COVER PAGE

Official title: Plasma and urine profiles of blueberry derived polyphenols after a short term intervention with blueberry in children with typical low fruit and vegetable consumption

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Study Title: **The Blueberry Study**

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Background and Rationale

Polyphenols are bioactive compounds that have gained research interest due to their protective effects against non-communicable diseases (NCD) (1,2). Blueberries are a rich source of polyphenols, particularly, anthocyanins (ACNs) (3). Their consumption has been linked with improved glucose levels in individuals at risk of type 2 diabetes (4), decreased blood pressure and arterial stiffness in women with hypertension, and decreased pain and disability in children with autoimmune arthritis (5). In addition, animal and *in vitro* studies from the Arkansas Children's Nutrition Center (ACNC) have shown that 2 weeks of wild blueberry consumption improves bone health by preventing loss of bone collagen by inhibiting bone forming osteoblast cell senescence (6).

However, the mechanism by which blueberry derived polyphenols exert their protective effects cannot be explained by their bioavailability as their systemic absorption is poor (7). In the past decade, studies have shown that dietary polyphenols undergo extensive metabolism by the gut microbiota and the liver, giving origin to bioactive derivatives that potentially mediate health benefits (8). To our knowledge, no study has characterized polyphenol derivative profiles in blood and urine in children after wild blueberry supplementation.

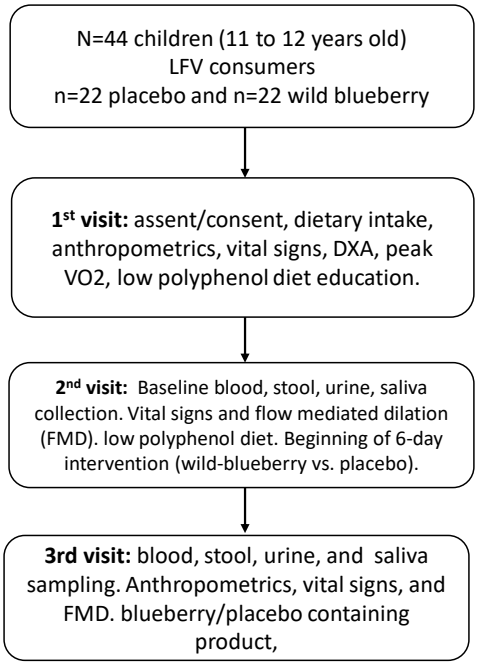
LFV is now considered among the top 10 risk factors for the development of NCDs and overall mortality (9). Alarmingly, in the USA only 40% and 7% of children meet the U.S. Department of Agriculture Food Patterns fruit and vegetable intake recommendation, respectively (10). It is not known if a polyphenol rich intervention with wild blueberry can improve markers of immune, cardiovascular and bone health in children with low fruit and vegetable consumption (LFV). Data from ACNC suggest that fitness (peak VO2) modifies the association between fruit and vegetable (FV) consumption and vascular health of children. Specifically, children with combined low FV intake and low peak VO2 have higher blood pressure values than children with low fitness but higher FV consumption. These results suggest, that children with low fitness would not only benefit from more exercise but also from a higher FV content in their diets. However, higher quality evidence is needed to inform clinical practice and dietary guidelines.

Objectives

1) Characterize plasma and urine profile of polyphenol-derived metabolites of school-age children supplemented short-term with wild blueberry powder.
2) Assess the effects of short-term wild blueberry supplementation on immune responses as well as on markers of bone metabolism and cardiovascular health in apparently healthy children who consume ≤2 cups of fruits and vegetables per day.
3) Evaluate if cardiorespiratory fitness (peak VO2) modifies the association between blueberry derived polyphenol consumption and markers of cardiovascular health.

