STUDY PROTOCOL

PREBIOTICS IN WOMEN'S HEALTH AND AGING: THE GUT-BONE CONNECTION

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1.0 BACKGROUND AND RATIONALE:

Each year in the U.S., an estimated 2 million women (~6,000 women each day) reach the critical stage of female reproductive aging known as menopause [1]. Menopause is characterized by the permanent cessation of menses and the loss of ovarian follicular activity brought about by the decline in 17β-estradiol (i.e., estrogen deficiency). Among American women, the median age of menopause is 51 y [1]. Based on a current life expectancy of 80.5 yrs [2], women in this country can expect to spend up to one-third of their lifetime in this postmenopausal stage.

The impact of decreasing 17β-estradiol on immune cell function, especially some T cell populations, has become the focus of much women's health research. It is widely accepted that after menopause women exhibit a significant increase in their risk for chronic diseases, including gynecologic and breast cancers, autoimmune diseases, cardiovascular disease, and osteoporosis [3, 4]. In terms of bone health, which is the focus of our laboratory's work, postmenopausal women are 4 times more likely to fracture compared to pre-menopausal women (age 20-50 yrs) [5]. Moreover, 1 in 3 women over the age of 50 y will experience an osteoporotic fracture [6]. The decline in 17β-estradiol that occurs with menopause is accompanied by immunological changes in $CD4^+$ effector T cells, most notably an increase in T helper cells expressing the pro-inflammatory cytokine, interleukin (IL)-17 (i.e., Th17 cells) relative to the immunosuppressive, T regulatory (Treg) cells [3, 7, 8]. Th17 cells are highly potent promoters of osteoclastogenesis and accelerate the bone resorption that occurs in estrogen deficiency [9-11]. In contrast, the osteoprotective role of Tregs, which are reported to either decrease or remain unchanged in with menopause, is less clear in humans beyond their anti-inflammatory properties. Consequently, it is the increase in Th17 cells that is the major determinant of the altered Treg/ Th17 ratio that is reported in response to declining estrogen and has a central role in the pathophysiology of postmenopausal osteoporosis [12, 13].

Recent evidence has revealed that the gut microbiota is a critical regulator of this osteoimmunologic (i.e., T cell) response; thus, establishing the gut-bone axis as a viable target to prevent estrogen deficiency-induced bone loss [14-16]. Clinical studies have shown that utilizing probiotics and prebiotics to alter the gut microbiota has beneficial effects on bone and mineral metabolism [17-23]. In animal models of postmenopausal osteoporosis, probiotics and prebiotics increase gut-derived metabolites (e.g., short chain fatty acids [SCFA]), program more tolerogenic or anti-inflammatory immune responses, while preventing bone loss [24-30]. Our laboratory's extensive work on dried plum and its unique ability to not only prevent, but also reverse bone loss in animal models, has revealed that it improves T cell function *ex vivo* and both its polyphenolic and carbohydrate components contribute to its prebiotic activity [31, 32]. These findings combined with strong clinical evidence in support of dried plum's ability to prevent postmenopausal bone loss [33-35] make it an excellent prebiotic candidate to test whether these gut-mediated changes in immune function observed in animal models translate to human subjects.

In addition to age-related changes in estrogen, other factors affecting the T cell biology of postmenopausal women (e.g., vitamin D status) could further compound their osteoporosis risk. Among U.S. women age

51-70 yrs, 28% are vitamin D insufficient and 10% are vitamin D deficient based on the Institute of Medicine guidelines [36]. Although vitamin D is known for its role in skeletal health, evidence of its important extraskeletal functions continues to emerge [37-40]. 1,25-$(OH)_2D_3$ simultaneously inhibits the pro-inflammatory Th17 cell and increases the anti-inflammatory Treg cell, number and function [40-44]. Furthermore, vitamin D's influence on immune function has been recently linked, albeit indirectly, to the gut microbiota [45, 46]. Thus, postmenopausal women who have compromised vitamin D status may face even greater risk of T cell-mediated pathologies (e.g., autoimmune diseases & osteoporosis), which further highlights the importance of targeting the gut microbiota.

