University of Arkansas for Medical Sciences (UAMS) Clinical Protocol

Study Title: **Maximizing the dietary pattern of older adults: the effects of protein intake on protein kinetics.**

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List of Abbreviations

3MH	3-methyl-d3-L-histidine stable isotope
AMDR	acceptable macronutrient distribution range
ANOVA	analysis of variance
AUC	area under the curve
BMI	Body mass index
CRF	Case Report Form
D2O	Deuterated water
DXA	Dual-energy X-ray Absorptiometry
EAA	Essential Amino Acid
GC-MS	Gas chromatograph-mass spectrometry
IRB	Institutional Review Board
MPS	Muscle protein synthesis
NB	Net balance (of protein metabolism)
NHANES	National Health and Nutrition Examination Survey
OPI	Optimal Protein Intake
PS	Whole-body protein synthesis
PB	Whole-body protein breakdown
RDA	Recommended Daily Allowance
RIOA	Reynolds Institute on Aging
UAMS	University of Arkansas for Medical Sciences

Study Schema

Recruiting will be performed via word of mouth, TRI subject database (ARresearch.org), and the UAMS social media platforms.

Visit 1: informed consent process takes place at the RIOA. Enrolled subjects undergo body composition test and DXA scan (or at Visit 2) using PI's equipment. Randomization (1:1:1) occurs.

Visit 2: subjects undergo body composition test and DXA scan if not done at Visit 1. Run-in meals are dispensed.

Visit 3: Subjects report fasted to the RIOA to begin a 13-hour visit that entails stable isotope ingestion, blood/urine/saliva sampling and study meal ingestion.

Visit 4: Subjects report fasted to the RIOA the morning after visit 3 to undergo blood, saliva, urine and muscle sampling approximately 24 hours after isotope ingestion.

Study Calendar

Procedure	Visit 1	Visit 2	Visit 3	Visit 4
Informed consent	X			
DXA scan	X[1]	X		
Body composition testing	X[1]	X		
Randomization	X			
Pick up run-in meals		X		
24-hour stable isotope procedure			Start	Finish

[1]Or at Visit 2.

1.0 Protocol Summary

This will be a randomized study to demonstrate how animal-based protein-rich food sources can be used by older adults to increase protein intake within pre-existing dietary patterns. In this regard, we propose to augment the current dietary pattern of older Americans by readily available quality protein sources. To this end, we will investigate the effects of recommended and common protein intakes on the maintenance of whole-body protein balance and potential for muscle protein anabolism. Plans to accomplish project goal(s): We will study 3 groups of 10 older subjects during daily protein intakes of 0.8 (recommended dietary allowance [RDA]), 1.1 (National Health and Nutrition Examination Survey [NHANES]), and 1.5 (optimal [OPI]) g/kg/d. Due to common eating patterns in older adults (1), these protein intakes will be structured within 2 meals per day. A 2-day dietary lead-in (matching the subject's intervention paradigm) will proceed each dietary intervention. Whole-body protein balance will be determined during metabolic study days by oral ingestion of 2 grams of the stable isotope 15N-alanine, and a 24-hr integrated value of muscle protein synthesis will be determined by ingestion of deuterated water. Oral ingestion of 10mg of 3-Methyl-d3-L-histidine and analysis of urine samples will permit measurement of muscle protein breakdown during this period. Integrated post-meal blood EAA will also be determined, as we have demonstrated a relationship between peripheral EAA response and protein anabolism.

Study duration per subject will be about 2 weeks.

2.0 Background

2.1 Rationale and Significance

Sarcopenia is the age-related loss of skeletal muscle mass and function. Sarcopenia is a complex multifactorial syndrome associated with increased risk of morbidity, mortality, and healthcare cost. Despite being associated with advanced age groups, the loss of skeletal muscle mass and function begin in the third and fourth decades of life, respectively (2). Thus, treatments that prevent age-related losses of skeletal muscle mass and function are essential for all aging populations.