Study Design and Procedures

Figure 1: Study design



This is a double-blind randomized control trial aiming to characterize the profile of polyphenol-derived metabolites in plasma and urine of children ages 11 to 12 years supplemented short-term with wild blueberry powder. Further, the study will assess if a short-term supplementation (up to 6 days) with wild blueberry powder impacts immune responses as well as markers of cardiovascular and bone health while assessing for the effect modification of peak VO2. Up to 44 children (boys and girls) will be recruited to participate. After telephone screening, the children and parents will come to ACNC for assenting/consenting and initial measurements. After this, children and parents will attend to 2 more study visits at ACNC (total of 3 study visits). A description of study procedures follow below. Children will be asked to stop taking any kind of nutritional supplement (e.g., multivitamins, vitamin D, fish oil, probiotics, prebiotics, immune boosters, and others) at least 2 weeks prior to the 1st study visit. Children who had an infection that required antibiotics will be scheduled after 2 months of finalizing antibiotic treatment.

Children with a viral infection such as diarrhea, cold or flu will be scheduled after 2 weeks of resolution of symptoms.

Study visit (s) and assessments: Three (3) study visits will be conducted at ACNC after an overnight fast. All assessments may be repeated if/when needed, and if the participant is willing. Following phone screening, qualifying participants will attend ACNC for further assessment of eligibility, informed consent and assent. Visit #1 will last up to 4 hours, visit #2 up to 4 hours, and visit #3 up to 8 hours. Visit #2 will take place if the participant has adhered to a low-polyphenol diet 2 days prior to the study visit. Adherence will be determined by research staff using the dietary logs provided to participants during visit #1. If participants have not been compliant, they will be given the option to re-schedule visit #2 upon completion of 2 days on a low-polyphenol content diet. The ACNC is a Well-Child facility separated from the ACH facilities with a separate entrance and is not used for clinical care which will limit exposures of the participants to illnesses. Parents will be instructed not to come to the study visit if they or their child is ill. The study visit will be rescheduled if that is the case to a time when both parent and child are well and asymptomatic.

Study measurements and procedures

Dietary Intervention

Low polyphenol Study Diet: Participants will be asked to adhere to a low polyphenol diet (i.e., less than ≤0.5cups of fruit and ≤0.5 cups of vegetables per day, and avoiding cocoa products, nuts, sweet potato, whole grains, caffeinated drinks, tea, and others). All participants will be instructed to follow a low polyphenol diet (a sample meal plan will be provided). Participants will start the low polyphenol diet 2 days prior to visit #2 up until the day prior to visit #3 (total of 7 consecutive days on a low polyphenol diet).

Table 1: Summary of study procedures

Procedure/measurement	Visit 1	Visit 2	Visit 3
Low polyphenol diet		x	x
Wild blueberry/placebo		x	x
Medical History	x		
Anthropometrics	x		x
DXA	x		
Vital Signs	x	x	x
Peak VO2	x		
Dietary Intake	x	x	x
Study diet coaching	x	x	
Stool, urine, and saliva collection		x	x
Endothelial function		x	x
Blood draw		x	x

Participants will still be eligible if they consume less than or equal to two (2) cups of fruits and vegetables combined per day before Visit #2. Visit 3 will continue even if the participant did not adhere to the low polyphenol diet.

Wild blueberry/placebo intervention: wild blueberry containing foods (i.e., wild blueberry powder or wild blueberry extract) or identical placebo-containing foods will be consumed starting on the day of study visit #2 until the morning of visit #3 (total of 6 days on blueberry or placebo). These food items have been designed in collaboration with the University of Arkansas Department of Food Science and may consist of oatmeal bars, beverages, ice pops, and cookies. Participants will receive product combinations that deliver up to 30g of wild blueberry powder per day (or the equivalent anthocyanin content in the case of products containing wild blueberry extract) or placebo. Parents and participants will keep a daily log which will be used to assess compliance with blueberry/placebo containing products and low polyphenol diet. Research staff may contact study participants between visits via phone, email, or text to inquire about their experience, perceived barriers with adhering to the intervention and to their goals. Participants will be asked to complete the Study Product Log book of what was consumed.