2.0 PROJECT GOALS AND OBJECTIVES:

The goal of this project is to understand how dietary supplementation with dried plums, a food with prebiotic activity, affects T effector cells (i.e., Th17 & Treg) and their function; and to explore whether vitamin D status of the host alters this response. The fundamental questions to be addressed are: 1) whether the findings of pre-clinical studies [26, 28, 47, 48] demonstrating the gut microbiota is a target to counter T cell changes associated with estrogen deficiency translate to postmenopausal women; and 2) whether the vitamin D status of the host influences the response to this prebiotic.

To accomplish this, we will recruit postmenopausal women, age 60-75 years, to participate in a randomized crossover control study with and without dried plum. Eligible volunteers who are willing to consume dried plum per day (5-6 dried plums/d), participate in an ~ 14-wk study, and meet all other inclusion/exclusion criteria, will be consented. We plan to enroll n>/=30 women.

The ***hypothesis*** is that dried plum will have anti-inflammatory effects on the gut-bone axis of postmenopausal women, characterized by a decrease in the abundance of Th17 cells (i.e., increase in Treg/Th17 ratio) and their activation; and this response will be more pronounced in women with higher serum 25(OH) D. To date, there are no reports indicating how dried plum affects the abundance and function of Th17 cells, which accelerate bone resorption by osteoclasts that leads to postmenopausal bone loss and the influence of vitamin D on this response. To test our hypothesis, we will perform a randomized crossover control study designed to:

Objective 1. Determine how dried plum supplementation alters circulating T cells (e.g., Th17 & Treg cells), other immune cells that regulate T cell function (e.g., B regulatory cells), and the inflammatory response of postmenopausal women compared to a control period in a crossover design study. *Peripheral blood mononuclear cells (PBMC) will be isolated from whole blood collected pre- and post- treatment of each arm of the study and the change in T cell populations and their function as well as inflammatory mediators in response to dried plum will be assessed.*

Objective 2. Assess the changes in the gut microbiota and gut-derived metabolites in response to dried plum compared to the control in stool and serum samples collected pre- and post each treatment arm. *Stool and serum samples will be collected and the change in gut microbiota taxa and fecal and serum metabolites in response to dried plum will be determined.*

Objective 3. Determine whether: a) serum vitamin D influences the immune, metabolite and microbiota response to dried plum; and b) the relationship of the immune, metabolite and microbiota outcomes to the bone resorption marker, c-terminal telopeptide 1 (CTX-1) and bone formation markers such as procollagen type 1 N-terminal peptide (PINP). *Serum 25(OH)vitamin*

D and bone biomarkers (e.g., CTX-1 and PINP) will be assessed and their relationship to outcomes from Objective 1 & 2 will be explored.

3.0 OUTCOME MEASURES AND ENDPOINTS:

The **primary outcomes** of this study will be change in circulating Th17 cells and their function in *ex vivo* experiments, and inflammatory cytokines (e.g., IL-6 & IL-17).

Secondary outcomes will include alterations in circulating Treg cells, their function and Th17/Treg ratio, other immune cells that affect T cells (e.g., B regulatory cells and national killer cells), serum and fecal metabolites, microbiota taxa, and serum 25(OH)D, cytokines (e.g., TNF-α & IL-10), and the bone resorption and formation markers (e.g., CTX-1 and P1NP).

4.0 ELIGIBILITY CRITERIA:

Inclusion Criteria.

- women 60-75 years of age at the start of the study;
- 12 or more consecutive months without a menstrual period;
- willing to include dried plums in their daily diet and collect fecal samples at four time points;
- ambulatory without assistance;
- capacity to give informed consent.

Exclusion Criteria.

