Beige Fat, Energy, and the Natriuretic Peptide System

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1.0 Background

Obesity and obesity-related complications are leading causes of morbidity and mortality among Veterans. Obese individuals experience a markedly increased risk of cardiovascular and metabolic ("cardiometabolic") diseases– including diabetes, hypertension, and coronary artery disease. The rising prevalence of these problems despite current therapies supports that obesity is a multifactorial disease and highlights the need for novel approaches to treat obesity-related cardiometabolic dysfunction.

Evidence from genetic studies in animals and humans indicate that the natriuretic peptide (NP) system may protect against cardiometabolic risk. The NPs are cardiac derived hormones, classically recognized for their role in the regulation of blood pressure and volume status. In recent years, animals studies and human genetic investigation have demonstrated that higher genetically-determined NP levels protect against development of hypertension, obesity, Type 2 diabetes, and metabolic syndrome.[1, 2] Further, administration of B-type natriuretic peptide (BNP) to wild-type mice leads to increased thermogenic gene expression in brown as well as white adipose depots, increased energy expenditure, and reduced fat accumulation.[3] The increase in thermogenic gene expression in white adipose tissue reflects the development of "brown-like" or "beige" adipose tissue (a process referred to as "beiging"). Brown and beige adipose tissue increase energy expenditure by dissipating energy as heat (thermogenesis) through the actions of uncoupling protein 1 (UCP1).[4] Promoting the development of a beige adipose phenotype could potentially lead to increases in energy expenditure, promote weight loss, and improve insulin sensitivity in obesity**.**

NP levels are lower in obese compared with lean individuals,[5] and in black compared with white individuals,[6, 7] in human epidemiologic investigations. This suggests that low circulating NP levels may reflect a relative NP deficiency in obese and black individuals. As the NP system appears to protect against cardiometabolic risk, a relative NP deficiency could contribute to adverse cardiometabolic outcomes observed in obese and black individuals. Whether a relative NP deficiency in these groups is associated with reduced thermogenic gene expression and lower energy expenditure is not well understood.

2.0 Rationale and Specific Aims

NP levels differ by obesity status and race in epidemiologic studies, and NPs appear to promote "beiging" of adipose tissue in animals. However, whether gene expression of beige adipose tissue differs by obesity status and race has not been established in humans. Human mechanistic studies investigating the relationships of beige adipose tissue markers with obesity status, race, and the natriuretic peptide system are limited. We propose an innovative physiologic study in which we will quantify adipose tissue gene expression and energy expenditure in states of relative NP deficiency in humans, namely in obese and black individuals. Our overarching postulate is that obese and black individuals have NP deficiencies that contribute to less beige adipose tissue and lower energy expenditure. Thus, we propose the following aims:

Aim 1: Determine whether beige adipose tissue markers differ between obese and lean individuals.
We plan to enroll and conduct subcutaneous adipose biopsies on approximately 155 adult participants (aged 18-55 years; approximately 50 lean and 105 obese participants). We will quantify expression of genes indicative of "beiging" in subcutaneous adipose tissue biopsies and determine whether gene expression differs based on obesity status. In a secondary analysis, we will determine whether these beige adipose tissue markers differ by race.

Aim 2: Determine whether beige adipose tissue markers and energy expenditure are associated with natriuretic peptide receptor expression in adipose tissue and circulating natriuretic peptide levels. We will examine these associations in approximately 155 adult participants, as described in Aim 1.

Secondary Aims:
-Determine whether adipose tissue gene expression profiles suggestive of beiging differ by race.
-Determine whether obesity-related differences in beige fat are attributable to differences in NP markers.
-Examine associations of adipose tissue phenotypes with cardiometabolic parameters.