Study Diet Coaching: Participants will be educated by a trained nutritionist on how to avoid polyphenol rich foods. A sample low polyphenol meal plan will be provided to the participants if needed.

Medical History: an interviewer-administered medical history questionnaire will be conducted.

Anthropometrics: Anthropometric measurements (body weight, height, waist and hip circumference) will be obtained using standardized techniques, while the children are wearing light clothing and without shoes. All anthropometric measures will be made in duplicate, or triplicate if not within acceptable agreement.

Body Composition: Body composition will be assessed using Dual-Energy X-ray Absorptiometry (DXA; Horizon-A with Advanced Body Composition™, Hologic, Bedford, MA). Measures of body composition are needed to standardize peak VO2 (see below) to lean body mass content. DXA technology involves very small amounts of ionizing radiation. Total body fat and fat free masses, bone mineral density, bone mineral content (whole body, femur,

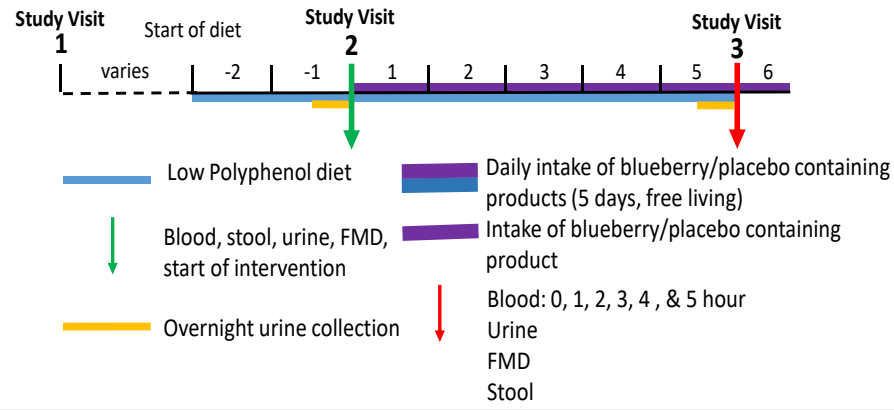
and spine) will be determined. This will use the newest DXA technology (Analysis with InnerCore™ Visceral Fat Assessment) which can separate the adipose tissue depot into visceral and subcutaneous fat mass by performing a scan of <3 min.

Vital signs: Blood pressure, heart rate, and body temperature will be assessed using standard techniques at each visit.

Fitness test (peak VO2): A graded exercise test will be performed to determine peak oxygen uptake (peak VO2). A bicycle fitness test will be conducted. During this test, the resistance of the stationary bicycle is gradually increased. The children will be told that they are free to stop the test at any time if they do not want to continue, but will be encouraged to perform maximally. During the test, the children will be required to wear an indirect calorimetry face mask in order to assess their oxygen uptake (VO2), carbon dioxide production (VCO2), and respiratory exchange ratio (RER; VCO2/VO2). Heart rate monitors will be worn to measure heart rate during the exercise test. Ample time will be given for familiarization with the stationary bicycle, face mask, and heart rate monitor. Children will be educated in how to assess their exertion levels, and will be asked to provide a rating of self-perceived exertion every minute during the test using the children's OMNI scale. This is a numerical scale from 0 to 10, with a score of 2 indicating "a little tired" and a score of 9 indicating "very, very tired," with associated pictures to represent perceived physical effort. Peak VO2 will be determined by the combined assessment of: heart rate of ≥185beats per min, and/or respiratory exchange ratio ≥ 1.0; and/or ratings of perceived exertion on the children's OMNI scale ≥ 8. The bike test will be conducted primarily by an ACSM registered clinical exercise physiologist (with M.Sc. in exercise physiology) with expertise in these procedures. Other research staff that have been trained in these measurements and certified by Dr. Elisabet Borsheim (Director of the Physical Activity Core at ACNC) may also conduct these measurements.