- women who have been on medications known to alter bone or calcium metabolism (e.g., oral bisphosphonates, raloxifene, denosumab, intermittent parathyroid hormone, growth hormone) within 12 months of starting the study. Prior use of intravenous bisphosphonates at any time.
- women who have been on hormone replacement therapy, steroids (i.e., glucocorticoids), biologics, or chronic NSAID within 3 months of starting the study.
- women with a previous diagnosis of osteoporosis (i.e., BMD T-score or history of vertebral fracture or fragility fractures of hip, wrist, humerus after the age of 50 yr) or other metabolic bone disease (e.g., osteomalacia or osteogenesis imperfect), renal disease, stroke, heart attack, type 2 diabetes, liver disease or autoimmune diseases (e.g., rheumatoid arthritis, systemic Lupus erythematosus, type 1 diabetes mellitus, IBD) that could affect bone metabolism or T cell biology will be excluded.
- women who have undergone treatment for cancer within 12 months of starting the study
- women who have been taking prebiotic or probiotic supplements or natural products that have estrogen-like effects in the past 3 months
- women who smoke, have a BMI >40 kg/m^2 or <18.5 kg/m^2, or consume $\geq$2 alcoholic drinks per day
- women who regularly consume dried plums or prune juice (>1 serving weekly).

5.0 RECRUITMENT:

Recruitment. We will use several different strategies to recruit study participants. One source of participants that we will capitalize on are women who meet the age criteria who have previously undergone assessment in the Indiana Center for Musculoskeletal Health (ICMH), Musculoskeletal Function, Imaging and Tissue (FIT) Resource CORE and have indicated that they are interested in participating other research studies. We will also utilize the All IN for Health TrialX iConnect platform offered through the CTSI. Additionally, participants will be recruited through flyers and e-mail announcements to area community wellness programs, senior centers, churches and local clinics and doctors serving older adult populations. We will promote the study to different racial demographic groups so that the distribution of the study participants is reflective of the overall population of Indianapolis, IN. Women who are interested in volunteering for the study will be able to contact the Study Coordinator for the initial phone screening.

6.0 STUDY DESIGN:

We will conduct a randomized crossover control design trial with postmenopausal women with or without dried plum (*See Overview of Study Design* **Figure 1**). The study design will require participants to complete 5 visits to the IU Health University Hospital Clinical Research Center. Postmenopausal women, age 60-75 years, will be recruited to participate and undergo an initial phone screen to determine if they are potentially eligible to participate in the study (*See Phone Screening Questionnaire)*. At Visit 1, a more extensive questionnaire involving relevant medical and health history will be completed focused on the inclusion/exclusion criteria, height and weight will be assessed. Volunteers who meet the inclusion/exclusion criteria and give informed consent to participate will be enrolled in the study. As a part of the consent process, volunteers will receive a separate informed consent that details the study tests and risk associated with participating in the Musculoskeletal FIT Core study (IRB study #1707550885). The FIT Core Study assessments will be completed at Visit 1 only. Importantly, this data will allow us to characterize the bone health and functional abilities of the study population. At the end of Visit 1, instructions will be provided for entering dietary data and stool sample collection, as well as a stool collection kit.

The primary study (Visits 2-5) is divided into three phases, with each lasting about 4 weeks (**Figure 1**). Prior to returning for Visit 2, participants will record their 24-hr dietary intake over three days using the Automated Self-administered 24-hour (ASA24) dietary assessment tool. At Visit 2, participants will return their stool sample, and baseline data collection will be performed that includes anthropometrics, questionnaires, any updates to the medical history questionnaire and a blood draw. Participants will then be randomly assigned to the AB or BA intervention sequence using a computer-generated list of random numbers. Instructions will be provided on the run-in protocol and incorporating dried plum into the diet (50 g/day or 5-6 dried plums per day) or not based on their sequence. Study personnel will provide the dried plums in snack packs at no cost. Note the snack packs will be provided by the California Prune Board. At Visit 3, participants will complete the data and sample collection as described for Visit 2 and then begin the washout period. Visit 4 will be scheduled following the washout period and the same procedures described in Visit 3 will be repeated with crossover to the second intervention. At Visit 5 (final visit), participants will complete the data and sample collection. At Visits 3 or 5, any remaining dried plums will be returned. All procedures will strictly adhere to the guidelines set forth by the Indiana University School of Medicine Institutional Review Board. The study will be registered with ClinicalTrials.gov.

