Safety and Pharmacokinetics of an Extract of Naringenin

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Clinical Safety and Pharmacokinetic Evaluation of Naringenin: Single Dose Escalation Randomized Double Blind Controlled Trial

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OVERVIEW

The discovery that humans possess functional depots of brown adipose tissue (BAT)[1-3] sparked exploration of ways to activate BAT and promote the conversion of white adipose tissue (WAT) to the brown phenotype known as beige or brown adipocytes. The accumulation of beige adipocytes in WAT, referred to as browning, has garnered interest in research on obesity because it engenders adaptive thermogenesis, a process by which the production of adenosine triphosphate (ATP) is uncoupled from the catabolism of lipids and carbohydrates. The process is mediated by uncoupling protein 1 (UCP1) which mediates resolution of the proton-motive force generated by substrate oxidation, without the synthesis of ATP. Rather, the proton gradient is liberated as heat.[4] This type of metabolically active and energy dissipating tissue offers a potentially effective solution to counteract excessive fat accumulation, especially since BAT is typically low in humans and correlates inversely with body mass index.[3,5,6] Among the gene regulatory pathways implicated in the thermogenic program is the nuclear receptor, peroxisome proliferator-activated receptor (PPARγ), its coactivator PGC-1β and the transcription factor carbohydrate response element binding protein (ChREBP).

The discovery and characterization of ChREBP unraveled the main mechanism by which the transcriptional response to glucose is mediated.[7,8] Increased *de novo* lipogenesis in fat provides a reservoir for excess energy and maintains glucose homeostasis.[9] The insulin-sensitive glucose transporter type 4 (GLUT4) is the rate limiting protein for shuttling glucose, the substrate for de novo lipogenesis, into adipocytes. Although adipose tissue is not the major site for glucose disposal, mice with adipose-specific GLUT4 overexpression display improved glucose control, whereas adipose specific GLUT4 deficient mice develop insulin resistance and hyperglycemia. Of interest is the upregulation of ChREBP that accompanies enhanced glucose flux in adipocytes of overexpressing GLUT4. High fat feeding causes an early reduction in the expression of ChREBP in mouse adipocytes and is due in part to a decrease in GLUT4 expression.[10] Increased ChREBP expression in adipose tissue and not liver mediates the effects on glucose homeostasis. Thus, *de novo* lipogenesis predicts metabolic health in a tissue-specific manner.

Strategies to enhance adipose tissue glucose uptake and restore depleted levels of ChREBP expression can be particularly beneficial to individuals with obesity and insulin resistance. Naringenin belongs to a class of flavonoids known as flavanones and is found in grapefruit, oranges, and limes. Naringenin has been shown to reduce diet-induced weight gain in rodents and improve glucose and lipid metabolism in a number of studies in animal and in vitro models. Activation of adenosine monophosphate-activated kinase (AMPK), regulation of insulin signaling pathways and lipid metabolism, and relief from oxidative stress in pancreatic beta cells are among the mechanisms that have been implicated.[11-14] Although most of the effects of naringenin have been shown in the liver, muscle, and islet cells, increases in energy expenditure and activation of brown fat has been demonstrated in mice fed a high-fat diet supplemented with naringenin.[15] Our in vitro studies in differentiated human subcutaneous adipose-derived stem cells from overweight and obese female donors show that naringenin stimulates mRNA and protein expression of UCP1, GLUT4 and ChREBP, key determinants of thermogenesis, systemic insulin sensitivity, and glucose homeostasis.[10,16]

The effects of naringenin have primarily been demonstrated in vitro and in animal studies but only through human intervention trials can the significance to human physiology be determined. The overarching aim of the study is to conduct a randomized controlled trial to determine the safety and tolerability of naringenin in humans.

BACKGROUND AND SIGNIFICANCE

Overexpression of ChREBP in mice fed a western diet enhances systemic insulin sensitivity, reduces fat mass and increases expression of the genes associated with thermogenesis

suggesting a role for ChREBP in whole body insulin sensitivity and brown adipose tissue function.[8,17] In this type of metabolically active tissue glucose uptake is stimulated.[18] ChREBP is a transcription factor that is known to be expressed as α and β alternatively spliced isoforms. In humans, expression of both ChREBPα and β isoforms in white adipose tissue correlates with insulin sensitivity; [9,10,19] but, obesity is associated with a reduction specifically in ChREBPβ expression.[9,19] ChREBP is highly expressed in active sites of de novo lipogenesis including the liver, and white and brown adipose tissue. ChREBP is a key mediator of the beneficial effects of enhanced glucose uptake in adipocytes through its specific control of lipogenic genes; however, the effects of adipose ChREBP on glucose homeostasis are not simply attributed to the clearance of glucose from circulation. In an elegant study published in 'Cell,' it was discovered that adipose tissue of mice overexpressing GLUT4 in adipocytes have branched fatty acid esters of hydroxy fatty acids (FAHAs). These novel fatty acids contribute to overall insulin sensitivity by enhancing glucose uptake in adipocytes and by stimulating direct and GLP-1-mediated improvement in beta cell function. FAHAs have been shown to act via the G protein coupled receptor120 (GPR120[20] (Figure 1)

