Study Protocol and Statistical Analysis Plan

Modulation of the Intestinal Microbiome by a High Protein Diet

NCT04812964

Document date: April 5, 2018

STUDY PROTOCOL: Modulation of the Intestinal Microbiome by a High Protein Diet

A. Design of randomized, controlled study comparing high and normal protein isocaloric calorie restriction diets for obesity.

Rationale and hypothesis: Based on my preclinical rodent model data, I hypothesize that a high protein diet will shift the composition of the microbiome of obese subjects, including increased *Akkermansia***,** and that this will be associated with alterations of the metagenome (bacterial genes) and metabolome.
Study design: This hypothesis will be investigated in a randomized controlled study of the intestinal microbiome of an estimated 216 obese Veterans assigned 1:1 to isocaloric high protein (HPD) or normal protein (NPD) diets. The study will be advertised by posting flyers in the West Los Angeles VA Medical Center MOVE obesity clinic. MOVE physicians will provide interested patients with information about the study. Participants will undergo an initial screening visit in the clinical research unit after their MOVE appointment or on a later date. Once enrolled, participants will return to the clinical research unit for a baseline visit and follow-up visits on weeks 2, 4, 6, 8, 12, and 16 for dietary counseling, physical measurements, phlebotomy, and dietary questionnaires. The recruitment rate is estimated as 7 per month based on the recently completed study led by Dr. Pisegna. Patient recruitment and sample collection will be performed during the first 3 years of the study.
Inclusion criteria: Men and women between 20 and 75 years of age, BMI 27 to 40 kg/m^2, non-smoker or stable smoking habits for at least 6 months prior to screening and agreement not to change such habits during the study; subjects on non-obesity prescription medication may be included.
Exclusion criteria: Weight change of > 3.0 kg in the month prior to screening, weight loss of > 10 kg in the 6 months prior to screening, calorie restriction diet (< 1500 kcal/day) for a period of 4 months or more in the 12 months prior to screening, use of any other investigational drug(s) within 8 weeks prior to screening, abnormal baseline laboratory parameters (serum creatinine > 1.6 mg/dl; ALT, AST, total bilirubin > 2.0 times the upper limit of normal; triglycerides > 500 mg/dl, total cholesterol > 350 mg/dl, TSH outside of normal range), consumption of more than 1 alcoholic beverage per day, pregnancy or intention to become pregnant.
Consent: Veterans will be consented during their screening visit. They must sign the VA GLAHS Institutional Review Board-approved written informed consent prior to the initiation of any study specific procedures or randomization.
Enrollment and randomization: Date of birth, sex, race, medical history, current medications, smoking and alcohol history will be obtained by the study coordinator during the screening visit. Eligible subjects will be assigned an enrollment number after signing the informed consent form then will be randomized to one of two diet groups.
Dietary intervention: Participants will be randomized to a HPD (30% protein, 40% carbohydrate, 30% fat) or a NPD (15% protein, 55% carbohydrate, 30% fat) with matched fatty acid profiles and fiber content. The diet will be implemented in two phases: an initial macronutrient standardized diet without calorie restriction for 2 weeks then the same macronutrient standardized diet with 1500 calorie restriction for 14 weeks. Subjects will receive dietary counseling during their baseline visit on their assigned macronutrient standardized diet. After 2 weeks of a macronutrient standardized diet, both groups will then receive further dietary counseling on how to restrict their intake to 1500 calories. Pea-based protein supplements will be provided to the HPD group to achieve the target of 30% calories from protein in the HPD group.

Nutritional assessments: Participants will complete a three-day food record (3DFR) and the Food Frequency Questionnaire (FFQ) at every study visit to assess adherence to the prescribed macronutrient composition and calorie limit of the two dietary groups. The 3DFR consists of detailed documentation of food eaten on three days. FFQ is a self-administered, semi-quantitative tool that queries the average frequency of consumption over the prior one month of a list of food items. It will be modified to cover only 2 weeks on study visits at weeks 2 and 4. This study will use the Diet History Questionnaire supported by the National Cancer Institute and previously validated against other FFQs. The extensive nutrient composition database that supports the questionnaire allows estimations of the intake of macronutrients (e.g. fats), micronutrients (e.g. carotenoids), and specific food groups such as fruits, vegetables and grains.