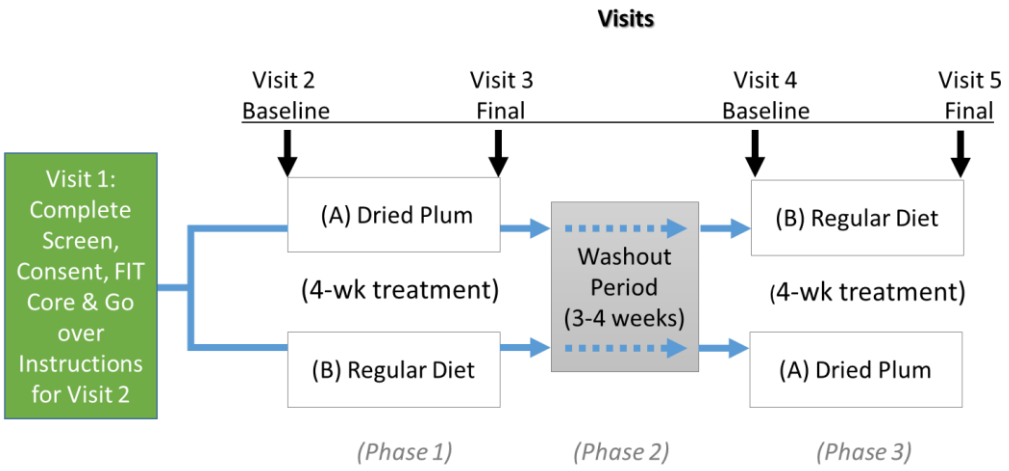

Figure 1. Overview of Experimental Design. Postmenopausal women (n>/=30) will be randomized to one of two treatment sequences (AB or BA) and complete 5 study visits (*details below*). Each treatment period (Phase 1 & 3) will consist of an approximately 1-wk run-in followed by 4 wks of treatment with a 3-4 wk washout in between.

7.0 OVERVIEW OF STUDY PROCEDURE AND SCHEDULE:

Procedures	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Explain the study, answer questions and consent	x				
Medical history questionnaire (form will be updated at Visits 2-5)	x	x	x	x	x
Anthropometric measurements (height and weight)	x	x	x	x	x
FIT Core (bone & muscle imaging, physical function testing, and questionnaires)	x				
Assigned to Group and provided instructions.		x		x	
Complete 3-day Food Record prior to visit		x	x	x	x
Return Stool Sample		x	x	x	x
Physical Activity Questionnaire		x	x	x	x
Sun Exposure Questionnaire		x	x	x	x
Bowel function and gastrointestinal tolerance questionnaires collected			x		x
Fasting Blood Collection		x	x	x	x
Compliance calendar collected			x		x
Honorarium ($50 gift card after each visit is completed)	x	x	x	x	x

8.0 STUDY PROCEDURES:

***Health and Medical History*.** Relevant medical history will be obtained at Visit 1 by the Study Coordinator. The information collected will include a brief medical history, questions related to health behaviors, reproductive history, bone health history (i.e., incidence of previous fractures), prior oral contraceptive and hormone replacement use, as well as the use of other medications and supplements (*See Medical History Form*). The health and medical history will be updated at each subsequent visit to account for any changes occurring over the course of the study. Any concerns raised will be brought to the attention of the CRC staff physician for recommendations.