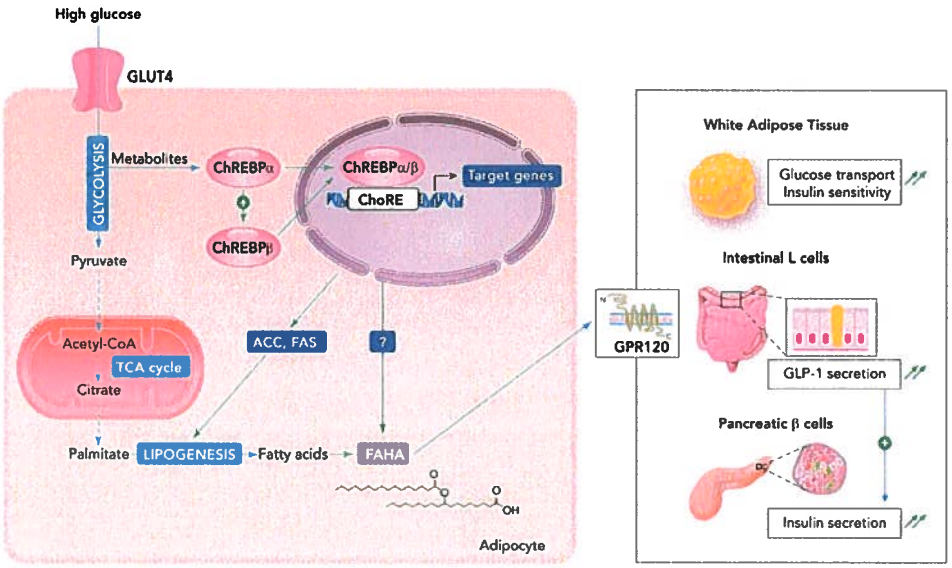

Figure 1. ChREBP links lipogenesis to insulin sensitivity in adipocytes. ChREBP is a key mediator of the beneficial effects of enhanced glucose uptake in adipocytes through its specific control of lipogenic genes. Branched fatty acid esters of hydroxy fatty acids (FAHAs), were found to contribute to overall insulin sensitivity by stimulating glucose transport in adipocytes and by enhancing both GLP-1 and insulin secretion. FAHAs were shown to act via the GPR120 receptor. *Physiology.* 2015;30(6):428-437.

In humans, grouped according to their two-hour blood glucose concentrations (<120mg/dL, 120-140 mg/dL, and >140 mg/dL) during an oral glucose tolerance test, the expression of GLUT4, lipogenic genes and ChREBP measured in subcutaneous fat biopsies was significantly decreased as blood glucose concentrations rose across the groups.[19] Interestingly, expression of adipose ChREBP correlated with expression of GLUT4, lipogenic genes, 2-hour blood glucose, and insulin sensitivity measured by the euglycemic hyperinsulinemic clamp method.[19] Although this study was done in obese adolescents, it provides preliminary evidence that ChREBP and GLUT4 gene expression correlate with physiologic outcomes related to glucose homeostasis.

Weight cycling is known to result in exacerbated post-dieting weight regain. In predisposed mice placed on a weight cycling protocol, naringenin attenuated weight regain. (Figure 2A).[15] Figure 2B shows weight loss in subjects given one-half of a grapefruit three times/day for 12 weeks.[21]

Clinical Implications

Lifestyle interventions promote weight loss, reduce the conversion of impaired glucose tolerance to diabetes and are recommended as the primary approach to prevent and treat obesity and its comorbidities.[22] However, for many people adherence to a diet and exercise regimen is particularly daunting, especially over the long term. Pharmacologic interventions such as metformin and pioglitazone reduce the risk of conversion to type 2 diabetes by 31% and 72% respectively;[23,24] but, according to the American Diabetes Association's assessment of trials in prediabetes pharmacologic interventions, these benefits are not without adverse effects.[25] Naringenin is a component of food with therapeutic potential to improve glucose metabolism. **In order to explore the mechanisms by which naringenin may increase energy expenditure and improve glucose metabolism in humans**, it is of vital importance that the safety and tolerability of naringenin is evaluated when administered to humans.

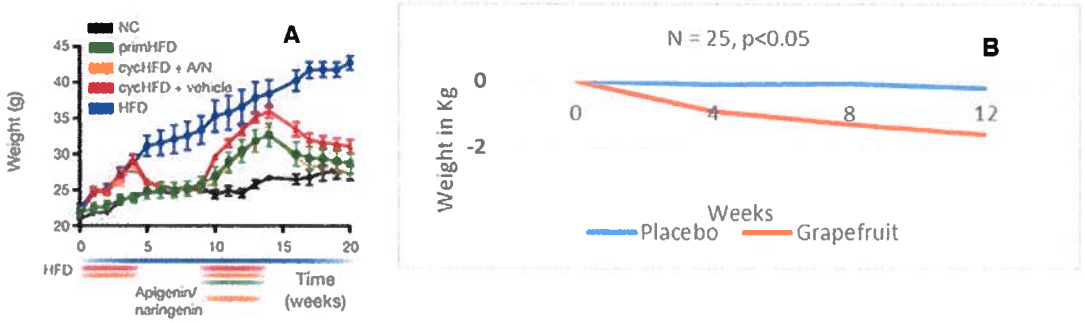

Figure 2 A. Weight loss in weight cycling mice. Mice placed on a cycling high fat diet (second cycle) supplemented with naringenin (orange line) gained as much weight as mice on the first cycle of a high fat diet (green line) and less weight than the mice on a cycling high fat diet (second cycle) that was not supplemented with naringenin (red line) (Nature 2016, 540:26-29). **B.** Weight loss in humans given grapefruit for 12 weeks (J Med Food 2006, 9;49-54).

