PI: Salvatore Scali

Study Title: Short-Term Dietary Protein Restriction Modulation of Skeletal Muscle Bioenergetics and Innate Immunity

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Protocol

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1. **Study Protocol: Short-Term Dietary Protein Restriction Modulation of Skeletal Muscle Bioenergetics and Innate Immunity**

2. **Investigator Info:**

Principal Investigator
Salvatore Scali, MD
Division of Vascular Surgery and Endovascular Therapy
Department of Surgery
University of Florida College of Medicine
(352) 376-1611
salvatore.scali@surgery.ufl.edu

3. **Abstract.**

Modulation of a patient's diet, specifically via short-term dietary protein restriction, can impact changes in cell energetics and dampen the normal subclinical inflammatory state. These changes can provide benchmarks for future research focused on using nutritional interventions aimed at improving the probability of a successful outcome following open aortic aneurysm surgery. We will conduct a short term dietary restriction study to characterize these changes in a normal control cohort. Knowledge gained from these experiments will be used in comparison to patients undergoing major surgery during a future study, as well as to identify the optimal time points for sample collection in our surgical population. These changes will be characterized by looking at inflammatory cells in the blood, proteins excreted in the urine, changes in microbiome signature of the stool and oral mucosal bacteria, as well as changes in skeletal muscle energetics derived from small biopsies.

4. **Background**

Our group is interested in developing a robust prospective translational research effort that focuses on patients undergoing open abdominal aortic aneurysm (AAA) repair since they have some of the highest rates of postoperative morbidity and mortality compared to other elective non-cardiac surgical patients. The underlying biologic basis of frailty in AAA patients that makes some subjects more vulnerable to postoperative complications is poorly understood. Short-term dietary protein restriction (stDPR) is a cost-effective, easily applied intervention that has strong pre-clinical data supporting efficacy in reducing surgical stress responses. Moreover, recent data supports the feasibility and safety of using stDPR to reduce adverse outcomes after cardiac surgery. These seminal observations offer a unique opportunity for application to other high-risk surgical patient populations known to have significant post-operative adverse event rates. Before initiating a clinical trial in AAA subjects, we need to characterize observations in healthy control, non-surgical subjects who are exposed to stDPR. Therefore, this pilot study is

designed to explore the ability of stDPR to impact the sub-clinical inflammatory milieu and skeletal muscle bioenergetics in a cohort of healthy, non-surgical patients.

5. **Aim**: Examine the baseline and early changes in muscle energetics, microbiome, and cell-mediated inflammation in healthy control patients receiving short-term dietary protein restriction.

6. **Research Plan**

Overview: Using targeted assays of mitochondrial energetics, 10X genomics, and the microbiome, we will assess changes in skeletal muscle bioenergetics and the leukocyte transcriptome induced by a prescribed 96-hour protein restriction diet, tracking the impact of these changes on the dynamic response patterns of these parameters in healthy control patients.

Study Time points Summary						
	Days -7 to -1	Baseline/Day 0	Day 2	Day 4	Day 7	Day 14
Consent	x					
Paffenbarger IQ	x					
IPR Study IQ		x	x	x	x	x
Stool Kit Distribution	x	x	x			
Diet Kit Distribution		x	x			
Blood		x	x	x	x	x
Urine		x	x	x	x	x
Feces		x		x	x	
Cheek Swab		x	x	x	x	x
Muscle Biopsy		x		x	x	
Meal Logger				Days 5 thru 14		

Health Questionnaires: We will collect height and weight information to be utilized in the diet equation, as well as 2 questionnaires. The first is a physical activity Paffenbarger questionnaire which is used for the diet equation (see below) and an IPR study questionnaire to track energy level, mental awareness, and digestive issues.