B. Measurement of clinical response to the dietary interventions.

Primary and secondary endpoints: The primary clinical endpoint for response to dietary intervention will be weight loss. Secondary measures of clinical response will include change in fat mass, liver steatosis, lipids, and HgbA1c.
Clinical data collection: The following parameters will be obtained on each of 7 study visits: baseline, week 2, week 4, week 6, week 8, week 12, and week 16.

1) Physical examination: The study coordinator will measure height, weight, and waist/hip circumference.
2) Bioelectrical impedance analysis (BIA): Electrodes attached to a BIA device will be placed on the wrists and ankles. A brief electrical current will be passed to measure resistance and reactance, which can be used to calculate lean body mass and fat mass. One BIA device will be dedicated to this study.
3) Liver transient elastography (FibroScan): Hepatic steatosis and fibrosis will be evaluated by transient elastography, a non-invasive test that can be performed in 5 minutes using equipment that will be available down the hall from the clinical research unit where study visits will occur. Steatosis will be assessed by the CAP score and fibrosis by elastography with stiffness measured in kilopascals.
4) Laboratory studies: Blood samples will be collected by venipuncture after 8-14 hours of fasting. Complete blood count, chemistry panel, lipids (total cholesterol, LDL, HDL, triglycerides), and hemoglobin A1c (HgbA1c) will be measured by the clinical laboratory at the West Los Angeles VA Medical Center.

Statistical analysis: Patient characteristics (physical measurements, lean body mass, laboratories, demographic traits) at baseline will be compared using a 2-sided t-test (for numerical variables) or chi-square test (for categorical variables) to evaluate the randomization. 3DFR and FFQ performed at each time point will be used to assess the predicted protein content of the patient's diet compared to baseline to evaluate the success of the dietary intervention. The significance of differences between the two groups in the primary and secondary outcome measures will be assessed using a 2-sided t-test.

C. Kinetic analysis of shifts in microbial composition in obese subjects (BMI 27-40) on a high protein diet compared to an isocaloric normal protein diet.

Fecal sampling: Participants will be provided with kits for home sampling. The subjects will urinate first then defecate into the Fisherbrand Commode Specimen Collection System. Subjects will use a spoon to transfer freshly defecated feces to a Para-Pak stool collection cup prefilled with 95% ethanol to fix the samples, allowing storage at room temperature for up to 2 weeks. Ethanol-fixed and fresh frozen samples give comparable metagenomics results to samples stored in preservatives such as RNALater while minimizing subjects' risk of exposure to toxic chemicals. The remaining feces will be frozen. This involves removing the cup from the

toilet hat, tightly closing the lid, and transferring the cup to a Ziploc bag for storage in the subject's freezer. Frozen samples will delivered to the VA clinic during scheduled study visits using a Styrofoam box containing U-tek freezer packs designed to maintain a temperature of -20°C as was performed for a recent study. Reminder calls will be made 4 days before each study visit. Subjects who forget to bring in samples will be asked to either bring them in at the next visit or send them via overnight FedEx delivery. Samples will be stored at -80°C.

Microbiome analysis: DNA will be extracted from fecal samples collected in ethanol at baseline, day 3, day 7, week 2, day 17 (3 days after calorie restriction), day 21 (7 days after calorie restriction), week 4, week 6, week 8, week 12, and week 16 using the MO BIO Powersoil kit. Sequencing of the 253 base pair V4 region of 16S ribosomal DNA will be performed using the Illumina HiSeq 2500 to a depth of 250,000 reads per sample. Microbial diversity (alpha diversity) will be compared between the two dietary groups at each time point using the Chao1 index, Shannon index, and Faith's phylogenetic diversity. Significance will be determined using a non-parametric test (Mann-Whitney U). Microbial composition (beta diversity) will be compared across all samples using weighted UniFrac (a phylogenetic distance metric) and visualized by principal coordinates analysis (PCoA). Significance of differences in composition at each time point will be determined using Adonis, a permutation-based method. Baseline data will be analyzed to confirm that randomization had successfully eliminated pre-existing microbial differences between the two dietary groups.