Protein turnover is an important regulatory process of muscle growth and function and is influenced by daily protein intake in order to supply precursor amino acids to activate protein synthesis and attenuate protein breakdown. The current RDA for protein for adults is 0.8 g/kg/d, which is the minimum protein intake necessary to avoid malnutrition (3). However, the acceptable macronutrient distribution range (AMDR) was developed to express dietary recommendation in the context of a complete diet. For protein the AMDR is 10 – 35% of total calories. Assuming a 2000 Kcal healthy US-style eating pattern, the AMDR provides 1.05 – 3.67 g/kg/d when reference body weights of 57 and 70 kg are assumed for women and men, respectively (4). In this regard, even the lower end of the AMDR intake is higher than the RDA. Moreover, many nutritional organizations (3,5) have recommended intakes above the RDA (1.2 - 2 g/kg/d) for older adults. Despite this level of support, an increase in the RDA for the general population, or for older adults, has not materialized.

Increasing the RDA would represent a physiologically accurate acknowledgment of an increased protein requirement with aging. The adequacy of protein intake is made further difficult as older adults tend to consume lower food/caloric intakes as they age. This age associated decrease in food/caloric intake has been attributed to reduced appetites, changes in chemo-sensory abilities, and deteriorations in dentition, manual dexterity, and gastro-intestinal function (6). Unfortunately, studies suggest that the consumption of animal-based protein-rich foods is impacted to a greater degree (6). This is of concern as animal-based protein-rich foods not only provide a better protein to

calorie ratio, requiring less total food intake to meet protein requirements, but also provide unique nutritional profiles that cannot be obtained through supplementation or substitution (7). Furthermore, we have demonstrated (Figure 1) that complete protein ingestion leads to a greater increase in peripheral EAA, and further, that this increase is directly related to the increase in muscle protein synthesis and whole-body net protein balance. It is apparent that the use of animal-based protein-rich food sources are imperative for older adults to meet their elevated protein requirements. Thus, it is crucial to identify nutritional strategies that require minimal changes in eating behaviors of older adults yet improve the potential for protein homeostasis.

Older adults have decreased motor abilities, which impacts their ability to cook and prepare their own food. Animal-based protein-rich food sources often require more time and effort to prepare than plant sources. This aspect contributes to inadequate protein intakes as plant sources have a lower protein to calorie ratio. Thus, an important strategy to increase daily protein intake is to improve the convenience and minimize the effort required to prepare animal-based protein-rich food sources. This can be readily accomplished by using minimally and further processed meat and poultry products within older adults' diet. The reduced effort and time required to incorporate these products into a pre-existing dietary plan would improve the potential of successful implementation.

One common approach to increase dietary protein in older adults has been to define an "optimal" within-day protein intake distribution. Based upon skeletal muscle responses to various doses of protein intake, it was concluded that an even distribution of protein intake, as opposed to an uneven distribution, was preferable (8). The primary issue here is that saturable protein dose limits are not well-defined, and arguably non-existent. Research consistently supports the concept that muscle protein synthesis is maxed out with around 10-15 grams of EAA when supplied in liquid format following resistance exercise (9–13). However, we have observed (Figure 1) that greater protein intake is required when provided in whole-food format as opposed to liquid drinks.

