve averages about 30 min and will not exceed 60 min. In optimal conditions the track of the nerve from superficial stimulation is accurate and the nerve remains in position during insertion. An uninsulated subcutaneous electrode will serve as the reference. Mechanoreceptor afferent activity can be recorded during light tapping on the muscle belly or passive muscle stretch. A suitable intrafascicular recording site for MSNA consists of regular bursts and synchronous with the cardiac cycle. The neural activity is amplified (2x104), filtered (0.5-5.0 kHz), rectified, and integrated. The amplified and filtered nerve signal is also led to an audio monitor and through a resistance capacitance circuit (time constant 100 ms). The integrated nerve signal is digitized at 400 Hz along with the ECG signal while respiratory and arterial pressure signals are also sampled at 400 Hz (MacLab 8s). Data for analysis is selected from periods of quiet breathing in which subjects were relaxed and not talking. Microneurography was approved under HIC protocols # 8079, #0512000875, #1609018353 and is currently used under # 2000020950.

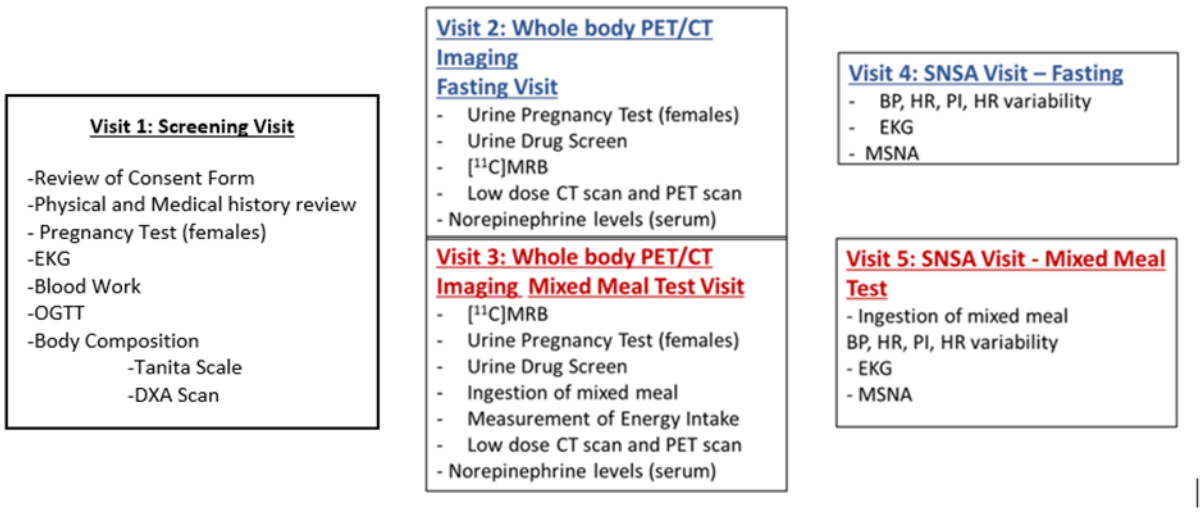
Cardiovascular variables. Recorded with a 16-channel computerized data-acquisition system at a sampling Speed of 400 Hz (ADI Instruments PowerLab 9, Castle Hill, Australia). Beat-to-beat DBP, SBP and pulse interval (PI) are determined using peak detection algorithms (44). Brachial artery BP and HR are measured with an automated sphygmomanometer (Colin Medical Instruments Corp, Komaki, Japan). Heart rate and pulse interval will be determined beat-to-beat from lead II of the EKG recording.

Visit 5 – Sympathetic Nervous System Activity (SNSA) measurement Visit – Mixed Meal Test (MMT)

After 2 weeks from visit 4, participants will be asked to return for a separated visit for measurements of SNS activity at rest and after intake of liquid meal. Participants will undergo similar procedures described on visit 4. However, MSNA will be obtained after MMT. The participants will come in the morning (~9am) and will be asked to fast for 10-hour and to maintain their regular exercise routine. A nurse will insert an intravenous (IV) catheter and subjects will ingest a liquid meal (65% CHO, 20% fat, and 15% protein, equal to 40% of daily energy expenditure, per ~16 ounces) to be prepared by the Metabolic Kitchen. Measurements of HR, BP, and EKG (beat-to-beat) will be obtained during the procedure. An intravenous catheter will be inserted into a forearm vein for blood sampling. Following the instrumentation period, resting HR, pulse interval (PI), BP, and MSNA will be recorded for 10 minutes. Following these measurements, plasma will be taken for measurements of insulin, glucose, free fatty acids, norepinephrine, and epinephrine levels. Approximately 40 mL of blood will be drawn at this visit.