Dietary intake: A food frequency questionnaire will be completed at visit 1. Parents (or caregivers) will be asked to keep a log of what their child eats while on a low polyphenol diet (7 days). Instructions will be provided to parents verbally, and/or via mail, and/or e-mail. Logs will be reviewed to assess compliance with the study diet before Visits 2 and 3.

Figure 2: schematic overview of study design



Stool, urine, and saliva sample collection: Stool samples will be collected and may be used to analyze the microbiome, polyphenol content, and polyphenol derived metabolites (table 1). Parents will be given the option to collect the child's stool sample at home. Instructions and supplies will be provided. Urine will be collected to measure wild-blueberry derived metabolites, markers of bone metabolism, and overall metabolic health. A urine sample will be collected the morning of visit #2. Urine will be collected before and following a single dose of blueberry or placebo containing foods on study visit #3. Saliva will be collected on visits #2 and #3 for metabolomics analysis. Supplies and instructions will be provided to participants.

Blood Collection After an overnight fasting, blood will be collected from a peripheral vein by a trained phlebotomist at visits #2 and #3. Up to 75 ml of blood will be collected during the entirety of the study. At visit #2, up to 16 ml (3.25 teaspoons) of whole blood will be collected following an overnight fasting. At visit #3, up to 24 ml (~1.6 tablespoons) of blood will be collected in the fasting state (baseline). After the initial blood draw, children will receive blueberry/placebo containing products. Participants will be free to drink water during this part. Eight ml (8 ml) of blood will be drawn 1 hour after the consumption of the study product while 6 ml will be drawn at 2, 3, 4, and 5 hours [Total 56 ml (24+ 32 mL) or 3.75 tablespoons on visit #3]. We may place a saline lock and administer a slow drip of saline

or lactate ringers at the first blood draw of visit #3 to minimize further needle sticks. If an IV catheter cannot be placed, and the participant agrees, standard venipuncture will be done to collect blood at baseline (fasting), and after 1- and 5 hours after eating the study product. If blood draws need to be repeated, the total amount of blood drawn for this study will not exceed 75 ml.

Plasma/serum may be used to measure levels of polyphenols, polyphenol-derived metabolites, and markers of cardiovascular health (e.g., lipids, glucose, etc.). Whole blood, plasma or serum from this study may be used in *in vitro* experiments to assess the immune response to blueberry compounds (e.g. blueberry phenolics and blueberry volatiles), or the response of cells that have been either purchased (e.g., mouse or human monocyte macrophages) or collected in other research studies (e.g., human derived macrophages) where immune responses in relation to nutrition in health and disease are an outcome of interest. The profile of innate and adaptive immune cells in children participating in this study enriched products will be measured with flow cytometry. In addition, immune stress tests in whole blood will be done to assess immune cell responsiveness to immune stressors. Markers of oxidative stress (e.g., plasma protein carbonyls, malondialdehyde, 8-Hydroxy-2'-Deoxyguanosine, and glutathione status, etc.), inflammation (C-reactive protein, erythrocyte sedimentation rate, and pro-inflammatory cytokines, etc.), platelet mitochondria function platelet activation/reactivity as well as metabolomics analyses (to characterize the metabolite signature associated with a dietary pattern rich in wild blueberries) may be conducted. Sexual hormones will be measured to complement assessment of pubertal status. We may conduct gene expression analyses on circulating immune cells and blood osteoblasts. We have previously shown that osteoblast senescence is delayed in mice supplemented with wild blueberries (11). Single cell RNA sequencing and/or gene expression tests such as GPR109A, osteocalcin, alkalin phosphoatase, NFkB, beta-catenin, among other. The proposed genetic analyses will not be used to diagnose disease but will be used as markers of bone, immune, and metabolic health.