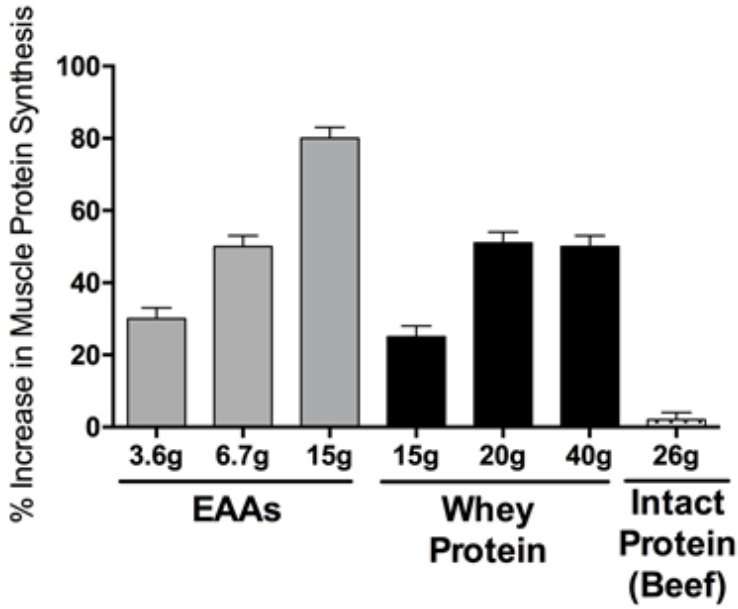


Figure 1: A greater digestibility requirement (whole-food protein sources) results in a lower stimulation of muscle protein synthesis. These data also demonstrate that greater peripheral EAA are required to increase muscle protein synthesis.

Basing protein distribution optimization upon saturable protein dose studies reflects the "muscle-full" effect theory, which recently was shown to be an artifact of the rapid and transient rise in circulating amino acids rather than a true physiological effect (14). Furthermore, protein recommendations are based upon whole-body protein requirements, not muscle requirements. This is an important consideration as we (15) and others (16) have demonstrated that whole-body protein balance does not plateau with protein intake; i.e., greater improvements in whole-body net protein balance are observed with increasing doses of protein.

The above highlight an incorrect underlying assumption of evenly distributing protein intake throughout the day. More pertinent to the issue is that studies directly testing protein distribution do not provide strong evidence for an even protein distribution being superior to an uneven protein distribution. Despite some promising data (17) in younger adults (36.9 ± 3.1 years of age), our recent studies (18,19) in older adults (50 – 75 years of age) demonstrated no superior effect of an even protein distribution on whole-body protein balance or muscle protein synthesis over a 24-hour period. More importantly,

our work indicated that total protein intake throughout the day has a direct influence on anabolism (18–21). The take home message from these studies is that total protein intake, not intake patterns, dictates protein homeostasis. This is an important message, as NHANES data indicates that only 17.3% of adults eat more than two meals a day containing ≥30 grams of protein per meal (1). Thus, the issue becomes one of increasing protein intake in older adults within their pre-existing dietary patterns. Incorporating minimally and further processed meat and poultry products into current dietary patterns provides an easily accessible strategy for older adults to meet higher protein requirements required to maintain protein mass. Therefore, demonstrating the efficacy of these products to meet body protein demands within a preexisting dietary pattern of two meals a day is the focus of this project.

3.0 Hypothesis

We hypothesize a stepwise increase, RDA<NHANES<OPI, in muscle protein synthesis and whole-body protein balance with increasing doses of daily protein intake.

4.0 Study Population

Up to 50 subjects of any gender or ethnicity will be enrolled with a target of n=30 for study completion. Subjects' duration of participation is approximately 2 weeks.

4.1 Inclusion Criteria

1. Men and post-menopausal women.
2. Ages 50-70 years.
3. BMI ≤ 35 kg/m^2.
4. Capable of providing informed consent.
5. COVID-19 negative and/or asymptomatic.

4.2 Exclusion Criteria

1. Subject who does not/will not eat animal protein sources.

2. Body mass index >35.
3. Complete blood count lab results that indicate anemia or abnormal white blood cell counts.
4. History of chemotherapy or radiation therapy for cancer in the 6 months prior to enrollment.
5. Use of insulin to control blood sugar level.
6. Currently receiving androgen (e.g., testosterone) or anabolic (e.g., GH, IGF-I) therapy.
7. Currently using prescription blood thinning medications.
8. Unable or unwilling to suspend aspirin use for 7 days prior to Visit 3.
9. Unwilling to avoid using protein or amino-acid supplements during participation.
10. Subjects who are unwilling to fast overnight.