Participants will be asked to maintain their regular exercise routine. The order of SNSA Fasting and MMT visits (visits 4 and 5) will be randomized. All study visits will be conducted within an 8-week period (including the two PET and SNSA scans).

6.3.1 Study Schedule



Abbreviations: BP=blood pressure, EKG=electrocardiogram, HR=heart rate, MSNA=Muscle sympathetic nerve activity, SNSA=Sympathetic Nervous System Activity

6.3.2 Informed Consent

All subjects are consented by one of the study investigators or research staff, as part of determining their study eligibility. The purpose, nature, and potential risks and benefits of the study will be explained to each potential subject in detail during the screening interview.

Prior to initiating the study, one of the study investigators or research staff will explain the planned study interventions, and each subject will be asked to read and sign a consent form approved by the Yale School of Medicine Human Investigation Committee (HIC).

All potential subjects are given ample time to ask questions and only after this are they asked to provide informed consent in the research study. Subjects are given a copy of the signed consent for their personal records.

6.3.3 Screening

Research team members will contact interested potential participants to assess eligibility and provide the participant with additional information about the study, including the study procedures, purpose and potential complications. The brief screen via email or phone will ask for the following information to confirm study eligibility:

- Name
- Age/Date of Birth

- Place of Birth
- Phone Number/Email
- Address
- Marital Status
- Race/Ethnicity
- Height/Weight
- Medical/Surgical History
- Medications
- Drug/Alcohol Use

In order to determine the potential participant's eligibility and avoid unnecessary screening and traveling efforts, it is necessary to collect this information prior to having the subject arrive for an in-person screening visit. This information will be protected according to HIPAA policies and will be destroyed if the individual is determined to not be eligible for the study during this correspondence, or is otherwise not interested in participating in the study, unless the subject consents for his/her screening information to be stored for future studies that he/she may be eligible for.

During the in-person screening visit, the participant will arrive to the Yale New Haven Hospital (YNHH) Research Unit (HRU). The study nurse will collect vital signs, EKG, and the Principal Investigator will review the participant's medical history and perform a physical exam. Fasting blood will be collected for HbA1C, creatinine, liver enzymes (ALT, AST), TSH, hematocrit, and lipid panel. A urine pregnancy test will be administered to females of child bearing potential.

Oral glucose tolerance test (OGTT) will be administered as follows: Following a 10-hour overnight fast, a nurse will insert an intravenous (IV) catheter. Subsequently, subjects will ingest 7.5 oz of Glucola, which contains 75 g of dextrose in orange flavored water. Blood samples will be taken at -15, 0, 10, 20, 30, 60, 90 and 120 minutes (after glucola ingestion) for the measurement of plasma glucose, insulin, and C-peptide concentrations, and calculation of measurements of insulin sensitivity index (Matsuda Index).

Body Composition, Percent Body Fat & Percent Body Water: will be assessed using bioelectrical impedance analysis Tanita® scale (FDA cleared); which is a special multi frequency seg-mental body composition analyzer that delivers a very mild electrical current that allows measurement of fat mass, percent body fat, fat free mass, total body water, and percent body water. This test will be performed at screening visit. For better accuracy, measurement of percent fat and percent body water will be obtained with whole body Dual X-Ray Absorptiometry (DXA) scan (Hologic®) (located in the HRU) on screening-OGTT visit. The scanner arm will move over the participant's body from feet to head. The machine uses a small amount of radiation (one tenth of the amount of radiation from a chest x-ray) to measure body fat, muscle and bone density.

6.3.4 Enrollment

HIC 2000030749

Research team members will enroll subjects who have been consented and screened during the in-person screening visit to ensure they meet eligibility criteria.

Recruitment Procedures:

Subjects will be recruited through flyers, by word of mouth, and through YCCI email blasts and JDAT.

a. Messaging to Study Participants through JDAT:

The following wording will be used for all applicable recruitment methods:

“You are receiving this [notification, email, letter, phone call] because you may qualify for and be interested in a research study conducted by Yale University investigators looking at whether obesity is associated with increased activity of the sympathetic nervous system.