STUDY DESIGN

This is a placebo-controlled, ascending treatment crossover design trial with washout periods of at least one week.

Primary objective: Evaluate the effect of single oral administration of naringenin at four escalating doses on safety and tolerability in healthy volunteers.

Secondary objective: Evaluate the pharmacokinetics of naringenin at low (150 mg) and high (600 mg) doses to test if changes in serum concentrations are proportional to the dose, by assessing naringenin drug exposure (AUC_{0-24}).

Rationale for Naringenin Dose

In a human subjects study, the pharmacokinetics of naringenin has been investigated after a 135 mg dose of naringenin, administered orally. The study population included six healthy adult volunteers (five males, one female). All subjects were in good health as assessed by medical history, clinical examination, blood pressure and standard biochemical health measures. After an overnight fast subjects each subject received a single oral dose (capsule) of 135 mg of naringenin. Blood was drawn to evaluate pharmacokinetics over a 12 hour period. All six subjects successfully completed the pharmacokinetic study and none reported undesirable or adverse effects after oral administration of naringenin. All subjects who participated in the study were discharged in good health.[26] A study completed by Harvard University and reported on ClinicalTrials.gov (NCT01091077) administered a single, 1000mg oral dose of naringenin mixed

with 16 grams of hydroxypropyl-β-cyclodextrin to enhance bioavailability. Eighteen subjects with hepatitis C viral infection were included in the study, with the primary aim of determining the safety and pharmacokinetics of naringenin. The study was conducted at the Massachusetts General hospital. There were no adverse events reported per communication with the principal investigator Arthur Kim, M.D., Harvard University School of Medicine (Appendix II).

Using the serum concentrations achieved in the only reported human pharmacokinetic study,[26] we conducted in vitro experiments in differentiated human subcutaneous adipose-derived stem cells from an overweight female donor and from an obese female donor and showed that naringenin (7-day treatment) acts in adipose tissue to stimulate thermogenesis and insulin sensitivity. Our methods have been validated using tissue from a donor matched for body mass index and cultured at the LSU Health Sciences System (New Orleans) in a microphysiologic system that allows the cells to be viable for two weeks (unpublished data). Due to the short half-life of naringenin ($t_{1/2}$ = 2.31± 0.4h),[26] we hypothesize that multiple dosing up to 600 mg will be necessary for effective duration of action. Thus, it is vital that the safety of naringenin is evaluated when administered to humans at higher doses.

The maximum recommended starting dose (MRSD) for naringenin may be estimated at approximately 135 mg since no adverse events were reported at this dose.[26] The dose escalation scheme recommended for first in human studies is rapid dose escalation initially (MRSD x 2) until the estimated effective dose (600 mg) is reached and then at a more cautious escalation (e.g. x 1.5).[27] The first dose will be 150 mg, the MRSD for the practical purpose of preparing the capsule of an extract from oranges which contains 30% naringenin (Gencor Lifestage Solutions, GE Nutrients Inc.,Irvine, CA). HPLC and mass spectrometry verification using a second standard to quantify the naringenin in the extract was conducted by the Rutgers University Botanical core. It was determined that the extract contains 27% naringenin by one method and 29% naringenin by a second method. Therefore, the amount of extract for the 150 mg, 300 mg, 600 mg, and 900 mg doses of naringenin will be calculated based on the average of 28%. According to the United States Department of Agriculture (USDA) database for the flavonoid content of selected foods 150 mg of naringenin could be found in a minimum of approximately four oranges and a maximum of 12 oranges.[28] The orange extract will be calculated to deliver 150 mg, 300 mg, 600 mg, and 900 mg of naringenin. The capsules will be prepared and dispensed by the PBRC pharmacist. The schedule for dosing is provided in Table 1.

Table 1. Ascending Oral Dose Schedule

	Visit 1	Visit 2	Visit 3
Cohort 1			
Sequence 1 (n=3)	150 mg	Placebo	300 mg
Sequence 2 (n=3)	150 mg	300 mg	Placebo
Sequence 3 (n=3)	Placebo	150 mg	300 mg
Cohort 2			
Sequence 4 (n=3)	600 mg	Placebo	900 mg
Sequence 5 (n=3)	600 mg	900 mg	Placebo
Sequence 6 (n=3)	Placebo	600 mg	900 mg

Study Subjects

This study will enroll 18 healthy adult male and female subjects. Subjects will be enrolled on the basis of the following eligibility criteria:

Inclusion Criteria

- Adult (≥18 years)

- Body mass index (determined at the screening visit) between 20 and 35 kg/m^2
- No evidence of diabetes (fasting blood sugar <126 mg/dL)
- Willing to refrain from consuming citrus fruits and tomato in any form, for 36 hours prior to each test day. Subjects will be provided a list of foods to avoid (Appendix I).