Endothelial function: Endothelial Function will be measured on visits #2 and #3 using ultrasound (GE Vivid 7 Ultrasound system with 5.0-13.0 mHz linear transducer, GE Healthcare, Chicago, IL, USA) to assess brachial artery flow-mediated dilation (FMD). This

technology uses sound waves (ultrasound) to visualize the diameter of the brachial artery. A baseline image of the brachial artery will be recorded at rest. Three ECG electrodes will be placed on the torso of the subject, such that ultrasound measurements will be captured at identical times during the cardiac cycle (i.e. ECG-gating of ultrasound images). A blood pressure cuff will be placed around the lower arm (the opposite arm that was used for measuring blood pressure) and the cuff will be inflated to 50 mmHg above systolic pressure for 5 minutes, followed by rapid deflation of the cuff. The diameter of the brachial artery will be measured using edge detection software (Vascular Tools, Medical Imaging Applications, IA, USA) from ultrasound images captured during the R wave of the cardiac cycle (via ECG-gating) throughout the 8-minute recording protocol. The advantage of using ultrasound for FMD measurements is that this is a painless imaging test that uses non-invasive sound waves for imaging. There may be some discomfort from the blood pressure cuff since it will be applied tightly on the forearm. If the measurement is uncomfortable for the participant, and he/she requests to stop, then the procedure will be immediately aborted. FMD measurements will be done primarily by a registered clinical exercise physiologist with expertise in this procedure. However, other research staff trained and certified by Dr. Keshari Thakali (Director of the vascular physiology laboratory at ACNC) may conduct this measurement. FMD will be measured in the fasted state in visits #2 and #3 and 1-2 hours after consuming a study product (visit #3 only).

Questionnaires: A food frequency questionnaire, a medical history questionnaire, selected questions form the National Survey of Children's Health, and a self-assessment questionnaire of pubertal status will be done at enrollment (12). Participants will have privacy to answer the pubertal status self-assessment questionnaire. Assessment of pubertal status is needed as measurements of bone metabolism are part of the present study. Puberty is known to dramatically affect bone turnover and metabolism (13). Physical examination for puberty staging is unacceptable outside the clinical setting thus self-assessment questionnaires have become the tool of choice in research studies.

Study Population

Recruitment of up to 44 children ages 11 to 12 years will be conducted by the research staff using IRB approved advertisements. Various advertisements will be distributed by direct

mail or to various locations, including but not limited to healthcare offices, health fairs, daycare centers, schools/universities, recreational centers, child retail stores, websites (ACH, ACHRI, ACNC, UAMS, and others as applicable) and churches. Also, print or digital ads may appear in newspapers, magazines, social media and circulars. On-hold phone messages, BoomText messages, screensavers, and radio/television advertisements may be used. In addition, ACNC research staff may contact parents who expressed an interest in our studies or who previously agreed to be contacted regarding future studies at ACNC. The ACNC research staff will educate the parents and children about the study. We will protect all personal information obtained during recruitment, enrollment, and testing processes.

Inclusion Criteria

- Ages 11 to 12 years
- Eats ≤ 2.0 cup of fruits and ≤ 2.0 cup of vegetables per day.
- Boys or girls
- All ethnicities
- All BMIs

Exclusion Criteria

- Known allergy to blueberries
- Attention deficit disorder (ADD) or attention deficit hyperactivity disorder (ADHD)
- Oppositional Defiant Disorder
- Conduct disorder
- Autistic spectrum disorder
- Asthma
- Diabetes mellitus (Type 2 and type 1)
- neurological disease (e.g. epilepsy or seizures
- lung diseases
- liver disease (e.g. hepatitis)
- kidney disease
- Hormonal disease (e.g., hypothyroidism and growth hormone deficiency).
- Autoimmune diseases (e.g., lupus, thyroiditis, juvenile idiopathic arthritis)
- Bleeding disorders (e.g., hemophilia)