Diet Modification: Subjects will follow a 4 day protein restricted diet using Scandishake® mixed with almond milk which will be provided. They may also drink water. No food, other beverages, or alcohol may be consumed. The amount of diet to be consumed will be calculated for each patient based upon resting energy expenditures plus additional energy needs using the Paffenberger IQ. REE = 9.99 x weight (KG) + 6.25 x height (cm) - 4.92 x age + 166 x sex (males, 1; females, 0) - 161. Patients will receive the study diet (ScandiShake® [any of 4 flavors] mixed

with almond milk), calculated individually for a total daily volume to achieve 30% caloric restriction and 70% protein restriction. These will be consumed by the study subjects at home in an unrestricted manner, in that they may be consumed at any point of time in the day.

Blood Collection and Cell Isolation and 10X Barcoding and RNA Sequencing. A 20 ml blood sample will be obtained via venipuncture before (Day 0), during (Day 2) and after completion of the 4 day protein restricted diet (Days 4, 7 and 14) for a total of 5 samples. These will be collected by Dr. Scali in the vascular clinic or vascular lab at the VA. Peripheral blood monocytes and neutrophils are isolated using a modified Ficoll method which can isolate both cell types. These samples are then subjected to bar coding with 10X reagents, followed by library preparation and RNA sequencing at approximately 30 million reads per sample (2 x 150 PE). Analysis of data will be primarily performed using 10X proprietary software, Cell Ranger, which is a set of analysis pipelines that process Chromium single-cell RNA-seq output to align reads, generate feature-barcode matrices and perform clustering and gene expression analysis. Cell Ranger includes four pipelines relevant to single-cell gene expression experiments.

Skeletal Muscle Mitochondrial Phenotyping: Collection of 3 tissue samples from the thigh; before and after the protein restrictive diet on Day 0, Day 4 and Day 7 will occur.
We will utilize a minimally invasive microbiopsy technique (skin puncture) to obtain skeletal muscle biopsies of the vastus lateralis muscle. These will be collected by Dr. Scali in the vascular clinic at the VA. Multiple aspects of mitochondrial function (basal and ADP-stimulated respiratory kinetics under multiple substrate combinations, H_2O_2 production and emitting potential, mitochondrial Ca^{2+} retention capacity) will be assessed in duplicate on permeabilized fiber bundles from freshly obtained muscle biopsy samples. The permeabilized fiber approach permits *in situ* study of mitochondrial function in real-time, retains the native reticular network structure of mitochondria in muscle, and requires very little tissue per protocol (~1.5 mg wet weight). All mitochondrial functional analyses are completed in real time within 2-3 h. Fibers are then freeze dried overnight, weighed, rehydrated, and freeze fractured for determination of citrate synthase activity. All data are expressed per dry weight and/or mitochondrial content.

Microbiome Sample Processing: Three stool samples will be collected on Day 0, Day 4, and Day 7. Stool collection kits and instructions will be given to patients at their screening visit. Patients will collect their feces in a toilet insert (similar to an upside down hat) and will take a walnut sized scoop of this feces using a provided tube/scooper and insert it in the collection container and bag. Stool samples may be collected at home within 24 hours of the clinic visit or during clinic visits on Day 0, 4 and 7. In addition, a swab of cheek saliva will be collected using a sterile swab and placed into a provided sterile tube. DNA will be extracted from these samples using DNeasy PowerLyzer PowerSoil Kit (Qiagen) and will be quantified using spectrophotometry. Amplification of 16S rRNA will be performed to create amplicons, after which PCR will be used to add adapters and dual-index barcodes to the amplicon target. These libraries will be pooled together for sequencing by Illumina MiSeq. Reads will be assigned to operational taxonomic units, which will enable us to identify phyla, classes, families, and genera in each sample. The MiSeq run output is approximately >20 million reads and can generate sufficient reads for metagenomic surveys.