Exclusion Criteria

Subjects will not be eligible for participation if they meet one or more of the following exclusion criteria:

- Report citrus allergies.
- Report a history of cardiovascular disease, diabetes, or cancer
- Subjects who have evidence of hepatic disease or dysfunction, e.g. ALT, or alkaline phosphatase twice the upper limit of normal; hepatitis; jaundice; cirrhosis.
- Current pregnancy or breastfeeding
- Women of childbearing potential who are not using an effective method of birth control (i.e., barrier method, intrauterine and cervical devices, oral contraceptives, hormonal injections (Depo Provera®), condoms with spermicidal gel or foam, contraceptive patch (Ortho Evra), diaphragm, or abstinence), are not surgically sterilized (including tubal ligation and hysterectomy), or not at least two years postmenopausal. All women of childbearing potential will have a pregnancy test performed prior to starting the study treatment in each cohort. If a subject becomes pregnant during the study, they will be dropped from the study.
- Chronic use of medications except over the counter medications and supplements that have been stopped 72 hours prior to the test day.
- Reported clinically significant GI malabsorption syndromes such as chronic diarrhea, celiac disease or malabsorption from bariatric surgery within one month of the study.
- Clinically significant abnormal laboratory markers (as determined by the medical investigator).
- Subjects with anticipated surgery during the study period.
- Subjects with a reported history of substance abuse or alcoholism or significant psychiatric disorder that would interfere with the ability to complete the study.
- Subjects who have donated blood during the month prior to study entry or planned during the study.
- Subjects who have participated in other studies using an investigational drug during the preceding three months.
- Subjects who are current smokers or have smoked within the previous three months. Smoking is not permitted during the study.

Recruitment and Screening

Subjects will be recruited through the use of printed material, targeted solicitation through the Pennington Biomedical Center email listserv and social media. The Pennington recruiting department will screen potential subjects over the phone, and those meeting basic eligibility criteria (age, self-reported BMI, medical and medication history) will be scheduled for a screening visit.

Subject eligibility criteria will be evaluated at a single screening visit. The screening visit will occur in the outpatient clinic, in the morning following confirmation of an overnight (at least 8 hours) fast. Subjects who provide informed consent will proceed with the tests and measurements of the screening visit. Subjects who satisfy the eligibility criteria will be enrolled in a study cohort. The schedule of assessments is provided in Table 2.

Randomization Procedure and Blinding

Each subject in the cohorts will be randomly assigned one of the relevant treatment sequences in order of enrollment. Treatment sequence assignments will be balanced so that each sequential block will have unique treatment sequences. As an example, the first three enrolled subjects in each cohort will be randomly assigned to one of three distinct treatment sequences, as will enrollees 4-6 and 7-9. As a result, three of the nine subjects in each cohort will be randomly assigned to each of the three treatment sequences. The randomization scheme will be prepared by Dr. Robbie Beyl under the supervision of Dr. William Johnson. Except for the pharmacist who will prepare and dispense the capsules, all study staff and the investigators will be blinded to the randomization.

Table 2. Schedule of Assessments

	Screening	Visit 1a	Visit 1b	Visit 2a	Visit 2b	Visit 3a	Visit 3b
Consent	x						
Height	x						
Weight	x	x		x		x	
Blood Pressure/Pulse/Temperature	x	x		x		x	
Medical History Questionnaire	x						
Pregnancy Test (Urine)*	x						
Concomitant Medications	x						
Chemistry Panel/CBC	x						
Adverse Events		x	x	x	x	x	x
Plasma naringenin			x			x	x
Chemistry Panel/CBC (0 hr)**		x		x		x	
Chemistry Panel/CBC (24 hrs later)**			x		x		x
Naringenin Administration		x		x		x	
IV Placement		x					
Blood Collection at 0, 2, 3, 3.5, 4, 4.5, 6, 8, and 12 hours		x					

* Women of Child bearing potential

** For Cohort 2 only BUN, AST, and Bilirubin added to Chem 15 panel

CLINIC VISITS

Subject in each cohort will complete one screening visit and three study visits.

Screening Visit (1½ hours)

Subjects will report to Pennington Biomedical in the morning following an overnight fast (except for water) that began no later than 8 hours prior to the study appointment. The screening visit includes explanation of the study purpose, procedures, and signing of the informed consent. If the participant agrees to participate by signing a consent form, the following tests and measurements will be performed:

- Self-report of personal and family medical history.
- Height, weight, vital signs (blood pressure, pulse, and temperature).
- Concomitant medication use.
- Urine specimen collection for urine human chorionic gonadotropin (HCG) (for pregnancy in women of child bearing potential).
- Blood collection for complete blood count (CBC) and Chemistry 15 panel.

Visit 1 (13 hours, fasting for at least 8 hours)

To start the study, eligible subjects will arrive at the clinic in the morning following an overnight fast (except for water) that began no later than 8 hours prior to the study appointment. The following tests and measurements will be performed:

- Weight, vital signs (blood pressure, pulse, and temperature).
- Blood collection for a CBC and Chemistry 15 panel.

- Subjects will be given a 150 mg dose of naringenin or placebo, administered orally.
- Blood collection for pharmacokinetic study of 150 mg dose of naringenin at 2, 3, 3.5, 4, 4.5, 6, 8, and12 hours.
- Subjects will be provided a meal 5 hours after naringenin administration and at the end of the pharmacokinetic testing.

Visit 1b (½ hour, fasting for at least 8 hours)

- Subjects will return 24 hours post naringenin administration and blood will be drawn for CBC, Chemistry 15 panel, and to evaluate serum naringenin concentrations.
- Adverse events will be assessed 24 hours after naringenin dosing.