4.3 Subject Recruitment

Recruiting will be performed via word of mouth and the UAMS social media platforms. Study staff will also utilize the ARresearch.org database through the UAMS TRI. Interested subjects will then be scheduled for Visit 1 where the informed consent process will take place.

4.4 Subject Compensation

Subjects will be compensated for participation as shown in the table below. They will be mailed a check to the address they provide through UAMS SAP program. Parking passes will be provided to all subjects for all visits at no cost to subjects.

Visit 1	$20
Visit 2	$20
Visit 3	$360
Visit 4	$120

4.5 Run-In Meals

Subjects will be provided with 3-days' worth of ready-to-eat meals designed and prepared by the study dietician. Subjects will be instructed to eat only the food that is labelled for each particular meal over the run-in days. They may add non-caloric condiments and seasonings and have their usual beverages. Caloric requirements are determined by the Harris-Benedict formula based on subject's weight and sex and are matched to the subject's intervention group (e.g. 0.8 or 1 or 1.5g protein/kg per day). Each meal's containers are placed into a labelled plastic zippered storage bag, with the meals for one day placed into a larger labelled bag. Subjects are asked to store them in a refrigerator.

5.0 Study Visits

Subjects will be required to wear a mask. If they do not have one, a mask will be provided to them by the study staff.

Visit 1: This visit will take place at the UAMS RIOA. At this visit, informed consent discussion will be held. This visit is expected to take less than one hour. If a subject consents, their height and weight will be measured to determine their BMI. Questions will be asked about their medical history and medication use. Eligible, enrolled subjects will be shown a menu of the foods they will be asked to eat to elicit any food allergies or intolerances. If the subject is fasted for 8 hours, has not had caffeine in 12 hours, and has not strenuously exercised in 24 hours, they will undergo body composition testing and DXA scan. If these criteria cannot be met during this visit, subjects will perform these two tests at Visit 2. A blood sample will be drawn for Complete Blood Count (Labcorp). Randomization will occur. Future study visits are scheduled.

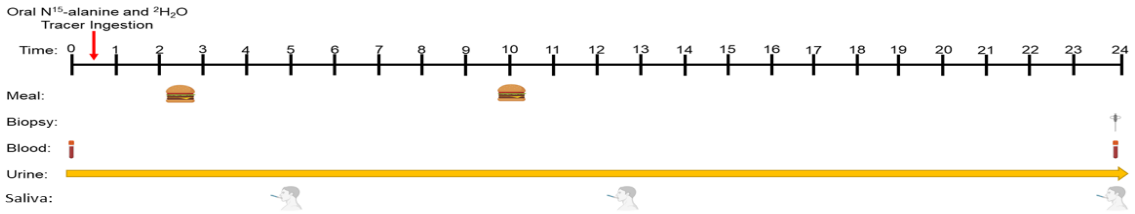
Visit 2: This visit will occur in a fasted state if the subject did not meet the body composition testing criteria at visit 1. Subjects will undergo body composition testing and DXA scan. Study meals eaten during the 2-day run-in phase will be dispensed. A

10mg dose of 3MH will be provided with instructions to mix it with water, ingest it at 1900, and record the time on the evening prior to study visit 3. Subjects are instructed to eat all of the provided food and add nothing but non-caloric seasonings and condiments. They may have their usual beverages but must not ingest any protein or amino acid supplements during this time. This visit will take place at the UAMS RIOA.

Visit 3: This visit will take place at the UAMS RIOA on the morning after subject completes the 2-day run-in meals. At this visit, subjects will report having fasted for 10 or more hours. This visit is expected to take about 13 hours. During this visit, subjects will be asked to provide blood/urine/saliva samples. They will ingest deuterated water (~3mL per kg body weight) at specific times (total dose divided into 10 aliquots and ingested every hour up to study hour 9:00) and 2g of a stable isotope of alanine (15N-alanine) dissolved in water. Two (group-specific) study meals will be served, one in the morning and one in the afternoon/evening. See **Figure 2** for a timeline. After the second saliva sample has been collected, subjects will go home and return to the study site the following morning having fasted overnight.

