Green tea confections to assess catechin pharmacokinetics and restore gut health in obese adults

I. Objectives

The *objective of this application* is to evaluate green tea catechin pharmacokinetic parameters and gut barrier function and gut microbiota composition in obese adults following chronic ingestion of a green tea extract (GTE)-rich confection. Our *central hypothesis* is that obese adults will have altered catechin pharmacokinetics compared to healthy adults and that chronic ingestion of a GTE-rich confection in obese adults will attenuate metabolic endotoxemia by limiting gut-derived endotoxin translocation and modulating gut microbiota composition. Metabolic endotoxemia is a condition characterized by elevated endotoxin in the blood, but at levels 10-50 times lower than during sepsis (1). To test this, we will complete the following objectives: 1) define alterations in catechin pharmacokinetics by health status (obese vs healthy adults), 2) evaluate gastrointestinal-level suppression of metabolic endotoxemia by GTE catechins, and 3) define changes in gut microbiota composition with GTE in relation to improvements in metabolic endotoxemia. Upon completing this study, we expect to provide translational evidence for the efficacy of a novel GTE-rich confection to attenuate metabolic endotoxemia and alter gut microbiota composition in obese adults. In addition, this study will provide preliminary evidence for future controlled trials investigating the beneficial impact of GTE in cohorts with underlying obesity, and other obesity-related metabolic disorders characterized by inflammation.

II. Background and Rationale

Obesity and type 2 diabetes are associated with nonalcoholic steatohepatitis. Nonalcoholic fatty liver disease (NAFLD) is the most prevalent liver disease in the U.S., affecting over 70 million American adults (2). NAFLD is the broad term used to describe a spectrum of progressive chronic liver conditions, which includes liver steatosis, nonalcoholic steatohepatitis (NASH) and cirrhosis. NASH is characterized by hepatic steatosis, hepatocyte ballooning, and inflammation (3). In the U.S., approximately 10-18 million Americans are diagnosed with NASH (4, 5). However, these statistics are likely underestimating the true prevalence since accurate diagnosis of the condition is complicated due to its asymptomatic presentation and requirement of a liver biopsy (5, 6). Further exacerbating this epidemic is that there are currently no Food and Drug Administration-approved treatments for NASH, other than weight management strategies. Major risk factors associated with NASH are obesity and type 2 diabetes (2, 7-9). Evidence suggests that between 25-94% (10-12) and 55-70% (13, 14) of obese and type 2 diabetics, respectively, have NASH. Therefore, intervening during obesity, in a prophylactic or preventative manner has the potential to mitigate the long-risk of developing NASH downstream of obesity.

Green tea rich confections are a novel catechin delivery system. Green tea is safe when consumed as recommended (15). Epidemiological evidence suggests that approximately 10 servings/d of green tea (equivalent to 1 g/d of GTE) lowers the risk of liver injury and inflammation (16). Freshly brewed green tea is an effective catechin delivery system, and has shown to deliver maximum plasma concentrations of catechins (0.19-0.73 μM) around 1.5-2.5 hours post-ingestion with levels rapidly declining after 3 hours (17, 18). To maintain a sustained plasma catechin level, it would require frequent ingestion of green tea, which is neither convenient nor practical for individuals. Previously, our lab, in collaboration with the Department of Food Science (Dr. Yael Vodovotz), developed a starch-based green tea extract (GTE)-rich confection that effectively delivered 1 g of decaffeinated GTE to adults (19). After ingestion of the GTE-rich confections, maximal plasma catechin concentrations (0.45-1.68 μM) were reached about 2.5 hours post-ingestion. However, there was no apparent decline in plasma catechin levels during the 3-hour post-ingestion period (19). Chronic exposure of green tea catechins may lead to intestinal adaptations that enhances catechin absorption

and metabolism (20). Not only are GTE-rich confections able to adequately deliver catechins, but are more appropriate for frequent ingestion and are palatable, and more acceptable compared with brewed green tea that tends to be perceived by consumers as astringent or bitter (19, 21). For this application, our previously developed GTE-rich confection will be modified to be low-carbohydrate and low-calorie compared to our prior formulation that contained starch (50 g) (19).