3.0 Animal Studies and Previous Human Studies

The natriuretic peptide (NP) system and metabolism

The NP system is classically known for its role regulating blood pressure and volume status. In response to increased cardiac wall stress, the heart secretes atrial natriuretic peptide (ANP) and BNP, which both cause natriuresis, vasodilation, and inhibition of the renin-angiotensin-aldosterone system. Thus, the biologic actions of the NPs serve to reduce blood pressure. Both ANP and BNP bind to their target receptor, NPR-A, and are cleared by the clearance receptor, NPR-C.[1] NP receptors are widely present throughout the cardiovascular system, as well as in adipose tissue, skeletal muscle, liver, brain, and gut.

Although the NP system is classically known for its role in blood pressure regulation, recent studies have shown that NPs have important metabolic actions as well. Administration of NPs stimulate lipolysis in animals and humans.[8-11] Transgenic mice that overexpress BNP are protected against weight gain, visceral fat accumulation, and insulin resistance induced by high-fat diets.[1] Moreover, Bordiccia et al. demonstrated in wild-type mice that BNP administration over 7 days significantly increases energy expenditure and oxygen consumption.[3] The increases in energy expenditure and oxygen consumption appear to be mediated by increased expression of thermogenic genes (including UCP1) in brown adipose tissue, as well as white adipose tissue.[3] The increase in thermogenic gene expression in white adipose tissue reflects the development of beige fat.

The NP system, obesity, and cardiometabolic risk

The NP system may protect against cardiometabolic risk. In humans, a common genetic variant resulting in ~10-20% higher NP concentrations (still within the physiologic range) is associated with lower prevalence of hypertension (by 15%),[2] obesity (by 46%), and metabolic syndrome (by 42%), and a lower risk of developing Type 2 diabetes (by 12%).[12, 13] Moreover, this genetic variant that causes higher NP levels is associated with favorable cardiovascular biomarkers and a lower prevalence of myocardial infarction.[12] Conversely, humans with a genetic variant leading to lower NP levels exhibit higher risk of hypertension, future T2DM,[12, 13] and evidence of adverse cardiac remodeling.[14] These data strongly suggest that the NP system may protect against cardiometabolic risk.

Obese and black individuals have low circulating NP levels,[5] which appears to reflect a relative "NP deficiency." Multiple large cohort studies have demonstrated that NP levels are lower in obese compared with lean individuals,[5] and in black compared with white individuals.[6, 7] Obese and black individuals have a propensity toward hypertension, which would be expected to cause higher circulating NP concentrations. Thus, the lower NP concentrations in obese and black individuals are physiologically inappropriate and likely reflect a relative NP deficiency. As the NP system appears to protect against cardiometabolic risk, a relative NP deficiency could raise susceptibility to the adverse cardiometabolic outcomes observed in obese and black individuals. The reasons for the low NP levels in obese and black individuals are not entirely known. One proposed mechanism for the low NP concentrations in obesity is enhanced NP clearance in adipose tissue. Obese individuals have been found to have higher expression of the NP clearance receptor in adipose tissue compared with lean individuals.[15] Despite these associations, thermogenic gene expression in adipose tissue and whole body energy expenditure in these relatively NP deficient groups are not well characterized and understood.

4.0 Inclusion/Exclusion Criteria

We will enroll and conduct subcutaneous adipose biopsies on approximately 155 participants (approximately 50 lean subjects ($18.5 \leq BMI < 25$ kg/m^2) and approximately 105 obese subjects ($BMI \geq 30$ kg/m^2)). Full eligibility criteria are listed below.

Inclusion Criteria:

- Men and women ages 18-55 years
- Body Mass Index (BMI) ≥18.5 and <25 kg/m^2 (lean), or BMI ≥30 kg/m^2 (obese)

Exclusion Criteria:

- Significant pulmonary, liver, or renal disease
- Heart failure (any type), unstable coronary artery disease currently, or history of significant cardiac event or stroke within 12 months
- Diabetes Mellitus (Types 1 and 2)
- Thyroid dysfunction