For JDAT queries only: The Yale New Haven Health electronic health record system has searched medical conditions to find people who may be good matches for research studies. No one has looked at your record and no information has been shared with any research doctor or research team member. Just because you received this message does not mean that you are in a research study or that you have to decide to be in this or any study.

To opt-out of research, including opting out of receiving future messages about research studies, please email optout@yale.edu or call 1-877-978-8348 and select option #3.

Title of study: Sympathetic Activation in Obesity

Principal Investigator: Renata Belfort de Aguiar, MD, PhD

Study Coordinator: Jacqueline Prinz

Phone # 203-785-5977

Description of Study:

The primary objective of this study is to determine whether sympathetic nervous system (SNS) activity in white adipose tissue (WAT) and brown adipose tissue (BAT), skeletal muscle and brain is altered in individuals with obesity in comparison to individuals with normal weight. Simultaneous multi-organ SNS activation will be obtained with a radiotracer for norepinephrine transporter (NET) for whole-body Positron Emission Tomography (PET) imaging and microneurography (peroneal muscle SNS activity).

Confidentiality and Privacy: Any personal health, financial data, and other information gathered in the study will remain confidential and will be stored on a password-protected computer, only accessed by study personnel. When the results of the research are published or discussed, no information will be included that would reveal your identity. We understand that information about you obtained in connection with your health is personal, and we are committed to protecting the privacy of that information. If you would like to learn more about participating in this study, please contact the study coordinator at 203-785-5977.

APPROVED BY THE YALE UNIVERSITY IRB 3/30/2022

HIC 2000030749

Possible Benefits: The study may have no direct benefit to you, however general advancement of scientific knowledge from this study may include a better understanding of the importance of SNS activation in obesity.

Participation in this study is completely voluntary. You are free to decline to participate, to end participation at any time for any reason, or to refuse to answer any individual question at any time. Refusing to participate will involve no penalty or loss of benefits to which you are otherwise entitled (such as your health care outside the study, the payment for your health care, and your health care benefits).

Questions: If you have any further questions about this study, you may contact the investigator, Dr. Renata Belfort de Aguiar, at 203-737-4777. If you would like to talk with someone other than the researchers to discuss problems, concerns, and questions you may have concerning this research, or to discuss your rights as a research subject, you may contact the Yale Human Investigation Committee at (203) 785-4688."

b. Describe how potential subjects are contacted. The subjects will be asked to call us at our main recruitment number or email if they are interested in participating in the research study. Subjects will be contacted back for a confidential phone screening which leads to an in-person screening if eligible.

c. Who is recruiting potential subjects? The PI, in collaboration with study investigators and trained research assistants, is responsible for subject recruitment.

6.3.5 On Study Visits

Screening

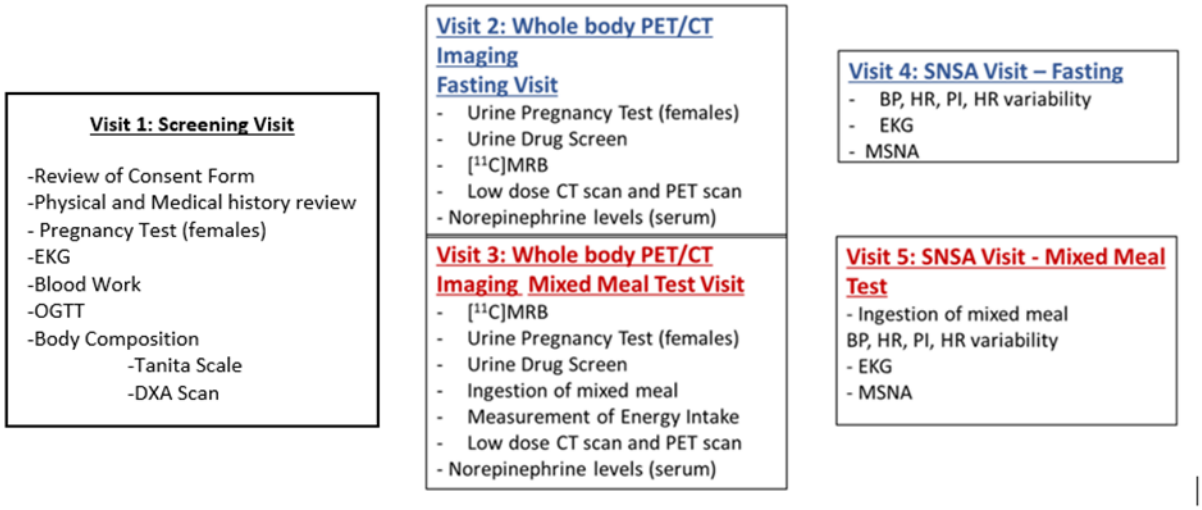
- Consent
- Urine Pregnancy test for women of child-bearing potential
- Height and Weight using Tanita Scale
- EKG
- IV Insertion and OGTT
- Blood draw for anemia, kidney and liver function and thyroid test and diabetes
- Physical & Medical History
- DXA Scan