Visit 4: This visit will take place at the UAMS RIOA on the morning after visit 3. Subjects will fast overnight between visits 3 and 4. Approximately 24-hours after isotope ingestion, the final biological samples will be collected and the subject offered a snack and beverage. They will then be dismissed.

Figure 2:



6.0 Blinding

The study is a single-blind, randomized, controlled trial (RCT). While participants cannot be completely blinded as to the amount of protein in their meals, key investigators and outcomes assessors will be blinded to treatment assignment, making this a single-blind RCT from the investigators' perspective.

7.0 Randomization

Subjects will be randomized equally to one of three groups after they are determined to be eligible using a generated list of group designations that corresponds to subject ID. Efforts will be made to balance the sexes within each group. The three groups utilized are:

1) recommended dietary allowance for protein (RDA; 0.8g/kg/day);
2) habitual protein intake consistent with population level norms (NHANES; 1.1g/kg/day);
3) optimal protein intake (OPI; 1.5g/kg/day).

8.0 Biological Samples

Samples will be handled exclusively by study staff. All samples will be identified using the subject's unique study code; the key to which is kept on a secure UAMS server

(\\rscoa\nmel\AAFerrando\262424 2Meal study 2021 folder). All specimens will be stored in appropriate freezers in the PI's lab on the 7th floor of the UAMS Reynolds Institute on Aging (a secured access floor). All specimens will be discard into biohazard receptacles within 7 years after conclusion of the study. With explicit written permission (on the ICF), muscle samples will be retained indefinitely for use in the PI's lab for future non-commercial uses that have been explicitly permitted by the UAMS IRB. The key to subject identity-study code will be deleted within 7 years of the conclusion of the study (e.g. closure of the study in Clara), regardless of whether any samples will be kept for future use.

8.1 Blood

Blood samples (~5mL each) will be collected via venipuncture at the specific times mentioned in Figure 2 below (+/- 10 minutes). The blood will be placed into an EDTA (purple top) vacutainer tube, gently agitated 6 times, and placed onto wet ice until it is processed in a refrigerated centrifuge. Plasma will be pipetted into a labelled cryo vial for each specific time point and frozen at < -60 degrees C in the PI's lab. Two samples will be drawn (~10 mL) during Visits 3-4 (1 each).

8.2 Urine

During Visit 3 through 4, all voided urine will be collected using standard plastic urine jugs. During visit 3, at each void 1mL of urine will be placed into a labelled cryo tube and the time of voiding will be documented on a table in the CRF. At the completion of Visit 4, all urine will be intermixed and aliquots placed into labelled cryo tubes and frozen at < -60 degrees C in the PI's lab.

8.3 Saliva

During Visit 3-4, three saliva samples will be collected at specific times (**Figure 2**) using absorbent collection devices (e.g. Salivette). They will be centrifuged and the saliva transferred into labelled cryo tubes and frozen at < -60 degrees C in the PI's lab.

8.4 Muscle

At Visit 4, qualified and credentialed study staff (M.D. or PA) will perform a vastus lateralis muscle biopsy of the subject's outer thigh using 1% plain lidocaine for anesthesia. Approximately 100mg of muscle tissue will be obtained using a Bergstrom needle. The tissue will be rinsed with normal saline and flash frozen using liquid nitrogen. The sample will be transferred into a labelled cryo tube and frozen at < -60 degrees C in the PI's lab.

9.0 Sample and Data Processing

All samples will be processed and analyzed in the PI's lab in the RIOA.

9.1 Blood

Plasma EAA concentrations will be determined by liquid chromatography-mass spectrometry using the internal standard method, as we have done previously (30). Briefly, analytes will be derivatized with 9-fluorenylmethoxycarbonyl and quantification of each peak will be determined using MultiQuant software (version 2.1: AB Sciex).