- Active malignancy
- Chronic inflammatory diseases, such as inflammatory bowel disease, hepatitis, rheumatoid arthritis
- Current use of medications likely to affect energy homeostasis, including amphetamines and beta blockers, and significant use of systemic glucocorticoids
- Currently pregnant or breastfeeding, or unwilling to avoid becoming pregnant or breastfeeding during study duration
- Significant claustrophobia that would prevent the use of the metabolic cart as part of the study protocol
- Hemoglobin A1c (HbA1c) ≥6.5%
- Liver Function Tests (LFTs) elevated >3x upper limit of normal
- Estimated Glomerular Filtration Rate (eGFR) <40 ml/min

5.0 Enrollment/Randomization

Recruitment will take place at Tennessee Valley Healthcare System (TVHS) outpatient clinics, community-based outpatient clinics, veteran centers, veteran service organizations, Fort Campbell, and Vanderbilt University Medical Center (VUMC). All patients coming for outpatient services at TVHS may be considered. We will place IRB approved flyers and brochures in target clinics, veteran centers, Fort Campbell, and community boards (electronic and physical) with basic study information and study team contact information for potential subjects to signal potential interest. Working with the VA Public Affairs office, we will utilize their advertisement services such as waiting lobby TV advertisements, VA web homepage announcements, newspaper, and email distribution lists to veterans. A link may also be posted on My HealtheVet. We will also screen medical records in CPRS system (including data linked to CPRS through JLV and VISTA). VA subjects, once identified through the CPRS system, will be sent recruitment letters about participation in the study. The recruitment letters may be followed by a phone call to the participant. Study personnel may also approach possible study participants in clinic once permission is received by that subject's provider. Providers and clinic teams members within the VA or non-VA medical centers may also send referrals to the study team. Subjects may also be recruited using My HealthEVet and the VINCI system. Subjects may also be recruited via the Vanderbilt Broadcast email system (research.notifications@vanderbilt.edu). ResearchMatch.org may be utilized as a recruitment tool for this protocol. ResearchMatch.org is a national electronic, web-based recruitment tool that was created through the Clinical & Translational Science Awards Consortium in 2009 and is maintained at Vanderbilt University as an IRB-approved data repository. Study personnel may also use a VA-issued phone to communicate with recruited subjects (including phone calls and text messages for appointment reminders and logistical information). Text messages will not include any patient identifiers.

Due to recruitment setbacks related to COVID-19, some samples and data previously collected from another study, Cardiovascular Effects of GLP-1 Receptor Activation (P.I., Dr. J. Matthew Luther (Vanderbilt University Medical Center (VUMC) IRB# 170213, ClinicalTrials.gov Identifier: NCT03101930) will be included to help meet our enrollment goals for the obese subject population. In this prior study (VUMC IRB# 170213), obese individuals underwent the same study procedures that we are performing in the present study as outlined below (subcutaneous adipose tissue biopsy, indirect calorimetry, DXA body composition scan), and thus the samples/data have already been collected. These samples were of similar inclusion criteria as the currently recruiting study, with the minor adjustment of age criteria of up to 65 years of age. For the prior study (VUMC IRB#170213), subjects signed a consent form which indicated their consent for their samples/data to be used for future research. Samples provided from this study will be de-identified for our use. There are approximately 122 obese individuals from this prior study with available baseline samples/data relevant to any of our endpoints, of whom approximately 80 individuals have adipose biopsy data (our primary endpoint). The inclusion of these samples will enable us to analyze baseline data for approximately 105 obese subjects with adipose biopsy data (primary endpoint) (from the subjects that we are able to personally recruit, as well as the previously collected samples/data from the prior study).