Subjects will return to the clinic to complete Visit 2 no less than seven days after the completion of Visit 1.

Visit 2a (½ hour, fasting for at least 8 hours)

Subjects will arrive at the clinic in the morning following an overnight fast (except for water) that began no later than 8 hours prior to the study appointment. The following tests and measurements will be performed:

- Weight and vital signs (blood pressure, pulse, and temperature).
- Blood collection for a CBC and Chemistry 15 panel.
- Subjects will consume 150 mg or 300 mg dose of naringenin or placebo, administered orally.

Visit 2b (½ hour, fasting for at least 8 hours)

- Subjects will return 24 hours post naringenin administration and blood will be collected for CBC, and Chemistry 15 panel.
- Adverse events will be assessed 24 hours after naringenin dosing.

Subjects will return to the clinic to complete Visit 3 no less than seven days after the completion of Visit 2.

Visit 3a (5 hours, fasting for at least 8 hours)

Subjects will arrive at the clinic in the morning following an overnight fast (except for water) that began no later than 8 hours prior to the study appointment. The following tests and measurements will be performed:

- Weight and vital signs (blood pressure, pulse, and temperature).
- Blood collection for a CBC and Chemistry 15 panel
- Subjects will consume 300 mg dose of naringenin or placebo, administered orally.
- Blood collection at four hours for measurement of serum naringenin.

Visit 3b (½ hour, fasting for at least 8 hours)

- Subjects will return 24 hours post naringenin administration and blood will be collected for CBC, Chemistry 15 panel, and serum naringenin concentrations.
- Adverse events will be assessed 24 hours after naringenin dosing.

The visits for Cohort 2 will be similar to Cohort 1 with the exception of oral naringenin dose to be administered (Table 1), and that BUN, AST, and Bilirubin will be added to the Chemistry 15 panel at visits 1a, 1b, 2a, 2b, 3a, and 3b.

STUDY PROCEDURES

Clinical Chemistry

Safety Assessments: Blood will be drawn at time 0 (before ingestion of each dose of naringenin, or placebo) and the subject will return 24 hours later (the next morning) for a repeat chemistry panel and CBC to assess safety of naringenin. Four ml of blood will be drawn for each of these tests. An additional 2 ml will be drawn with the 24 hour specimen to measure serum naringenin concentrations.

- Chemistry 15 Panel will be performed according to standard procedures for blood draws and the relevant chemistry panel.
- Comprehensive Blood Count (CBC) will be performed according to standard procedures.

Pharmacokinetic Testing: To evaluate the pharmacokinetics of naringenin, blood (two ml) will be drawn prior to administration of naringenin and at 2, 3, 3.5, 4, 4.5, 6, 8, 12 and 24 hours post-dose of 150 and 600 mg naringenin, and at four hours after the 300 and 900 mg doses. The serum will be separated from the blood, frozen and sent on dry ice to University of Tennessee for measurement of serum naringenin concentrations. This time course stretches over what is estimated to be five half-lives so a complete pharmacokinetic profile can be described including the maximal concentration achieved, time to peak, half-life, area under the curve, and apparent oral clearance (C_{max}, T_{max}, $T_{½}$, $AUC_{0\text{-}24hr}$ and $Clearance_{oral}$ respectively).

Adverse Events

An Adverse Event (AE) is defined as any untoward medical occurrence in a patient or clinical investigation subject, temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product. We define AE as any unfavorable and unintended sign (including a clinically significant abnormal laboratory finding), symptom, or disease (new or exacerbated) temporally associated with the use of a medicinal product.

We will define a serious adverse event (SAE) as any untoward medical occurrence that results in death, is life threatening, requires inpatient hospitalization or prolongation of existing hospitalization, results in persistent or significant disability/incapacity or in a congenital anomaly.

Safety will also be assessed by recording all adverse events. The study team including coordinator and investigators will inquire regarding adverse events while minimizing the chance for bias when detecting AEs/SAEs. The study team will employ open-ended and non-leading verbal questioning of the subject as the preferred method to inquire about AE occurrence. For examples, appropriate questions include: "How are you feeling?", "Have you had any (other) medical problems since your last visit/contact?", or "Have you taken any new medicines, other than those provided in this study, since your last visit/contact?"

It is the responsibility of the investigator to attempt to establish a diagnosis of the event based on signs, symptoms, and/or other clinical information. In such cases, the diagnosis will be documented as the AE/SAE and not the individual signs/symptoms.

Assessment of Intensity

An assessment of intensity for each AE and SAE reported during the study will be provided by the investigator and the investigator will assign it to one of the following categories:

Mild: An event that is easily tolerated by the subject, causing minimal discomfort and not interfering with everyday activities.

Moderate: An event that is sufficiently discomforting to interfere with normal everyday activities.

Severe: An event that prevents normal everyday activities.

An AE that is assessed as severe will not be confused with an SAE and both AEs and SAEs can be assessed as severe. Severe Adverse Event is a category utilized for rating the intensity of an event when it meets at least one of the pre-defined outcomes as described in the definition of an SAE.

Assessment of Causality

The investigator is obligated to assess the relationship between study treatment and the occurrence of each AE/SAE. A "reasonable possibility" is meant to convey that there are facts/evidence or arguments to suggest a causal relationship, rather than a relationship cannot be ruled out. The investigator will use clinical judgment to determine the relationship. Alternative causes, such as concomitant therapy, other risk factors, and the temporal relationship of the event to the study treatment will be considered and investigated.