9.2 Urine

Following ingestion of [15N] alanine, 24-hr dietary nitrogen intake and excretion will be measured. Isotopically labeled nitrogen from the urine samples will be used to determine nitrogen flux according to Fern et al. (24). Whole-body protein synthesis (PS) and breakdown (PB) are calculated from 24-hr urinary urea enrichment according to Stein et al. (25) and used to determine net protein balance and flux. The ingestion of 3MH will increase the naturally-occurring 3MH in the body by less than 5%. This molecule is not reutilized in the body, but only excreted via the urine. Measurement of it in urine will allow determination of muscle protein breakdown.

9.3 Saliva

Body water enrichments following D2O ingestion will be determined through isotope exchange with acetone using GC-MS (29).

9.4 Muscle

MPS will be determined by oral D2O ingestion to assess 24-hr muscle protein synthesis using the single biopsy approach (26). In the interest of brevity, the sample preparation methods are standard in the PI's laboratory and have recently been outlined in detail (27). Muscle preparations will be analyzed for incorporation of deuterated alanine by GC-MS using partial integration (28). The daily rate of muscle protein synthesis will be determined according to Gasier et al. (27).

9.5 Dual Energy-Xray Absorptiometry

This test will be used to measure body composition (fat mass, lean mass, and percent body fat). Subjects will be asked to remove all metal, thick clothing, and heavy plastic which could interfere with the DXA scans. The subject's ID, age, ethnicity, height and weight will be entered into the computer prior to the scanning. The subject will be asked to lie down on the DXA table in the supine position. The participant will be centered on the table within the scanning area. The subject's shoulders and hips will be centered, and the hands will be placed by the side of the legs in a pronated position. Subjects will be instructed to remain still for the duration of the full-body scan. A trained DXA technician will perform and analyze all scans. Subjects will be asked to fast for 8 hours, avoid caffeine and alcohol for 12 hours, and avoid strenuous exercise for 24 hours prior to this procedure.

9.6 Multi-frequency Bioelectrical Impedance Analysis

This test will measure total body and segmental water, to be combined with the DXA measures for a more accurate body composition estimates. To obtain measurements, subjects are asked to stand barefoot on a calibrated scale to obtain subject weight. Once subject height, sex, and age are entered into the device, subjects are instructed to

hold onto two handles and remain still while an imperceptible electrical current runs between electrodes in the handles and scale. Total assessment time is <2 minutes. Subjects will be asked to fast for 8 hours, avoid caffeine and alcohol for 12 hours, and avoid strenuous exercise for 24 hours prior to this procedure.

10.0 Statistical Analysis – Data Use

10.1 Statistical Analysis

Means by which experimental data will be analyzed or interpreted: Changes in whole-body protein synthesis, breakdown, flux, net balance, muscle protein synthesis, and plasma EAA area under the curve (AUC) between groups will be assessed with a one-way analysis of variance (ANOVA). In the event of a main effect of group, appropriate post-hoc comparisons will be carried out. Statistical significance will be accepted at an alpha level of $p \leq 0.05$.

10.2 Dissemination of Data

The results of this project will demonstrate that daily protein intake from minimally and further processed food sources will serve as a pragmatic and efficient means for improving body protein status. Results of this study may be used for presentations, posters, or publications. The publications will not contain any identifiable information that could be linked to a subject.

10.3 Data Handling and Recordkeeping

The Principal Investigator will carefully monitor study procedures to protect the safety of research subjects, the quality of the data and the integrity of the study. Paper source documents and Case Report Forms (CRFs) will be stored in a secure area of the PI's laboratory. Access will be limited to study personnel. Documents will be archived according to UAMS policies regarding destruction of research records. Data (without identifiers) will be entered into an electronic spreadsheet or database (e.g. REDCap) to facilitate analyses. These files are maintained on secure password-protected UAMS

servers. Approximately 7 years after study completion, paper and electronic records will be destroyed per UAMS disposal guidelines. At no time shall Protected Health Information be released to non-study personnel.