FIT Core. At Visit 1, participants will complete assessments at the FIT Core (IRB study #1707550885). This will include but is not limited to providing additional information related to their medical history, medications, and body composition (height, weight). They will be asked to answer questionnaires regarding their physical function and physical activity and will undergo a DEXA or bone density test. Participants will also be asked to complete a battery of tests related to their physical capacity, including hand grip strength, repeated chair stands, gait speed, timed up-and-go, a six-minute walk test, and a balance assessment.

Blood Sample Collection. Participants will provide fasting blood specimens, collected between 7:30-10:00 AM at baseline and final time points in each period (Visit 2-5). Whole blood (~21 mL) will be collected in CPT tubes (BD Biosciences) for FACS analyses and *ex vivo* experiments with PBMCs to characterize Th17 and Treg abundance and function. One additional tube (~5 mL) will be collected and processed for serum, and stored at -80°C for metabolomics, cytokines, 25(OH)D and bone biomarkers.

Fecal Sample. Participants will be asked to collect 2 stool samples prior to each visit (Visits 2-5) using a sample collection kit (DNA GeneTek). They will be also asked to rate their stool consistency using the Bristol Stool Scale, as well as ease of passage using a 5-point Likert scale (1 = very easy, 2 = easy, 3 = neither easy nor difficult, 4 = difficult, 5 = very difficult) as we have previously reported [49].

***Anthropometric Measurements*.** Height and weight will be assessed at each visit and body mass index (BMI kg/m^2) calculated.

***Physical Activity Patterns*.** Physical activity patterns will be assessed Visits 2-5 using the validated CHAMPS Physical Activity for Older Adults Questionnaire. This questionnaire is designed to elicit information on leisure, occupational, and home activities and classifies the activities based on duration. Participants will be asked to refrain from altering their physical activity from Visit 2 until the completion of the study at Visit 5. Physical activity data will be analyzed to determine usual activity level, consistency over time and deviations from baseline.

Bowel Function and Gastrointestinal Tolerance. At Visits 2 through 4, participants will be instructed to complete a validated questionnaire [50] about their bowel function and gastrointestinal tolerance each day throughout the treatment period. They will be asked to rate the severity of gastrointestinal symptoms—such as abdominal distention and nausea—on a 4-point scale (1 = absent, 2 = mild, 3 = moderate, 4 = severe). Participants will also record the date and time of each bowel movement. Stool consistency will be assessed using the Bristol Stool Scale, and ease of stool passage will be rated on a 5-point Likert scale (1 = very easy, 2 = easy, 3 = neither easy nor difficult, 4 = difficult, 5 = very difficult) [49].

***Dietary Assessment*.** Prior to Visits 2-5, participants will enter their 3-day diet intake using ASA24 to include 2 weekdays and 1 weekend day. They will be instructed to measure (using standard measuring cups/utensils) and record all food, beverages and supplements consumed. At least two of the three days

should include the days immediately prior to their visit to coincide with fecal collections. Dr. Biruete, who is an RD, will assist with developing these instructions. Participants will be asked to refrain from making changes to their routine diet or supplement intake throughout the study. They will also be instructed to try to consume the same foods in the days preceding their final visit of each study period as they reported at baseline to minimize the influence of dietary factors on outcomes. The 3-day record will be analyzed by study personnel using the ASA24 tool. Dietary assessment will be performed to assess dietary pattern, energy intake, and macronutrient and select micronutrient composition.