Visit 2 & 3 PET Scans

- Subject will arrive to PET Center, fasted
- Urine Pregnancy Test for women of child bearing potential
- Urine Drug Screen
- A-line and IV insertion
- Mixed Meal Test on Visit 3
- A low dose CT scan will be completed immediately before or after each PET scan.
- Up to 3-4 hour PET scan
- Collection of Blood Sampled during PET scan
- Light Lunch given to subject after PET Scan

Visits 4 & 5

- Subject will arrive fasted to the Pierce Laboratory for measurements SNS activity
- Urine Sample Collection
- Collection of Weight
- Collection of the following: Microneurography, BP, HR
- EKG
- IV Insertion
- Blood Sample
- Measurement of skin blood flow
- Sympathetic Nervous System Activity (SNSA) measurement



Abbreviations: BP=blood pressure, EKG=electrocardiogram, HR=heart rate, MSNA=Muscle sympathetic nerve activity, SNSA=Sympathetic Nervous System Activity

6.3.6 End of Study and Follow-up

Follow Up Procedures:

A member of the study team will reach out to the subject 1-2 weeks upon study completion for a brief follow-up telephone call. During this phone call, the subject will be asked how they are feeling since completing the study assessments and if they have any questions or concerns.

6.3.7 Removal of subjects

Participating in this study is voluntary. Subjects are free to choose not to take part in this study. Refusing to participate will involve no penalty or loss of benefits otherwise entitled to the subject (such as health care outside the study, the payment for health care, and health care benefits).

Subjects may withdraw permission to use and disclose health information at any time by telling the study staff or by writing to;

APPROVED BY THE YALE UNIVERSITY IRB 3/30/2022

Yale University School of Medicine

300 Cedar street, TAC S-135

PO Box 208020

New Haven, CT 06520

The researchers may withdraw a subject's participation in the research if deemed necessary. The subject may be asked to withdrawal from the study if they experience significant discomfort during the procedures, become pregnant during the study intervention, are not able to follow instructions during the procedures, or are not compliant with scheduled appointments.

No new health information identifying the subject will be gathered after the date of study withdrawal. Information that has already been gathered may still be used and given to others until the end of the research study, as necessary to ensure the integrity of the study and/or study oversight.

6.4 Statistical Method

6.4.1 Statistical Design

Primary endpoints: Group differences in 1) [^{11}C]MRB-binding in brown adipose tissue (BAT), white adipose tissue (WAT), skeletal muscle, and norepinephrine-rich brain regions (hypothalamus and thalamus); and 2) microneurography (muscle sympathetic nerve activity (MSNA)). Correlations between NET in the brain and peripheral NET and MSNA.

Secondary endpoints: Plasma hormone levels, heart rate, blood pressure, electrocardiogram (beat-to-beat)..

Statistical Analysis: Before statistical testing, all continuous variables will be tested for normality of distribution. Summary statistics will be computed. For between-groupcomparisons of patient characteristics, two sample t-test or Mann-Whitney U test will be usedfor continuous variables, and Fisher's exact test for categorical variables. Comparisons of repeatedly measured MSNA will be made using the mixed model analysis. Fixed factors include group and sex and their interactions. Linear contrasts comparing differences between groups or different time points within a group will be estimated.

6.4.2 Sample Size Considerations

Statistical Considerations: sample sizes of 6 per group (normal BMI x obesity, and women x men) will achieve 90% power to detect a between-group difference. To account for up to 10% missing data due to drop out or poor-quality data, we will need to enroll a total of 40 subjects (10 per group) for this study.