- Cancer (e.g. leukemia)
- Chronic infections (e.g., HIV, hepatitis B, hepatitis C).
- Pre-existing medical conditions or medications as determined by the investigators to affect the outcomes of interest.
- If the participants/parents/invesitgators perceive barriers with adhering to low-polyphenol diet. (e.g., on vegetarian diet)
- Parent/child refusal to stop nutritional supplements (e.g., multivitamins, vitamin D, fish oil, probiotics, prebiotics, immune boosters, and others) 2 weeks prior to study visit #1.
- Parent/child refusal to have blood drawn.
- Parent / child unwillingness to consume the study product offered by investigators on study visit 3.

Note: Children who had an infection that required antibiotics will be scheduled (visit #1) after 2 months of finalizing antibiotic treatment. Children with a viral infection such as diarrhea, cold or flu will be scheduled (visit #1) after 2 weeks of resolution of symptoms. Children will be screened (phone screening) for usage of antibiotics and occurrence of infections and study visits scheduled as described above. This step is necessary as immune responses and microbiome analyses are part of the studied outcomes and infections and antibiotic intake may affect these measurements

Risks and Benefits

We do not foresee any potential major adverse events associated with this protocol. Most procedures are minimal risk. Risk to confidentiality of personal information will be minimized by applying the appropriate steps to secure the collected data. Dual-Energy X-ray Absorptiometry (DXA) scanners use low-dose X-rays during the measurement, which may concern some participants/participants' parents; however, the ionizing radiation exposure during a DXA scan is among the lowest found in medical procedures of this kind (about one tenth of a clinical chest X-ray), and is equivalent to less than two days of natural background radiation. There is a small risk that the participants may have an unknown allergy to the

blueberry / placebo enriched products. These food items (blueberry enriched only) were used in a sensory testing study involving 7 to 10 year old children, and no complications were reported (14). During the fitness test, participants may experience discomfort associated with exercise or the mask that is worn which is normal. If the participant does not tolerate the mask and requests to stop the test, the study visit will be terminated and the participant will not be allowed to continue in the study. There is a minor risk that participants will encounter bruising and/or infection after having blood taken, however, the use of well-established blood draw techniques, and antiseptic procedures, by trained phlebotomists, will ensure minimal risk. Participants may also experience sickness or dizziness associated with blood draw and fasted state. However, this and any other procedures can be aborted at any time at the request of the participant. Genetic testing may present a unique risk to human research subjects. These involve medical, psychosocial and loss of confidentiality. To minimize these risks, participate names will be removed from the samples, and kept in secure and badge access only laboratories. In addition, the proposed tests are for research purposes only, that is, they will not be used as diagnostic tests for any disease or illness and will not be placed in medical records or shared with treating physicians.

In the event of an adverse event or unanticipated problems, this will be reported to the study PIs, the IRB, and the study sponsor in accordance with IRB Policy 10.2.

There will be no direct benefits to the study participants; however, knowledge gained from the study could potentially benefit patients in the future.

Data Handling and Recordkeeping

All investigators and study staff will complete and maintain appropriate CITI training. All data and communications will be recorded in either electronic or paper copy standardized case report forms. Survey and questionnaire data will be entered directly into REDCap or Remark Web Survey Professional on study iPads when possible by participants, with paper versions of all forms to be used as back-up in the instance that iPads are unavailable. Remark Web Survey Professional will be used with participants ID only and therefore no identifiers will be available on the Remark Web Survey Professional. All data

and communications will be recorded in standardized case report forms or directly in the ACNC clinical relational database (MS Access, Redmond, CA). Data will be collected and managed using the ACNC clinical relational database (MS Access, Redmond, CA). The data integrity will be password protected. Data analyses will be performed from queries which will not change and/or modify the original data from the tables. This database will be housed on a shared drive backed up nightly off-site Monday – Friday. Access to our server is password protected, as is access to the study database(s). Only faculty and staff who have had appropriate CITI training have access to study database(s) with participant identifiers.