Consumption of Dried Plums and Compliance. The test product will be provided by the CA Dried Plum/Prune Board in commercially prepared snack bags. During the 1-week run-in to the dried plum intervention period, participants will be instructed to gradually increase their consumption of dried plum over 1 week following a schedule of 2 dried plums/d for 2 days (1 after breakfast and 1 after dinner), 4 dried plums/d for 3 days (1 after breakfast, 1 after lunch and 2 after dinner), followed by 6 dried plums/day (2 each after breakfast, lunch and dinner). Participants will be instructed to try to continue to spread the consumption of 50 g of dried plum throughout the day during the 4-wk intervention period and to consume their final dried plums at ~9:00 PM prior to their study visit. Suggestions will be provided with the assistance of Dr. Biruete about ways to incorporate dried plums into the diet, without cooking. To assess compliance with the dried plum, each participant will be given a calendar to record the date and time that the fruit was consumed. The Study Coordinator will contact participants weekly by text or email to encourage compliance and note any adverse events. At the final visit, participants will return their calendar and any unused dried plum as indicators of compliance.

9.0 POTENTIAL RISKS AND PROCEDURES FOR MINIMIZNG RISK:

Risk Associated with Blood Draws

The risks of drawing blood might be pain at the needle site, bruising, and feeling faint. Rarely is there a risk of infection. To reduce potential risks, blood will be drawn by experienced technicians using standard institutional practice and guidelines.

Risk from Physical Ability Tests

Risks during or following the FIT Core physical ability testing (i.e., hand grip strength, chair stands, gait speed, walk test, isokinetic dynamometer, and balance assessment) include muscle soreness or cardiac ischemia due to physical activity. However, the protocol is not effort dependent, and participants are able to terminate the test at any time. In addition, the test is terminated if heart rate reaches 180 bpm in order to minimize the cardiac risk.

Risk from DXA Scan

Exposure to a very small radiation dose occurs with DXA scanning. This amount of radiation is less than one tenth that of a chest x-ray and less than the natural radiation that one is exposed to during a normal day. Therefore, DXA scans are very unlikely to have a significant impact on health risks, but participants will be made aware.

Risk of Gastrointestinal Issues/Distress

There is a chance that participants will experience changes in their bowel habits, the consistency of stools, and experience gas and bloating when consuming the dried plum. To minimize these effects, participants will be given details instructions about the run-in period for the dried plum arm of the study. This

approach has been shown in previous studies to almost eliminate the chance of these effects with the 50 g/d dose [35].

Risk of Developing an Allergy to Plums
There is a remote possibility that a participant will develop an allergic reaction to consuming dried plums. Plums are related to cherries, peaches, nectarines, and apricots. The most common allergic reaction is itchy mouth and throat; however more severe reactions such as rash, difficulty breathing, nausea and vomiting, and anaphylactic shock can occur. If participants have known allergies to cherries, peaches, nectarines, or apricots, a small sample will be provided at the initial visit to monitor possibility for reaction.

Risk of Loss of Confidentiality
There is a slight risk of loss of confidentiality of subject information. Every effort will be made to keep subject information confidential. Information collected will be maintained on secure, password protected electronic systems. Paper files that contain personal information will be kept in locked and secure locations only accessible to the study team. Samples that are collected will be identified by a participant study number assigned at the time of enrollment. Any material issued to researchers will be anonymized and only identified by a coded number.

10.0 STUDY WITHDRAWAL/DISCONTUATION:

Participants may elect to withdraw from the study at any time and for any reason by notifying the research personnel. Participants may also be withdrawn for incomplete data or failure to perform the visits as outlined within the specified protocol period.

11.0 STATISTICAL CONSIDERATION:

Power Calculation. Although we anticipate a low attrition rate due to the utilization of an effective run-in period as previously described [35], we conservatively incorporated a 10% attrition rate in our power calculation. Our sample size has 80% power to detect an effect size of 0.8 standardized mean difference in the immune response between the treatment with dried plum and control (i.e., no dried plum). Based on a previous study of the anti-inflammatory effect of dried plum in a similar population of postmenopausal women consuming 50 g/d [51], an effect size of approximately 1.5 SD was observed for serum IL-6 when comparing the dried plum group to the control group. Thus, our study sample size (n=30) provides adequate power to detect expected treatment effects. Power estimation was conducted our by biostatistician and co-PD, *Dr. Ren*, using PASS v19.