Abundance of individual bacteria will then be fitted to multivariate models using DESeq2. This algorithm performs normalization using size factors estimated by the median-of-ratios method, employs an empirical Bayesian approach to shrink dispersion, and fits the data to negative binomial models. Diet group, time point, interaction between diet and time point, baseline BMI, age, sex, and smoking status will be covariates in the model. Diet composition parameters derived from the nutritional instruments (3DFR, FFQ) may also be included. This model will be compared to a reduced model that does not include the diet:time point interaction. A likelihood ratio test will be used to identify microbes with differential abundance by diet at time points after baseline. Estimates of significance will be adjusted for multiple hypothesis testing to calculate q-values, which represents the false discovery rate. Q-values less than 0.05 will be considered significant. Microbes that are differentially abundant on a HPD will be grouped by hierarchical clustering to highlight distinct kinetic patterns of microbial change.

D. Risk/benefit analysis

1. RISKS TO SUBJECTS

   a. Human subjects involvement and characteristics
      Human subjects will be recruited from the MOVE Obesity Clinic at the West Los Angeles VA Medical Center within the VA Greater Los Angeles Healthcare System (GLAHS). They will be referred for study enrollment if they meet the inclusion criteria and do not meet any provisions of the exclusion criteria. The inclusion criteria include: men and women between 20 and 60 years of age, BMI 27 to 40 kg/m2, non-smoker or stable smoking habits for at least 6 months prior to screening and agreement not to change such habits during the study. The exclusion criteria include weight change of > 3.0 kg in the month prior to screening, weight loss of > 10 kg in the 6 months prior to screening, calorie restriction diet (< 1500 kcal/day) for a period of 4 months or more in the 12 months prior to screening, use of any other investigational drug(s) within 8 weeks prior to screening, abnormal baseline laboratory parameters (serum creatinine > 1.6 mg/dl; ALT, AST, total bilirubin > 2.0 times the upper limit of normal; triglycerides > 500 mg/dl, total cholesterol > 350 mg/dl, TSH outside of normal range), consumption of more than 1 alcoholic beverage per day, pregnancy or intention to become pregnant. Following

informed consent, subjects will undergo randomization to either the normal protein diet or high protein diet groups. Both dietary interventions are consistent with clinical practice for management of obesity and do not pose any appreciable risk to the patient. Subjects will be asked to collect stool eleven times and to visit the VA clinical research unit seven times over 16 weeks.

b. Sources of material

Data and materials collected during the study will be used only according to the protocol approved by the VA Institutional Review Board (IRB). All information will be retained electronically behind the VA's electronic firewall except for regulatory documents and printed informed consent forms. All subjects will receive a unique research identifier number with the identities of the subjects retained only in a code key kept in a secure directory behind the firewall. Stool will be collected by techniques described in Aim 1 of the Research Plan. Demographic and medical data will be collected for all of the subjects according to the study protocol. Only the principal investigator (PI, Jacobs) and study coordinator will have access to confidential patient information.

c. Potential risks

1. Venipuncture - There may be some discomfort associated with obtaining peripheral blood. Rarely, venipuncture may result in the development of a hematoma. Venipuncture will be performed by a certified phlebotomist in the VA's Laboratory Medicine department.
2. Stool collection - This will be done privately by the study patients in their homes using materials provided to them by the study coordinator. There are no risks associated with self-stool collection.
3. Confidentiality - Aspects of the subjects' medical history will be obtained by the study personnel. All data will be retained within the VA's electronic firewall to ensure the greatest confidentiality.

2. ADEQUACY OF PROTECTION AGAINST RISKS

a. Recruitment and informed consent - Subjects will be recruited from patient populations being followed clinically at the VA's MOVE Obesity Clinic. Approved flyers will also be distributed. Inquiries by potential subjects will be initially directed to the study coordinator. Informed consent will be performed in a confidential and private manner. The PI and study coordinator will be able to answer any questions or concerns by the potential subject prior to obtaining informed consent. Although most patients being seen at the VA speak fluent English, in the event that a translator is needed one will be provided.

b. Protection against risks

1. Venipuncture - Collection of blood at baseline and during the course of the study will be conducted by trained and experienced phlebotomists in the VA Laboratory Medicine department. Every effort will be made to minimize the number of times that venipuncture is performed. Following disinfection of the skin surface, standard venipuncture techniques will be performed using a 22 or 25 gauge needle. A topical anesthetic can be made available should the subject have this preference. Following the blood draw, the area of the needle entry will be covered with gauze and pressure applied to minimize the development of a hematoma. A bandage will then be applied to the area. Should the patient experience a hematoma, the patient will be examined by a physician and standard clinical care will be employed to treat the hematoma.
2. Stool collection - Since there are no risks associated with collecting their own stool, patients will be instructed to perform the collection in the privacy of their home. The stool will be stored either in the subjects' freezers or at room temperature (after

fixation with 95% ethanol). Subjects will be warned not to consume the ethanol in the specimen cups. Samples will be returned in person to the study coordinator.