Operational taxonomic units (OTUs) for bacterial species at each time point will be determined for all patients. Both alpha-diversity (overall diversity) and beta-diversity (composition of species) measurements will be obtained from these OTUs. Mann-Whitney tests will be used to compare alpha diversity in patients who developed post-operative complications to those who did not. One-way analysis of variation (ANOVA) or Kruskal-Wallis tests will similarly be conducted to compare beta-diversity [21]. Heatmaps with clustering for relative abundance of species at the genus level will be created for all samples and pairwise differences between pre-operative and post-operative samples will be identified. The response will be measured as a change from baseline, and the baseline response will be included as a covariate in the subsequent multivariate analysis. In addition to this, we will perform principal component analysis (PCA) to create a mixed multivariate model with the intent of combining laboratory values for IL-10, LXA_4, TNF-α, and annexin-1 with OTUs.

Urine sample processing for ketones: Collection of 5 urine samples will occur on Day 0, 2, 4, 7 and 14. Urine will be collected in a cup and approximately 1 ml of urine will be stored for later analysis. While on diet and immediately following completion of the diet, ketones are a marker for diet compliance, as well as urinalysis will attempt to understand the physiologic impact of the diet on renal function can occur through processing for serum ketones (that get generated on diet). Commercially available assays can quickly evaluate these parameters which will further assist in determining the time-dependent, physiologic effects of this nutritional intervention in healthy control subjects.

De-identified biologic samples will be stored in refrigerator/freezers within the PI's designated research space for the duration of the study. Unused samples will be discarded in the appropriate safety disposal container.

Mealogger App – This is a free app available on smartphones in which patients take a picture of the meals they ingest to approximate their normal food intake following the study. This will begin day 5 and will complete on day 14 of the study. During clinic visits, the patients will open the app so that study coordinators can record information about what foods consumed and the caloric/protein content patients exposed themselves too after completion of the study diet. This information will be recorded with the other study data in a de-identified manor.

Subject Selection

A. Inclusion criteria:
    - Healthy volunteers between 18 and 70 years of age
B. Exclusion criteria:
    - Age less than 18 years.
    - The presence of any significant medical condition that might significantly confound the collection of biological data in the study including cancer, diabetes, IBD, Advanced Renal Disease, Nut Allergy
    - Unwilling to follow protocol

- Participation in another interventional clinical trial.
- Prisoners, pregnancy, or direct employees of the investigative team.

Subject Enrollment

We anticipate enrolling 10 patients for up to a year following study initiation. Patients who express interest in learning more about the study will meet with a member of the study staff who will describe the study, review the informed consent form, give them a copy of the informed consent to read and answer any questions. Potential participants will be allowed to take a copy of the consent form, as well as written information about the study if they so desire. When possible, signing of the consent shall occur at least one day prior to diet initiation to allow adequate time for patient to make consideration without any stress. Any questions will be answered by the Principal Investigator or other study team members.

Compensation

The study payment and time schedule:

Baseline Visit	(1-1.5 hours)	$100.00
Day 2 Visit	(30-40 minutes)	$ 50.00
Day 4 Visit	(1 hour)	$100.00
Day 7 Visit	(1 hour)	$100.00
Day 14 Visit	(1-1.5 hours)	$150.00
Total		$500.00

Statistical Analysis This will be performed in a blinded fashion (no patient identifiers) by the UF Surgery Department Statistical Support Group.

7. Possible Discomforts and Risks:

Microbiopsy: There is a small risk of muscle damage, infection, or prolonged bleeding but these are very rare. Subjects may experience muscle soreness and bruising at the biopsy site. No sutures are required to heal the puncture but they will be left with a small scar at the puncture site.

Blood draw: The risks of drawing blood from a vein include discomfort at the site of puncture; possible bruising and swelling around the puncture site; rarely an infection; and, uncommonly, faintness from the procedure.

Protein Restrictive Diet: Subjects may experience a change in bowel habits, constipation, loose stool and cramping and possible weight loss. Subjects may experience a change in energy level during the 4 days of the protein restricted diet.

8. Possible Benefits:

Participation in this research will not provide any additional benefits to the subjects. Our hope is that discoveries from this study will eventually lead to a better understanding of how nutritional interventions may improve the probability of successful outcomes following open AAA surgery.

9. Conflict of Interest:

No conflict of interest exists for this project.

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