Fasting: A Power analysis was performed based on previous study published from our group in lean and obese female individuals: (lean 1.15 ±0.19 vs obese 0.80 ± 0.12 BAT DVR, p = 0.004; two-sided independent sample t-test) [2]. In our previous cross-sectional comparison,

using the mean and standard deviations between lean and obese individuals, we expect an effect size of d=2.20 and with a power of 0.90, to account for possible smaller differences in males, we need 6 individuals per group. For reference, d=2.20 and 0.80 power would be 5 per group.

MMT: Postprandial whole-body energy expenditure per fat free mass (FFM) differences were shown for low-BAT (32±2 kcal/day/kg FFM) and high-BAT (37±2 kcal/day/kg FFM) [45]. Based on these data, we can calculate an effect size of d=2.50. With a power of 0.80 required, we will need 8 individuals (4 per group) determine EE differences between low- and high-BAT individuals. Increasing the power to 0.95 will increased the number of individuals to 12 (6 per group). Due to the technical challenges of performing microneurography on multiple occasions in obese women, we overestimate and account for ~ 10% failure rate, so we will recruit 10 subjects per group.

6.5 Planned Analyses

6.5.1 Primary Objective Analysis

The primary objective of this study is to quantify sympathetic nervous system (SNS) activity by [^{11}C]MRB PET imaging and microneurography (MSNA). [^{11}C]MRB binding will be quantified in adipose tissue (WAT and BAT), muscle; and whether [^{11}C]MRB binding and sympathetic bursts are altered in individuals with obesity in comparison to individuals with normal weight.

Statistical Analysis: Before statistical testing, all continuous variables will be tested for normality of distribution. Summary statistics will be computed. For between-groupcomparisons of patient characteristics, two sample t-test or Mann-Whitney U test will be usedfor continuous variables, and Fisher's exact test for categorical variables. Comparisons of repeatedly measured MSNA will be made using the mixed model analysis. Fixed factors include group and sex and their interactions. Linear contrasts comparing differences between groups or different time points within a group will be estimated.

6.5.2 Secondary Objectives Analyses

1) to evaluate whether gender differences affect peripheral SNS in healthy normal weight and obese men and women in adipose tissue (WAT and BAT) and muscle (resting/fasting); 2) To investigate the relationship between peripheral and central SNS activity (in obesity, by correlating SNS activity in peripheral tissues (WAT, BAT, and muscle) and brain; and 3) To investigate NET CNS and peripheral SNS activity before and after a high carbohydrate mixed meal in lean and obese men and women.

6.5.3 Exploratory Objectives Analyses (if applicable)

N/A

6.5.4 Safety

The principal investigator Renata Belfort De Aguiar, MD will be responsible for monitoring the data, assuring protocol compliance, and conducting the safety reviews at the specified

frequency, which must be conducted at a minimum of every 6 months (including when reapproval of the protocol is sought). During the review process, the principal investigator will evaluate whether the study should continue unchanged, require modification/amendment, or close to enrollment. Either the principal investigator, the HIC, the NIH (NIDA), or the RSC have the authority to stop or suspend the study or require modifications.

6.5.5 Analysis of Subject Characteristics

The goal of this study is to measure in 20 individuals with normal weight (BMI 18-25 kg/m2) and 20 individuals with obesity (BMI 30-50 kg/m2)

6.5.6 Interim Analysis (if applicable)

N/A

6.5.7 Health economic evaluation

Subjects will paid $25 for the screening OGTT visit, regardless of the outcome, $200 for completion of each PET scan sessions (2 in total, $400), $100 for completion of each SNSA visit (2 in total, $200), $25 for each Mixed Meal Test (2 in total, $50),

and $100 bonus for completion of all 5 visits.

Subjects will be sent a Bank of America reloadable card that will be sent to their confirmed home address upon completion of each study visit as detailed above. In addition, subjects will be provided with a light lunch on PET day.

2. Costs for Participation (Economic Considerations): Clearly describe the subject's costs associated with participation in the research, and the interventions or procedures of the study that will be provided at no cost to subjects.

No cost to subject related to participation in this research study.