Catechins undergo extensive biotransformation with varying bioavailability. Green tea is a dietary rich source of catechins, comprising up to 30% of the dry weight (22). The four major catechins found in green tea are epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC) (23, 24). Catechin pharmacokinetics in humans have been thoroughly studied (17, 18, 25-30). Maximum plasma catechin concentrations range from 0.07-1.8 μM at approximately 1-3 hours post-ingestion with half-lives of about 2.5-5.7 hours (17, 30). Additionally, catechins undergo extensive biotransformation, mainly through glucuronidation, sulfation, methylation, and ring-fission pathways in the liver and small and large intestines (31-35). EGCG appears primarily in the free form (unconjugated) in the blood (18, 26), while more than 80% of the catechins are in conjugated forms (25). Suggesting that the conjugated forms are more bioavailable than their parent compounds. Furthermore, unabsorbed catechins are metabolized by gut microbiota to ring fission products (e.g. valerolactones) and phenolic acids (e.g. hydroxybenzoic acid) that appear in the blood and urine of humans (32, 34, 36). Although these microbial metabolites are mostly prevalent in urine (34), maximal plasma concentrations have been found in levels greater than 0.2 μM (37). The bioactivity of conjugated and catechin-derived microbial metabolites have been limitedly study, but evidence suggests potential antiinflammatory properties (33, 38, 39). Although the metabolism and pharmacokinetics of green tea catechins are well studied, most of these studies have been conducted in healthy adults. The extent to which catechin pharmacokinetics and metabolism are influenced by obesity and altered by chronic green tea ingestion remains unknown and will be addressed in this application.

Green tea attenuates metabolic endotoxemia and improves gut barrier function. Obesity-mediated NASH is implicated through a mechanism involving the gut-liver axis. A key mechanism involves binding of the ligand endotoxin to Toll-like receptor 4 (TLR4), which in turn induces pro-inflammatory signaling leading to NFκB activation and hepatic inflammation. The gut microbiota is the major source of endotoxin, where it is found in the outer membrane of Gram-negative bacteria (40). Translocation of gut-derived endotoxin to the liver is primarily due to increased intestinal permeability, mediated in part by alterations in gut microbiota composition (41, 42). Our preclinical studies show that GTE treatment attenuates hepatic NFκB activation and lowers systemic and portal vein endotoxin, otherwise increased in obese mice with NASH (43). Additionally, we show that GTE improves gut barrier function by restoring expression of small intestinal tight junction proteins (occludin, zonal occuldin-1, claudin) to similar levels of healthy controls (43). Studies in humans support our findings, showing obese and NASH patients have increased endotoxin and gut permeability compared to healthy individuals (44-46). Thus, rationale exists to examine GTE ingestion as a dietary strategy to mitigate metabolic endotoxemia by restoring intestinal barrier function.

Green tea alters gut microbiota composition. Emerging evidence has highlighted the important role of the gut microbiota in the development of NASH (47, 48). Green tea catechins and the host microbiota exhibit a reciprocal relationship, where catechins are metabolized to more bioavailable metabolites by the microbiota and those catechins also alter the microbiota composition (49). Studies in mice showed that a green tea infusion and fermented green tea mitigate obesity and NASH symptoms (50, 51). Both studies showed distinct alterations in microbiota composition in the obese mice, specifically increases in the *Firmicutes/Bacteroidetes* ratio, typically associated with obesity

and type 2 diabetes. However, only the fermented green tea restored the *Firmicutes/Bacteroidetes* and *Bacteroidetes/Prevotella* ratios to that of control mice. Green tea catechins modulate the gut microbiota possibly through prebiotic (52-54) and/or antimicrobial (55-57) mechanisms. *In vivo* and *in vitro* studies have shown the beneficial impact of green tea in inhibiting and reducing pathogenic bacteria populations (e.g. *Clostridium perfringens*, *E. coli*) and promoting beneficial/commensal bacteria (e.g. *Bifidobacterium* spp., *Lactobacillus* sp.) growth (55, 56). These data support the health-promoting effects of green tea on modulating gut microbiota composition.

In ***conclusion***, evidence shows that 1) obesity and type 2 diabetes are associated with NASH, 2) green tea-rich confections are an effective catechin delivery system, 3) catechins are considerably biotransformed, and 4) green tea is effective in attenuating metabolic endotoxemia, improving gut barrier function, and modulating gut microbiota composition. Our team has the expertise and experience to safely conduct pharmacokinetic trials and perform controlled interventions to assess metabolic parameters, gut barrier permeability, and gut microbiota composition. Thus, conducting this innovative study will further advance the scientific knowledge of green tea in humans and help to establish timely dietary recommendations that reduce inflammation in association with obesity.