6.0 Study Procedures

Screening Visit

After informed consent has been obtained, the subject's medical history and medications will be discussed and documented. A physical exam, including measurement of height, weight, and vital signs, will be performed. Blood will be collected for a comprehensive metabolic panel, complete blood count (CBC), Hemoglobin A1c, thyroid stimulating hormone (TSH), and free T4. A urine or serum pregnancy test will be completed on female subjects of child-bearing potential. Whenever possible, we will combine these research screening tasks and procedures with standard of care tasks and procedures to decrease the burden placed on the patient. This information will be acceptable from standard of care visits as far back as 6 months prior to the day the patient is identified as a possible candidate. Even if the patient has had these assessments completed during a standard of care visit, it is up to the discretion of the Principal Investigator to use them or require an updated set of assessments. The Study Investigator may also request a second screening blood draw to confirm eligibility. Inclusion/exclusion criteria will be reviewed to confirm that the subject meets study eligibility requirements. Patients may be asked to stop spironolactone and eplerenone, if the participant is using them for cosmetic or excessive hair growth, 4-6 weeks prior to the study visit at Vanderbilt's CRC, as these medications may affect the results of aldosterone and renin.

Study Visit

Subjects will arrive at the Vanderbilt Clinical Research Center (CRC) after having fasted for at least 8 hours. We will collect a urine sample, including urine sodium and

creatinine. Women of childbearing potential will undergo a pregnancy test. Subjects will then lie supine (flat on their back). After approximately 30 minutes of lying supine, baseline resting energy expenditure will be assessed using indirect calorimetry (metabolic cart, described below). At the completion of the metabolic cart, and while subject is still lying supine, we will collect a blood sample, including basic metabolic panel, measurements of NPs and measurements of the RAAS system (renin and aldosterone), insulin, glucose, and markers of lipolysis (glycerol, free fatty acids). Subjects will then undergo a subcutaneous fat biopsy, as described below. Subsequently gene expression will be assessed for markers of "beiging" (including UCP1), as well as other cardiometabolic pathways including the natriuretic peptide pathways. Protein expression and histology may also be assessed if sufficient tissue is obtained. Subjects will also undergo a dual-energy x-ray absorptiometry (DXA) scan to assess body composition. In the event of scheduling issues, the DXA scan may be performed within about 2 weeks before or after the main study visit. Subjects will need to remain lying supine for the majority of the study visit (about 2 hours, before and during the metabolic cart, blood draw, and fat biopsy).

For women, these study visits will be scheduled to coincide with the follicular phase of the menstrual cycle to minimize the impact of hormonal changes on energy expenditure (EE) and on measurements of renin-angiotensin-aldosterone (RAAS) system.

Other study procedures

Phlebotomy: Blood draws will occur as outlined above.

Subcutaneous adipose tissue biopsy: Participants will undergo percutaneous subcutaneous adipose tissue biopsies on the abdomen (peri-umbilical region). This procedure will be performed by a trained study physician or nurse practitioner. With the subject laying in a supine position, an approximately 10 cm diameter area will be cleaned with a chlorhexidine swab lateral to the umbilicus. A fenestrated sterile drape will be placed to establish the sterile field. Approximately 20 ml of 1% lidocaine will be applied to anesthetize the skin. A small (approximately 0.5 cm) incision is made through the skin with a scalpel, within a few centimeters lateral to the umbilicus. Upon examination of the participant's body habitus, the trained proceduralist may choose to first infiltrate normal saline into the subcutaneous adipose tissue if the proceduralist feels that this may improve the success of obtaining sufficient adipose tissue. Then, a TULIP 2.7mm liposuction cannula with a syringe attached is inserted and moved parallel to the skin at a rate of approximately 1 Hz without breaking suction and with a twisting motion. The sampling continues until a goal of approximately 10 ml of tissue (approximately 9.5 g) is collected. The syringe is then removed, antibiotic ointment applied, a steri-strip is placed, and the incision is covered with an adhesive bandage. The tissue will be rinsed from blood. Then, tissue samples will either be placed immediately in liquid nitrogen and stored at -80 degrees Celsius until analysis, or processed immediately. The bandages will be removed after 24 hours, and steri-strips are left in place until they fall off.

Indirect Calorimetry (Metabolic Cart): Resting energy expenditure will be determined by indirect calorimetry, using a metabolic cart (a ventilated hood system).