3. In Case of Injury: This section is required for any research involving more than minimal risk, and for minimal risk research that presents the potential for physical harm (e.g., research involving blood draws).

a. Will medical treatment be available if research-related injury occurs? Yes

b. Where and from whom may treatment be obtained? Subjects would be assessed by PET Center practitioners and referred as appropriate to medical services.

c. Are there any limits to the treatment being provided? No

d. Who will pay for this treatment? Subject or subject's insurance

e. How will the medical treatment be accessed by subjects? Subjects would be assessed by PET Center practitioners and referred as appropriate to medical services.

Medical treatment will be offered to the subjects for any physical injuries that they receive as a result of participating in this research. However, the subject or his/her insurance company is responsible for the cost. Federal regulations require that subjects be told that if they are physically injured, no additional financial compensation is available.

6.5.8 Other

N/A

6.5.9 Subsets and Covariates

N/A

6.5.10 Handling of Missing Data

Analyses will be conducted on available data. Adjustments to statistical models will be performed, as appropriate and necessary.

7 Trial Administration

7.1 Ethical Considerations: Informed Consent/Assent and HIPAA Authorization

7.2 Institutional Review Board (IRB) Review

The protocol will be submitted to the IRB for review and approval. Approval of the protocol must be obtained before initiating any research activity. Any change to the protocol or study team will require an approved IRB amendment before implementation. The IRB will determine whether informed consent and HIPAA authorization are required.

The IRB will conduct continuing review at intervals appropriate to the degree of risk, but not less than once per year.

A study closure report will be submitted to the IRB after all research activities have been completed.

Other study events (e.g. data breaches, protocol deviations) will be submitted per [insert institution's] IRB's policies.

7.3 Subject Confidentiality

Required private identifiable information about individuals, such as their medical history, current medications, psychiatric problems, and family history, will be collected by research staff and be used for research purposes and charting after consent is obtained.

HIPAA identifiers:

☒ Telephone numbers

☒ E-mail addresses

☒ Medical record numbers

☒ All elements of dates (except year) for dates related to an individual, including: birth date, admission date, discharge date, date of death, all ages over 89 and all elements of dates (including year) indicative of such age, except that such ages and elements may be aggregated into a single category of age 90 or older

The data are collected and recorded by trained research personnel. The data will be recorded in Excel spreadsheets that will be saved onto a University encrypted computer or a

secure server, or will be in the form of questionnaires that are filled out by the subject or the researcher. These paper research materials containing confidential information are stored in locked filing cabinets. Additional brain data is collected during the brain imaging scans by trained technologists and is stored on password-protected and encrypted computers with identifying information carefully in compliance with HIPAA regulations.

This research is covered by a Certificate of Confidentiality from the National Institutes of Health. The researchers with this Certificate may not disclose or use information, documents, or biospecimens that may identify you in any federal, state, or local civil, criminal, administrative, legislative, or other action, suit, or proceeding, or be used as evidence, for example, if there is a court subpoena, unless you have consented for this use. Information, documents, or biospecimens protected by this Certificate cannot be disclosed to anyone else who is not connected with the research except, if there is a federal, state, or local law that requires disclosure (such as to report child abuse or communicable diseases but not for federal, state, or local civil, criminal, administrative, legislative, or other proceedings, see below); if you have consented to the disclosure, including for your medical treatment; or if it is used for other scientific research, as allowed by federal regulations protecting research subjects

7.4 Deviations/Unanticipated Problems

If the study team becomes aware of an anticipated problem (e.g. data breach, protocol deviation), the event will be reported to the IRB.

7.5 Data Collection

All digital data will be stored either on a secured server or on University encrypted desktops or laptops. All staff members that come into contact with the data are fully trained to the current HIPAA regulations and are informed as to the proper use of all data. Identifiable paper information is kept in locked file drawers and password protected computer files. Results are published as group data without the use of characteristics that would identify individual subjects. We quote information only by number in conference discussions, scientific reports, or publications, in order to maintain anonymity. Identifiable research data, including recruitment and screening information and code keys, are stored on a secure database located on the internal PET Center Network. The PET network is protected by a Cisco PIX firewall operated by ITS. All research data are backed up nightly to a Dell PV-136T library wit 4 IBM Ultrium-TD2 tape drives using the backup software Legato Networker 7.3 from EMC. Human subjects enrolled in the study are assigned a subject-specific random identifier. Subject identifiers and the means to link the subject names and codes with the research data are stored in separate locations within the database. The software of the database limits the ability to connect the random identifier to the actual subject identification information to research team members only. Access to the database is password protected and each research team member is required to have a unique ID and password to gain access to the database. Authorized users employ their netid and authentication is performed using Yale's central authentication server. Users always access research data through the random identifier only. Direct identifiers belonging to subjects who withdraw from the study, will be stripped from the key.