Events meeting the definition of an AE:

- Any abnormal laboratory test results (clinical chemistry) or other safety assessments (e.g., vital signs measurements), including those that worsen from baseline, and felt to be clinically significant in the medical and scientific judgment of the investigator.
- Exacerbation of a chronic or intermittent pre-existing condition including either an increase in frequency and/or intensity of the condition.
- New conditions detected or diagnosed after study treatment administration even though it may have been present prior to the start of the study.
- Signs, symptoms, or the clinical sequelae of a suspected interaction.
- Signs, symptoms, or the clinical sequelae of a suspected overdose of either study treatment or a concomitant medication (overdose per se will not be reported as an AE/SAE).

Events that do not meet the definition of an AE:

- Any clinically significant abnormal laboratory findings or other abnormal safety assessments that are associated with the underlying disease, unless judged by the investigator to be more severe than expected for the subject's condition.
- Medical or surgical procedure (e.g., endoscopy, appendectomy); the condition that leads to the procedure is an AE.
- Situations where an untoward medical occurrence did not occur (social and/or convenience admission to a hospital).
- Anticipated day-to-day fluctuations of pre-existing disease(s) or condition(s) present or detected at the start of the study that do not worsen.

Reporting Procedures

The reporting of SAE's will follow the standards of practice as outlined by the PBRC IRB in the investigator's responsibility policy (pages 4 and 5). Once the investigator determines that an event meets the protocol definition of an SAE, the SAE will be reported to the IRB within 24 hours. If the investigator does not have all information regarding an SAE, the investigator will not wait to receive additional information before notifying the responsible parties of the event and completing the appropriate forms. An assessment of causality at the time of the initial report will be provided. Email transmission of the SAE data collection tool will be the preferred method to transmit this information followed by notification by telephone and/or fax. A copy of the SAE report will also be sent to the institution officials. New or updated information will be recorded in the originally completed data collection tool. The investigator will submit any updated SAE data within 24 hours.

Data Safety Monitoring Plan

For each AE, the seriousness, intensity, and relationship to study product will be assessed, documented, and supported by an entry in the subject's medical records. During the study, each

subject will be carefully monitored for any adverse events. After the initial AE/SAE report is completed and sent, the investigator is required to follow each subject at subsequent visits/contacts. As currently practiced, it is planned that all AEs and SAEs will be followed in clinic until: 1) resolution; 2) the condition stabilizes; 3) the event is otherwise explained; or 4) the subject is lost to follow-up.

Drs. William Johnson and Robbie Beyl, LaCATS Center biostatisticians, will be responsible for all blinding procedures and for subject randomization. Except for the pharmacist who will prepare the capsules, randomization will be blinded to the staff performing the physical measures, metabolic testing, performance of assays, entering or calculating data related to the study and study investigators to assure minimal potential sources of bias. Additionally, sealed randomization envelopes will be sent to the pharmacist for emergency breaking of the blind. The randomization envelopes will be maintained in a secure location with access limited to authorized personnel. Blinding is critical to the integrity of this clinical trial. However, in the event of a medical emergency or pregnancy in an individual subject, in which knowledge of the investigational product is critical to the subject's management, the blind for that subject may be broken by the medical investigator of the trial. This will be done by a request to the pharmacist to break the blind and report the treatment assignment to the medical investigator.

Before breaking the blind of an individual subject's treatment assignment, the investigator should have determined that the information is necessary, i.e., that it will alter the subjects' immediate management. In many cases, particularly when the emergency is clearly not investigational product-related, the problem may be properly managed by assuming that the subject is receiving active product without the need for breaking the blind.

In case of an emergency, the investigator may open the emergency randomization envelope to reveal the identity of the medication for that subject. If such breaking of the blind occurs, the investigator shall notify the IRB immediately. This information, including the reason for the blind being broken must be recorded in the participant chart and regulatory files.

STATISTICAL CONSIDERATIONS

As comparative efficacy of the doses of the extract is not the primary issue in this protocol, power to detect efficacious effects is not specifically evaluated to justify sample sizes. A sample of 18 subjects (three subjects in each of three treatment sequences in each of two cohorts) will be enrolled in the ascending single dose evaluation. Thus, all nine subjects in Cohort 1 will receive the placebo and both 150 and 300 mg doses of the extract; similarly all 9 subjects in Cohort 2 will receive the placebo and both 600 and 900 mg doses of the extract. Before the next cohort is commenced, subjects in the preceding cohort must have received all three treatments and the safety and tolerability data must have been assessed showing tolerance to the doses of naringenin. Based on the pharmacokinetic study done in humans,[26] the sample size (n=6, at each dose) is sufficient to provide a pharmacokinetic profile of naringenin in serum.

The primary outcome will be to determine the effect of the naringenin extract on safety and tolerability based on clinical and biochemical assessment.

The secondary outcome will be to determine the pharmacokinetics of naringenin at the 150 mg and 600 mg doses. Serum concentrations of naringenin will be obtained to assess drug exposure.

Safety and tolerability.