Hemodynamic monitoring (blood pressure monitoring and heart rate monitoring by cardiac telemetry): We will monitor heart rhythm and rate during the study. This will be done by placing sticky pads (electrodes) on the chest. The sticky pads are connected to wires (leads) that hook up to a machine and display heart rhythm, rate and blood pressure.

Substrate oxidation (lipid oxidation and carbohydrate oxidation): Respiratory exchange ratio will be determined using VO2 and VCO2 measurements, which is determined via indirect calorimetry, and collected at the same time points as energy expenditure.

Biomarkers: Blood, urine, and tissue samples will be coded for subject confidentiality. These samples will be measured for a wide array of cardiometabolic parameters and inflammatory markers. Possible techniques include RNAseq and metabolomics techniques. Additional samples will be frozen and stored for possible future investigation.

Table Of Events:

Procedure	Screening Visit	Main Study Visit
Informed Consent	X	
Medical History & Physical exam	X	
Vital Signs, height, weight	X	X
Pregnancy test	X	X
Clinical Laboratory sample collection	X	X
Fat Biopsy		X
Metabolic Cart		X
DXA scan		X

7.0 Risks

Fasting: Inconvenience and hypovolemia. To minimize risks of hypovolemia, subjects will be instructed to drink plenty of fluids the day before the visits, and we will measure subjects' blood pressure and heart rate upon arrival for their study visits and during the study visits. Also, there is a potential risk of hypoglycemia while fasting. However, the risk of hypoglycemia in healthy individuals while fasting for this amount of time is low.

Subjects will be monitored for signs and symptoms of hypoglycemia throughout the study visits.

Phlebotomy: The risks associated with phlebotomy in healthy individuals are minimal. Risks include pain, bruising, inflammation, and rarely fainting and infection. Other possible risks include low fluid status in the body and anemia (low blood counts). Risks will be minimized by excluding subjects with anemia or history of hypotension. Subjects will be advised to drink plenty of the fluids the night before the study visit, and after the study visit.

Hemodynamic monitoring (blood pressure monitoring and heart monitoring by cardiac telemetry): A non-invasive technique with no symptoms aside from possible minimal discomfort.

Percutaneous subcutaneous adipose tissue biopsy: The biopsy procedure will be performed by a study physician or nurse practitioner trained in this technique. The percutaneous subcutaneous adipose tissue biopsy procedure is associated with low risk. Possible risks of the biopsy procedure include pain, local skin irritation, bleeding, bruising, and hematoma at the site. There is potential risk for local or systemic infection, more severe bleeding, or a small scar; however, the risk of these events is extremely low.

Lidocaine: May cause local discomfort during injection, or a rash, redness or soreness at the injection site. In rare cases, lidocaine could potentially cause hypersensitivity reactions, confusion, or induce a transient alteration in heart rhythm; to reduce this risk, suction will be applied to the syringe prior to injection to ensure the lidocaine is not being injected into a vessel.

Metabolic cart: This is a non-invasive technique which involves placing a ventilated hood over the subject's head for approximately 30 minutes. The technique carries no risks other than possible minimal discomfort. Subjects with history of claustrophobia will be excluded from the study.

Dual Energy X-ray Absorptiometry (DXA): Radiation risk is very minimal. DXA uses very low dose radiation, and the expected total exposure for each participant is in the range of 7 millirem, which is equivalent to approximately 9 days of background radiation.

Subjects will be offered lunch after completing study procedures that must be performed in a fasted state (metabolic cart, blood draw, and abdominal fat biopsy).

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

Participants should and will be instructed to notify study personnel regarding any adverse events or unanticipated problems. Adverse events will be reported to the IRB per IRB policy. Also, all serious and unanticipated adverse events or problems involving risks to subjects that may possibly be or are known to be related to the research activity will be reported promptly to the IRB office per IRB policy.