Anticipated Results

Our clinical studies showed that a novel GTE-rich confection was an effective delivery system for catechins (19). We expect the GTE-rich confections to retain its effectiveness, even after modifying its composition to create a low carbohydrate and low calorie snack food product. Pharmacokinetic studies of green tea catechins have been extensively studied in healthy individuals. However, the extent to which catechin pharmacokinetics are altered in obese individuals has not been reported. We anticipate that obese individuals will have impaired catechin pharmacokinetic parameters (e.g. bioavailability, half-life) compared with healthy adults. Furthermore, prior studies in our lab show that GTE ameliorates hepatic inflammation in obese mice with NASH by improving gut barrier function and limiting gut-derived endotoxin translocation (43, 58). Based on these preclinical findings, we expect that obese individuals will have increased endotoxin levels and gut permeability compared to healthy controls and that chronic GTE ingestion will mitigate these parameters. Finally, evidence in animals (59, 60) and humans (61, 62) suggest a reduced microbial diversity and increased *Firmicutes/Bacteroidetes* ratio is associated with an obese phenotype. In addition, obese and NASH patients showed increased abundance of Gram-negative bacteria and higher levels of endotoxin compared to healthy individuals (63). Therefore, we expect that obese individuals will exhibit a less diverse microbiota composition, greater abundance of *Firmicutes* compared to *Bacteroidetes*, and increased abundance of Gram-negative bacteria and chronic GTE consumption will modulate these effects.

Pitfalls and Alternatives

Our lab has successfully conducted previous controlled intervention studies, and the techniques proposed for this study have been well established in our laboratory. Therefore, we do not expect to experience any technical problems or difficulties in this application.

Significance of the Research and Potential Benefits

NAFLD is the most prevalent liver disease in the U.S. affecting 80-100 million Americans (64), which is largely associated with obesity and type 2 diabetes (6, 10-14, 65). Although several mechanisms are involved in the development of NASH from NAFLD, metabolic endotoxemia due to increased gut barrier permeability and alterations in gut microbiota are highly implicated. Findings of this application are expected to provide the first evidence using a novel GTE-rich confection (i.e. snack food) to assess catechin pharmacokinetics in association with improvements in metabolic endotoxemia, gut permeability, and gut microbiota composition to improve gut health, otherwise

impaired in obese individuals. Thus, GTE-mediated improvements in obese subjects are expected to provide support for future translational studies in NASH patients.

III. Procedures

A. Research Design

We will conduct a randomized, double-blind placebo controlled trial in obese and healthy individuals. We will enroll overweight/obese (BMI = 28-40 kg/m^2) and age- and gender-matched healthy (BMI 19-24 kg/m^2) men and women (18-50 y; n = 20/group). Participants will be block randomized to receive GTE-rich (1 g GTE) or placebo confections and will be asked to consume the confections daily for 4 wk. Prior to (0 wk) and following 4-wk intervention, we will collect fasting blood, 24-hour urine, and 3-d fecal samples. Additionally, at the beginning (0 wk), participants will perform a sugar probe test to assess gut permeability and a 12-h pharmacokinetics trial to assess green tea catechin absorption, metabolism, and elimination. At the end of the intervention (4 wk), participants will perform a second sugar probe test to assess gastrointestinal changes associated with chronic GTE supplementation. Blood collected at wk 0, 2, and 4 will be used to assess blood chemistries (i.e. liver function, glucose, insulin, insulin resistance, markers of inflammation). In addition, blood pressure, anthropometrics, and 3-d diet records will be measured and collected.

GTE-rich Confections. The Bruno and Vodovotz labs have previously developed a starch-based GTE-rich confection (19) as a successful catechin delivery system. For this application, we will modify this confection to produce a GTE-rich confection that is low calorie and carbohydrate while delivering 1 g of *decaffeinated* GTE. Decaffeinated GTE will be used to minimize potential side effects such as excess caffeine consumption and caffeine/drug interactions that may occur. One confection will provide about 18 calories. Thus, 6 confections will provide about 110 calories per day, while providing 1 g of decaffeinated GTE. The catechin profile 82% catechins (w/w); 63% EGCG, 18% EGC, 10% ECG, 9% EC as we verified (19) is similar to that of freshly brewed green teas. All ingredients used will be food grade, meaning they are food certified safe for human consumption. The formulation consists of water, sucrose, gelatin, citric acid, and lime flavoring for treatment blinding. Sucrose is the main carbohydrate source because sugar alcohols alter microbiota composition (66). Placebo confections will be made identical to GTE-rich confections, but without GTE. Confections will have a similar sweet taste, soft texture, and consistency as commercially available lime-flavored Jell-O®. To deliver 1 g GTE, 6 confections (~1 cm^3) will be consumed each day throughout the intervention; 2 with each meal. Confections will be packaged into single-serve, oxygen impermeable containers, and coded for study blinding. Participants will be instructed to refrigerate the confections to maintain freshness and quality.