Dose safety will be investigated by compiling by treatment (e.g. 150 mg dose, 300 mg dose, placebo) a list of adverse events such as frequency of headaches, nausea, vomiting. The study physician in consultation with the coordinator will review and determine safety and tolerability. The frequency of these events will be counted and compared with the placebo group. The

cohorts will be run in series. There will be an interval of up to four weeks between the two cohorts. During this time an interim analysis will be conducted and a safety summary of adverse events for Cohort 1 will be compiled and reviewed by the investigators.

Stopping Rules

Dosing within a cohort will be stopped and further dosing will be halted until un-blinded safety information can be reviewed in the event that:

- A death occurs
- Two or more subjects experience the same SAE following administration of study product.
- Two or more subjects in each cohort experience the same severe study product-related AE following administration of study product
- Based on AEs, laboratory findings, or clinical findings the Investigator determines that review of pertinent safety information is required.

Dosing may only resume if, after review of safety information, both the Investigator and IRB agree that it is safe to proceed.

Pharmacokinetics of naringenin extract. Pharmacokinetics of orally administered naringenin will be analyzed using a non-compartmental model. The maximal concentration (C_{max}), time to peak (T_{max}), half-life ($T_{½}$), area under the serum concentration versus time curve ($AUC_{0\text{-}24hr}$) and apparent oral clearance ($Clearance_{oral}$ = $Dose/AUC_{0\text{-}24hr}$) for each subject will be determined and the means for subjects given 150 mg and 600 mg doses will be calculated. Pairwise comparisons of 150 mg, 600 mg and placebo will be tested with a linear model for each of the parameters (C_{max}, T_{max}, $T_{½}$, $AUC_{0\text{-}24hr}$ and $Clearance_{oral}$) across all time points. All analyses will use SAS Version 9.4 software (SAS Institute Inc., Cary NC).

DATA COLLECTION AND QUALITY ASSUARANCE

The only people who will know that these patients are research participants are members of the research team. No information about them, or provided by them during the research, will be disclosed to others without their written permission, except if it is necessary to protect their rights or welfare (for example, in case of injury or emergency care), or if it is required by law. When the results of the research are published or discussed in conferences, no information will be included that would reveal the identity of these patients. All data will be kept in locked files, and subjects will be identified by codes when the data gathered in this procedure is presented or published. Authorized representatives of the National Institutes of Health may need to review records of individual participants. As a result, they may see their name; but they are bound by rules of confidentiality not to reveal the patients' identity to others. Blood samples for measurement of serum naringenin concentrations will be shipped by the Clinical Chemistry Core to Dr. Hector Castro at the University of Tennessee. Serum naringenin concentrations will be measured at the University of Tennessee by a validated high performance liquid chromatographic method using solid phase extraction.[26]

Privacy

The subjects will be interviewed in the privacy of an exam room and their records will be protected by a secure medical records area and a password-protected electronic database monitored by the Pennington Research Computing group. Subjects will be asked to sign a written consent after reading it, having it reviewed with them by the study staff and having all their questions answered. The consent conversation will be conducted in the privacy of an exam room and the subject will be allowed to take the consent home to discuss their decision with their family or counselor, if desired. Study procedures will be conducted by trained staff in accordance with PBRC outpatient and inpatient clinic standards of practice and with subjects' informed consent. Confidential subject information including medical records and test results will

be available only to persons authorized by the Pennington. Information collected from subjects will be the minimum amount of data necessary to accomplish the research purposes.

Data and Specimen Management

Study participants will be assigned unique subject identification (ID) numbers. Study subject ID numbers will be used on all data collection instruments, to include questionnaires, data collection forms, biological specimen tubes, and computer records. A master list linking the participants' names and ID numbers will be kept in a password-protected computer file with access restricted to the PI and co-PI's. Biological samples that are moved off-site for analysis will not contain any personally identifiable information and will be labeled with only the unique subject ID numbers. Staff at these sites will not have access to the master list at any time.

Data collection forms will be kept under lock and key, or password-protected if computerized, and under the control of the PI, co-PIs, and medical investigator. Only personnel assigned to the research study by the PI will have access to the data. Hard-copy data records will be stored for a minimum of 3 years.

The PBRC has a fully integrated, campus-wide, automated data management system. All data are entered into a central database using existing methodology that has been fully validated and undergoes continuous quality assurance by the PBRC Research Computing Core and the nutrition and obesity research center (NORC). All data are backed up daily, and the Research Computing Core at the PBRC oversees all data management. The research team has extensive experience using the procedures and methods required to conduct this study. Standard operating procedures in place throughout the units at Pennington Biomedical will be utilized for repeatable, valid data collection and quality.

In accordance the standards of practice followed by the Clinical Chemistry core, blood samples will be stored frozen at PBRC until analysis can be completed. Specific blood samples will be shipped to University of Tennessee for further analysis. Packaging and shipping of biological samples will be overseen by the PI, shipped by the laboratory and will be completed in accordance with International Air Transport Association regulations to ensure that viable biological samples reach their intended destination.

WITHDRAWAL OF SUBJECTS

We will attempt to retain program participants once randomized for study completion through the end of study visit. It is our desire to analyze results on all participants who were enrolled in the study. In accordance with the declaration of Helsinki/Tokyo/Venice/Hong Kong, participants have the right to withdraw from the program at any time for any reason. The investigator also has the right to withdraw participants from the program treatments in the event of inter-current illness, adverse experience, treatment failure, protocol violation, or other reasons. Should a participant decide to withdraw from treatment, all efforts will be made to complete and report follow-up observations as thoroughly as possible.