Statistical Analyses. De-identified data will be analyzed using PC SAS version 9.4 and SAS PROC MIXED, GLIMMIX, and GLM. Descriptive statistics will be calculated for all variables and will include means, SD, medians, and minima/maxima. Distributions of outcome variables will be examined graphically using histograms and box plots for symmetry and for outliers. Logarithmic transformation will be applied to non-normally distributed data. For our primary outcomes, we will use linear mixed effects models to determine if the level of serum IL-6 and T cells (i.e., Th17) at the end of each period is significantly different for dried plum compared to the control. Baseline measures, treatment and period indicators,

vitamin D (i.e., serum 25-OH D), and important demographic characteristics (e.g., age, BMI, years post menopause) will be included as fixed effects. Random participant effects will be included to accommodate the correlations among repeated measures. We will test for a carry-over effects, but we assume there will be no effect after adjusting for baseline and period effects. We will perform a per protocol analysis on the primary outcomes, which includes only those who completed the intervention and who were compliant ($\geq$85%) with the study protocol. Hypothesis tests for the treatment effect will be carried out following standard procedures for mixed-effects models [52]. Similarly, for secondary outcome measures (i.e., gut microbiota, metabolites, and bone resorption markers), we will use separate mixed effects models, adjusting for baseline, period, and demographic covariates. Categorical variables will be assessed using the generalized linear mixed-effects models. Importantly, to gain a better understanding of the gut-bone axis response, we will also integrate the immunologic outcomes (i.e., T cell), metabolomics, microbial taxa and bone biomarker data. These analyses will include regression analyses to correlate key outcomes. In addition, we will explore if dried plum consumption modulates the immune response via alterations in metabolite and microbiota profiles by conducting exploratory mediation analysis. For all statistical analyses, alpha will be set at 0.05.

12.0 DATA SECURITY:

This study will utilize the secure, web-based, Research Electronic Data Capture (REDCap) system for data input. REDCap was developed by Vanderbilt University and is provided by Indiana University through their community license. REDCap is managed by the Indiana University Department of Biostatistics and secured by University Information Technology Services Advanced IT Core. Access to the password protected database will be limited to the investigators of this study, and any data that is distributed will be de-identified.

13.0 PARTICIPANT CONSENT:

The protocol and informed consent form for this study must be approved in writing by the appropriate Institutional Review Board (IRB) prior to any participant being enrolled on this study.

Changes to the protocol, as well as a changes of principal investigator and study personnel, must also be approved by the Board. Records of the IRB review and approval of all documents pertaining to this study will be kept on file by the investigator (housed in the PI's Office) and are subject to inspection at any time during the study. Status updates will be submitted to the IRB as required by the board, as well as notification of completion of the study.

14.0 DATA SAFETY AND MONITORING:

Although minimal risk is involved with this study, investigators will conduct continuous review of data and participant safety. Quarterly review meetings will be held and include the principal investigator,

clinical research coordinator and other members per principal investigator's discretion. Quarterly meeting summaries should include review of data, the number of subjects and responses observed. The meeting minutes and attendance will be kept on record from these meetings.

Adverse Event Reporting:

Adverse events related to the study intervention will be captured throughout the study. All adverse events related to blood draw, gastrointestinal distress or allergic reactions to dried plums, or injuries associated with the physical function testing will be followed until resolution, a return to baseline, or deemed clinically insignificant.

Reporting to the IRB

Unanticipated problems involving risks to participants or others will be reported **promptly** to the IRB if they:

- are unexpected;
- are related or possibly related to participation in the research; and
- suggest that the research places subjects or others at a greater risk of harm than was previously known or recognized.
  If the serious adverse event does not meet all three (3) criteria listed above, the event does not have to be promptly reported to the Indiana University IRB. However, it will be reported at the time of continuing review.

  Prompt reporting of unanticipated problems to the IRB is defined as within 5 days from becoming aware of the event.

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