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. All study subject material will be assigned a unique identifying code or number (e.g. participant ID). The key to the code will be kept in a shared network folder with access granted only to study staff who have had appropriate CITI training. Only study investigators/staff will have access to the code and information that identifies the subject in this study. At the conclusion of the study, the data will be stored in the database and/or secure server.

Specimen Handling and Storage

During the consent process, parents will be given the option to allow banking of specimens obtained from their child over the course of the study. Participants' names will be removed from specimens. Specimens will be stored indefinitely for potential use in future research studies that are within the scope of the current one. Urine, stool, and blood samples will be stored in freezers in locked laboratories at Arkansas Children's Nutrition Center and Arkansas Children's Research Institute. Participants may choose to withdraw permission for banking at any time they decide. A letter or e-mail addressed to the principal investigators expressing their desire to withdraw their consent would suffice. Consent for banking will be separate from consent to participate in the current study. Choosing not to have their specimens stored for future analyses will not affect their eligibility for this study. Participants may call the investigators or research staff to obtain more information if needed.

Data Analysis

Statistical analysis: Based on means and standard deviations observed in previous similar studies, our primary outcome will have in excess of 80% power to detect statistical significance among the study groups testing at the 5% level of significance. This calculation assumes that we have at least n=9 subjects in each of our four combinations of gender and blueberry status. This being the case, we propose to recruit n=11 subjects in each combination of gender and blueberry status (n=44 total, 22 per study arm) to compensate for a reasonable level of dropout that may occur. Power and sample size calculations were performed by SAS 9.4 Proc Power. David Williams, PhD, ACNC statistician will be in charged randomization process. Prior to analyses, data will be checked for potential outliers and aberrant measurements. Additionally, the data will be also checked for conformity with distributional assumptions of the proposed statistical models. Analysis of variance (ANOVA) or its non-parametric variants will be used to assess differences among gender and blueberry diet combinations. Significance will be declared at the 5 percent level of significance. To learn whether peak VO2 indeed modifies the effect of WBB supplementation, the potential modifier will be added into the ANOVA thus making an analysis of covariance (ANCOVA). The slope of the modifier will be allowed to depend on WBB status; that is, there will be a slope for the placebo group and one for the WBB group.

Reimbursement

The compensation per visit are $50 for visit #1, $50 for visit #2, and $175 for visit #3 (Total $275). If the participant decides not to complete the study, is unwilling, or unable to complete an assessment, a $25 gift card or check will be provided.

In the event that blood draws or other measurements need to be repeated and re-scheduled due to technical problems (but not participant refusal to blood draw), an additional $50 gift card or check will be provided.

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. Formal consent and assent,

using the IRB-approved consent/assent form, will be obtained before the subject is submitted to any study procedure. Parents may also be contacted before study visit (s) to confirm appointments and ensure eligibility. All parents and children for this study will be provided with a consent/assent form describing this study and providing sufficient information in language suitable for them to make an informed decision about their participation in this study. A copy of the consent/assent may be mailed or e-mailed to them for information after successful telephone screening. Upon arrival to the ACNC, the consent/assent process will take place in a quiet and private room, and children and parents 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 or assent process. Potential participants will be asked if their information can be saved for consideration for future studies and their responses will be documented. The person obtaining consent and assent will outline the risks, benefits, and requirements of the study as well as review all study procedures and the informed consent/assent documents with the child and parent. If eligible and interested in enrollment, informed consent will be obtained from the parent and documented, and assent will be obtained from the child and documented. A copy of the signed consent and assent will be given to the participant, and the informed consent process will be documented in each subject's research record.

Dissemination of Data

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 participant. Parent may also receive a report containing some of his/her child's results. The study will be listed on clinicaltrials.gov in accordance with (journal or FDA) requirements.

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