The data will be stored in locked filing cabinets and on the password-protected secure database on the internal Yale University PET Center Network for at least 7 years after the close of the study, accessed only by authorized personnel. After 7 years, the data will be de-identified. Data may be kept indefinitely.

7.6 Data Quality Assurance

See 6.5.4

7.7 Study Records

- Regulatory Documents
- EKG
- Consent Forms
- Subject Medical Records
- PET imaging data (and related scan day paperwork)
- Biospecimens (and related lab request paperwork – e.g. blood insulin measurements)

7.8 Access to Source Documents

See 7.5

7.9 Data or Specimen Storage/Security

See 7.5

7.10 Retention of Records

The data will be stored in locked filing cabinets and on the password-protected secure database on the internal Yale University PET Center Network for at least 7 years after the close of the study, accessed only by authorized personnel. After 7 years, the data will be de-identified. Data may be kept indefinitely.

7.11 Study Monitoring

The principal investigator Renata Belfort De Aguiar will be responsible for monitoring the data, assuring protocol compliance, and conducting the safety reviews at the specified frequency, which must be conducted at a minimum of every 6 months (including when reapproval of the protocol is sought). During the review process, the principal investigator will evaluate whether the study should continue unchanged, require modification/amendment, or close to enrollment. Either the principal investigator, the HIC, the NIH (NIDA), or the RIDC have the authority to stop or suspend the study or require modifications.

7.12 Data Safety Monitoring Plan

The principal investigator Dr. Belfort De Aguiar. will conduct a review of all adverse events upon completion of every study subject. The principal investigator will evaluate the frequency and severity of the adverse events and determine if modifications to the protocol or consent form are required.

Research staff will call subject 1-3 days after the PET scan to check in and address any adverse events that may have arisen post PET scan.

The study will be stopped if either of the following occur:
1) If 1 or more subjects experiences a serious adverse event (SAE), which is considered by the investigator to be possibly or probably related to the radiotracer.

2) If 2 or more subjects experience AEs of severe intensity, which are considered by the investigator to be possibly or probably related to the radiotracer and of clinical concern.

After review of the safety data, SAEs and AEs confirmed to have been related to the radiotracer will be reported to the FDA and the appropriate regulatory committees at Yale. The study will be placed on hold until a determination is made as to whether the study may continue as written, if modifications are needed, or if the study will be discontinued.

7.13 Study Modification

Study modifications will be submitted to the IRB review and subjects will be contacted if necessary.

7.14 Study Discontinuation

If the study is prematurely terminated or suspended, the Principal Investigator (PI) will promptly inform study participants, the Institutional Review Board (IRB), and sponsor and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule.

Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Demonstration of efficacy that would warrant stopping
- Insufficient compliance to protocol requirements
- Data that are not sufficiently complete and/or evaluable
- Determination that the primary endpoint has been met
- Determination of futility

7.15 Study Completion

When participants have completed study and analysis has been complete, or if any other factor would lead to the shutdown of the study i.e. loss of funding or safety concerns, the IRB will be notified via their respective reporting channel..

7.16 Conflict of Interest Policy

The independence of this study from any actual or perceived influence, such as by the pharmaceutical industry, is critical. Therefore, any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed. Furthermore, persons who have a perceived conflict of interest will be required to have such conflicts managed in a way that is appropriate to their participation in the trial. The study leadership in conjunction with the appropriate conflict of interest review committee has established policies and procedures for all study group members to disclose all conflicts of interest and will establish a mechanism for the management of all reported dualities of interest.

All investigators will follow the applicable conflict of interest policies.

7.17 Funding Source

NIH/NIDDK 1R56DK129344-01

7.18 Publication Plan

These studies are likely to produce novel results relating to obesity and SNS that will merit publication, and the PI (Renata Belfort De Aguiar, MD, PhD) will be responsible for publishing any manuscripts resulting from these efforts.

8 Appendices

Appendix #	Title	Section	Topic

9 List of Tables

10 References:

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