RISKS/BENEFITS

The study involves the following risks:

- **IV Procedures/Blood Draws:** There is the possibility of discomfort, pain, and bruising at the vein on the arm where the needle is inserted. There may also be a small risk of bleeding and a very small risk of infection at the site of the blood draw. Sterile technique and trained personnel minimize these risks.

- **Dietary Supplement**. Naringenin is a component of food and is marketed in the US as a dietary supplement. [29,30] The naringenin extract is from Gencor Lifestage Solutions, GE

Nutrients Inc. (Irvine, CA), a reputable supplier of botanical extracts and supplements. The extract from *Citrus Senensis* (sweet oranges) has been characterized using high performance liquid chromatography, (HPLC), thin layer HPLC (HPTLC), and Liquid chromatography-mass spectrometry (LC-MS). The details of the extraction process, characterization of the extract, certificate of analysis, specifications, and materials safety data sheet are provided in the Protocol Supplemental Info. Cellulose, used for the placebo, will be encapsulated in identical opaque capsules as the naringenin extract. The naringenin extract and placebo containing capsules will be prepared and dispensed by the PBRC pharmacist.

The literature is replete with the beneficial effects of naringenin in obesity, cardiovascular disease, and cancer.[31] However, grapefruit has been shown to increase the bioavailability of drugs administered orally. Several compounds in grapefruit including the flavonoids (naringin and naringenin), furanocoumarins (bergamottin) and sesquiterpens have been implicated.[32] Naringenin being polyphenolic and high in electrons, can theoretically inhibit cytochrome P 450 enzymes and enhance the bioavailability of medications such as statin drugs. Although inhibition of the cytochrome P 450 enzyme system has been demonstrated in vitro, the results of in vivo studies suggest that naringenin is not the main inhibitory compound in grapefruit.[33,34] Additionally, naringin has been shown to inhibit the enteric organic anion-transporting polypeptide in vitro, and reduce the bioavailability of an antihistamine.[35] The supplement may also adversely affect subjects with citrus allergies. However, subjects taking chronic medications or having citrus allergies are excluded from the study, and over the counter medications must be stopped 72 hours prior to the test day.

- **Meals.** There is no risk associated with the meals provided by the Pennington Metabolic kitchen. Additionally, subjects will be asked to inform staff about any food allergies or intolerances. A dietary questionnaire is routinely administered at the screening visit when the study involves a meal service. Research dietitians are responsible for managing the dietary component of specific study protocols. A continuous quality assurance program is followed to check food item weights, recipe procedures, packed meal and tray assembly, and food temperatures. Documentation is maintained for each study. All Metabolic Kitchen staff members receive training in food sanitation and in research diet preparation.
- **Anthropometric Measurements and Vital Signs.** The PBRC outpatient and inpatient clinic staff are trained to perform these procedures in accordance with PBRC standards of practice. There are no risks involved.
- **Urine Test.** The staff of the phlebotomy unit collect the urine in accordance with standard operating practices. There are no risks involved.

In addition to the risks listed above, participants may experience a previously unknown risk or side effect.

Minimizing risks

Continuous monitoring by the PI and/or the medical investigator of the study will minimize all potential risks and discomforts. Research participants will be immediately withdrawn from the study upon evidence of any significant adverse event.

Potential Benefits

There is no direct benefit to the volunteers but knowledge may be gained that will benefit others.

PAYMENT FOR PARTICIPATION

At the completion of all study visits and procedures, participants in each cohort will be paid $225 for the time spent in the clinic. The number will be broken down into two payments of $100 after visit 1 and $125 at the completion of the study. This compensation is in line with all the other studies conducted at the Pennington Biomedical Research Center.

EMERGENCY CARE AND COMPENSATION FOR RESEARCH-RELATED INJURY

No form of compensation for medical treatment is available from the Pennington Biomedical Research Center. In the event of injury or medical illness resulting from the research procedures the research volunteer (from any group) will be referred to their physician/surgeon or a treatment facility. The Pennington Biomedical Research Center is a research facility and provides medical treatment only as part of research protocols. Should a volunteer require medical treatments, community physicians and hospitals must provide them to him/her.

SHARING OF RESULTS WITH SUBJECTS

The screening lab will be shared with the subjects on their first testing visit. At the end of the study, a manuscript will be prepared for submission to a peer-reviewed journal. A summary of the results will be posted on ClinicalTrials.gov which subjects may access.

RESOURCES AVAILABLE

The outpatient and inpatient research units are well equipped and staffed to carry out the requirements of this study and appropriate standards of practice are in place to ensure appropriate research procedures.

ECONOMIC BURDEN TO SUBJECTS

There are no costs for which the subjects will be responsible.

CONSENT PROCESS

Written informed consent will be obtained in the outpatient research clinic by the coordinators and one of the physicians will be available to answer questions if needed. A waiting period will be allowed, if desired by the participant. The coordinators and investigators will be available for questions throughout the study.

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Appendix I

Foods to Avoid During the Study

Please do not eat the following foods in any form (whole fruit or juice) at least 36 hours prior to the test day

- Sweet oranges: Blood orange, kumquat, navel, cara cara, valencia
- Mandarins: Clementine, tangerine, tangelo
- Limes
- Lemons
- Grapefruit
- Pomelo
- Tomato

Please be careful to avoid tomato paste, sauce, and ketchup.