10.4 Data Access

All subjects will be assigned a unique identifying code or number. The key to the code (the instrument associating the data with subject identity) will be kept on a password-protected UAMS server. Only study staff members will have access to the code and information that identifies the subject in this study. This file will be deleted approximately seven years after data analysis is completed.

10.5 Power Calculations

We propose a group size of 10 per group, based upon an ANOVA model to compare the nutrition interventions. With this sample size, the ANOVA model has 80% power to detect effect sizes of f = 0.484 or larger. This estimate assumes that the basal covariate explains 50% of the variation in the response and a 5% α-level is used to determine statistical significance.

11.0 Risks and Benefits

There will be no direct benefits to the study participants; however, knowledge gained from the study could potentially benefit patients in the future. Anticipated risks associated with this protocol are described in detail below. All experimental procedures will be performed by appropriately trained and credentialed personnel. The PI and study physician will be responsible for oversight of study procedures and evaluation of adverse events. There are no known risks of body composition testing (InBody770, BioSpace, Seoul, South Korea), urine or saliva collection.

11.1 Stable Isotope Ingestion

The risks of drinking 70% deuterated water are temporary dizziness, nausea and possibly vomiting. Each dose is approximately 20-40 mL (1 ½ - 2 tablespoons). There are no known risks of ingestion of the stable isotopes of alanine or methyl histidine.

11.2 Blood Sampling

Up to 10 mL of venous blood will be drawn during visits 3 and 4 in total. This is less than a tablespoon. The risks of drawing blood include pain, bruising, bleeding, and small chance of infection from the needle sticks. One ~5mL sample will be drawn during visit 1 to determine eligibility.

11.3 Percutaneous Vastus Lateralis Muscle Biopsy

The risks of this procedure include pain, bruising, bleeding, scar formation, and infection. This procedure will be done using aseptic technique by licensed study staff (MD or PA) after injection of local anesthesia (1% plain lidocaine). Approximately 100mg of tissue will be removed from the vastus lateralis muscle (subject's choice of left or right leg).

11.3 Study Meals

The menu for study meals will be presented to subjects during Visit 1 to elicit whether any food allergies or intolerances are present.

11.4 DXA Scan

Subjects will undergo a DXA scan for whole-body composition. The radiation exposure for one DXA scan is approximately equal to ½ of the radiation from a chest x-ray.

11.5 Confidentiality

A potential risk to study subjects is the loss of confidentiality. Measures to protect the confidentiality of study subjects will be implemented as described in the Data Handling and Recordkeeping section above.

11.6 Data Safety Monitoring Plan

- Study staff will ask subjects about the occurrence of adverse events during visits.
- The PI and study physician are responsible for reviewing and evaluating adverse events.
- Adverse events will be recorded in the CRF and on an excel spreadsheet that is submitted to the IRB at required intervals.
- Serious adverse events will be reported to the IRB within 24 hours of their discovery.

12.0 Ethical Considerations

This study will be conducted in accordance with all applicable government regulations and University of Arkansas for Medical Sciences (UAMS) research policies and procedures. This protocol and any amendments will be submitted and approved by the UAMS Institutional Review Board (IRB) to conduct the study.

The formal consent of each subject, using the IRB-approved consent form, will be obtained before that subject is submitted to any study procedure. All subjects for this study will be provided a consent form describing this study and providing sufficient information in language suitable for subjects to make an informed decision about their participation in this study. The person obtaining consent will thoroughly explain each element of the document and outline the risks and benefits, alternate treatment(s), and requirements of the study. The consent process will take place in a quiet and private room, and subjects may take as much time as needed to make a decision about their participation. Participation privacy will be maintained and questions regarding participation will be answered. No coercion or undue influence will be used in the consent process. This consent form must be signed by the subject and the individual obtaining the consent. A copy of the signed consent will be given to the participant, and

the informed consent process will be documented in each subject's research record.

13.0 References

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