A Data Safety Monitor will monitor the study. The Data Safety Monitor will provide ongoing evaluation of studies' progress including patient accrual and retention and monitoring of adverse events. Reports will be submitted to the Data Safety Monitor for review approximately every 12 months.

9.0 Study Withdrawal/Discontinuation

Subjects may withdraw from the study at any time and should notify study personnel in writing if they wish to withdraw from the study. Subjects may request their biological samples to be destroyed at any time. However, any data or biological samples that have already been used for research cannot be destroyed.

Subjects may be discontinued from the study at the discretion of the investigators' (possible reasons listed below).

Possible reasons for withdrawal/discontinuation from study include, but are not limited to:

- Noncompliance with procedures
- Decision by participant/participant withdraws consent
- Loss to follow-up
- Change in patient's health status that makes them no longer eligible for study.
- Development of a significant medical condition specified in the exclusion criteria
- In female subjects, becoming pregnant during study
- Significant adverse event deemed by investigator to preclude continued participation

10.0 Statistical Considerations

Sample size justification: A total of 100 subjects will provide 80% power to detect a minimum difference between lean and obese groups of 0.7 units of UCP1 mRNA expression normalized for housekeeping gene mRNA gene expression (primary analysis). This represents a metabolically meaningful difference of approximately 0.45 standard deviations between lean and obese groups. These calculations are based on a Type 1 error rate of 0.05, and interindividual variations in UCP1 gene expression reported in prior studies.[16] Since there is only 1 main study visit, dropouts should not be an issue in this study. However, there is a low likelihood that some adipose tissue samples may be inadequate quantity or quality. Even if some adipose tissue samples are of insufficient quantity or quality, a sample size of 80 would still provide 80% power to detect a minimum difference between groups of 0.75 units in UCP1 normalized mRNA expression, which represents a metabolically meaningful difference of approximately 0.48 standard deviations between lean and obese groups.

Of note, the above sample size calculation was based on the original enrollment goal of 100 subjects. Now that we have the benefit of including some samples from a previously completed study, our statistical power to answer our scientific questions will be improved.

Statistical Analysis: The primary analysis will determine whether adipose tissue gene expression of UCP1 differs between lean and obese individuals. To assess this, we will use multivariable linear regression, with UCP1 gene expression as the outcome variable, and BMI as the independent variable. The multivariable linear regression will be adjusted for covariates that may impact markers of beige adipose tissue, including age and sex. The null hypothesis is that UCP1 gene expression will not differ based on BMI. Secondary analyses include whether UCP1 gene expression differs by race and whether expression of additional "beiging" markers differ by obesity status. Like the primary analysis, the secondary analyses are analyzing a continuous outcome variable. Thus, the statistical approach for the secondary analyses will be similar to that described for the primary analysis.

11.0 Privacy/Confidentiality Issues

Strict confidentiality will fully be maintained possible by the research team, including keeping all data in a secure location. All specimens will be de-identified and coded after they are obtained, and the code key kept in a secure location. Only study personnel will access to the data and specimens. Samples may be shared with third parties outside of the VA and Vanderbilt for future testing but will remain anonymous to the recipient. Subjects may contact the principal investigator at any time to request that samples be destroyed. However, any data or biological samples that have already been used for research cannot be destroyed.

The Research Electronic Data Capture (REDCap) database is a secure web application housed on the TVHS VA secured computer network and is password protected. REDCap offers secure institutional data hosting and includes full audit-trails in compliance with HIPAA security requirements. Only members of the study team will have access to this database.

Adverse event reports and annual summaries will not include subject-identifiable material but only the assigned study identification numbers.

12.0 Follow-up and Record Retention

Anticipated study duration is 40 months. Research data will be maintained by the PI after study closure. After study closure, research data will be maintained for a minimum of 6 years and possibly indefinitely. Data will be stored on a secure computer network in a password-protected database. Only members of the study team will have access. Pertinent paper documentation will be kept in locked office and only study personnel will have access. Only personnel directly involved with the study will have access to source data and the electronic database.

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