3. Confidentiality - All study related data will be managed in a confidential manner. The Department of Veterans Affairs has instituted a secure server system with 256-bit encryption that requires the use of a Personal Identity Verification (PIV) card to access the secure server or computer systems. In addition, our IT department will create space on the server for the entry and management of secure patient data. Only the PI and a study coordinator will have access to the secure data through their PIV cards. Each of these individuals have been certified by the VA's Research and Development Committee to access human data following the completion of mandatory training, including HIPAA training.

3. POTENTIAL BENEFITS OF RESEARCH TO HUMAN SUBJECTS AND OTHERS

The proposed research may directly benefit Veterans participating in the research by providing them with nutritional counseling on macronutrient standardized weight loss diets (normal or high in protein) that may result in weight loss. The study will benefit others by providing insight into how the intestinal microbiome contributes to the efficacy of a high protein diet. This knowledge may result in new strategies to improve the effectiveness of dietary intervention for obesity and the development of novel microbial therapies for obesity.

4. IMPORTANCE OF KNOWLEDGE TO BE GAINED

Obesity is a national health care crisis that is a critical issue for the VA as over three quarters of Veterans are overweight or obese. Strategies to help overweight and obese Veterans lose weight would decrease their long-term morbidity and mortality and would save the health care system the costs of treating the medical complications of obesity.

E. Data and Safety Monitoring Plan

a. Adverse events – Patients will be monitored and questioned regarding the occurrence and nature of any adverse events. An adverse event is any change in the physiological or psychological state, other than the primary condition, that qualifies the patient for this study. Adverse events are first graded according to seriousness and then severity. Additionally, the PI must decide if the occurrence of the adverse event is related to the study product. A description of the event and its relationship to study product must be reported on the adverse events case report form for each adverse event recorded in the patient's chart.
b. Definition of serious adverse event – A serious adverse event, as defined in Title 21 of the code of federal regulations, Part 312, subpart ID, Section 312.32, means any adverse experience occurring at any dose that results in any of the following outcomes: death, a life-threatening adverse drug experience, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require in-patient hospitalization may be considered a serious adverse experience when, based on appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. An adverse event is usually considered to be serious if severity for the event is graded as 3 (severe) or 4 (immediately life-threatening or fatal).

c. Data safety monitoring procedure – Any adverse event (AE) will be recorded as described below. All AEs will be reviewed by the PI. If it is deemed to be a serious AE, these will be recorded and submitted to the IRB for review.
    1. Severity – For evaluation and reporting purposes, the clinical severity of the event is stratified into four classes. The four classes of severity are 1-Mild, 2-Moderate, 3-Severe, or 4-Immediately Life-Threatening/Fatal.
    2. Life-threatening adverse event – A life-threatening adverse event is defined as any adverse experience that places the subject, in the view of the investigator, at immediate risk of death from the reaction as it occurred, i.e., it does not include a reaction that, had it occurred in a more severe form, might have caused death.
    3. Association with the use of study product (high or normal protein calorie restriction diets) – Association with the use of the study product means that there is a reasonable possibility that the adverse event may have been caused by the product under investigation. All adverse events are graded with regard to their association with the use of the study product. The classifications used include Not Related, Unlikely Related, Probably Related and Definitely Related.
    4. Unexpected adverse event – An unexpected adverse event is any adverse product experience that is not consistent with the current nutritional product insert in specificity or severity.
    5. Adverse event reporting procedure – The PI must report the occurrence of any serious adverse events, regardless of relationship to study product, or the first occurrence of any unexpected or previously unknown clinical event (regardless of Grade). A written report (Report for Serious/Life Threatening Events) of the event must be faxed within 5 working clays to The VA GLAHS Institutional Review Board.