B. Sample

Enrollment Criteria. Overweight/obese men and women are the ideal study population because of its close association with NASH (6, 10-14, 65). Obese/overweight participants will be required to meet these ***inclusion criteria***: a) overweight/obese (BMI = 28-40 kg/m^2), b) 18-50 y of age, c) fasting glucose < 126 mg/dL, d) normotensive (blood pressure < 140/90 mmHg), e) non-dietary supplement user, f) nonsmoker. We will also enroll age- and gender-matched healthy adults who meet the following criteria: a) normal weight (BMI 19-24 kg/m^2), b) 18-50 y of age, c) normoglycemic (< 100 mg/dL), d) normolipidemic (total cholesterol < 240 mg/dL and triglyceride < 150 mg/dL), e) normotensive (blood pressure < 140/90 mmHg). Those having any of these ***exclusion criteria*** will not be enrolled: a) regular tea drinkers (*Camellia sinensis* or herbal varieties; ≥ 2 cups/wk), b) vegetarians, c) use of any medications to manage diabetes, hypertension, or hyperlipidemia (e.g. statins, metformin, ACE inhibitors), d) use of any medications known to be contraindicated for use with green tea ingestion (e.g. antipsychotic medications [Clozapine, lithium, Diazepam]), blood

thinning medications [Warfarin]), e) user of dietary supplements, prebiotics or probiotics, f) recent use of antibiotics or antiinflammatory agents, g) women who are pregnant or lactating, or have initiated or changed birth control in the past 3-months, h) individuals with any gastrointestinal disorders or surgeries, i) hemochromatosis, j) alcohol intake > 3 drinks/day, k) any history of cancer.

Recruitment. We will recruit participants through posted flyers, e-mail, electronic and newsprint advertisements (e.g. campus student and faculty/staff newspapers, local and regional newspapers), word of mouth, and through use of ResearchMatch (OSU CCTS). The posted advertisements will instruct interested participants to call the study center (Bruno Laboratory, Department of Human Sciences) to obtain further information. During the phone-in hours, a trained individual (i.e. project coordinator or graduate assistant) will be available to describe the study and determine preliminary qualification by conducting a scripted phone interview (e.g. do you take dietary supplements?, do you smoke?; see *Phone Script* attachment). The individual will record answers and assess whether or not the person calling is likely or not to be an acceptable study participant. If the caller and the interviewer agree that the caller should participate, the prospective participant will be invited to a screening meeting, where the study will be fully described and the individual will be provided a consent form to complete prior to any involvement in the study procedures. Data collected during this phase will include participant's age, health status, physical activity, and contact information (see *Informed Consent* attachment). Recruiting efforts through ResearchMatch will utilize a strategy whereby registered individuals in the ResearchMatch database can be searched for against their non-identifiable volunteer profile in the system. Unidentified individuals meeting search criteria will then be forwarded an electronic recruitment message (see *ResearchMatch Recruitment Message* attachment) that identifies them as a potential match for study participation. The secure ResearchMatch clearinghouse will route this standard notification that provides specific study content (i.e. content similar to that of a posted advertisement) to each of these potential ResearchMatch volunteers who will then have the option of replying "yes", "no", or not respond through a set of quick links available in this notification. ***Note:*** This message will not include the study's direct contact information (e.g. email, phone) as ResearchMatch will measure the response rate through the clearinghouse's quick links made available in this electronic message. The response rate metrics will be made available to researchers through their ResearchMatch dashboard as well as the Institutional Liaison dashboards. By responding "yes", the volunteer has authorized ResearchMatch to release their contact information to the researcher responsible for the study. This information will be made available on the researcher's ResearchMatch study dashboard. The researcher will be responsible for managing this contact information as specified in the IRB-approved study protocol. ResearchMatch will also be collecting aggregate data regarding the status of ResearchMatch volunteers within the study. ResearchMatch volunteers consent to this within the ResearchMatch volunteer agreement. The ResearchMatch enrollment continuum will allow researchers to indicate where the volunteer currently stands within the recruitment process and thus helps researchers monitor the utility and effectiveness of this recruiting tool. Research access to recruit through ResearchMatch will last only as long as the duration of IRB-study approval with the expiration date of ResearchMatch being identical to the end-date of OSU IRB approval. Researchers will be able to submit current IRB-approval letters for the lifetime of the study and thus provide evidence of successful continuing review applications. If an unintentional lapse in time occurs and the research is not able to submit this continuing review evidence via ResearchMatch, stored ResearchMatch data will not be deleted, but the researcher will not have access to this information until a current IRB-approval letter is uploaded and routed to the Institutional Liaison for review. A complete description of ResearchMatch along with the most current IRB approval from Vanderbilt University (i.e. this is the site where ResearchMatch was developed and its secure computer servers are housed) has been attached.

Power Calculation and Data Analysis. A power calculation was performed using serum endotoxin (lipopolysaccharide; LPS) as the primary outcome. Studies in obese (44, 67, 68) adults and individuals with metabolic syndrome (69, 70) show increased serum LPS or LPS-binding protein compared to healthy controls. The known difference in metabolic endotoxemia between obese and healthy adults was used to predict an expected 50% improvement by GTE treatment. Based on a linear model with treatment and health status, we will have >90% power to detect the primary contrast of interest (GTE vs. placebo in obese individuals) and additionally 80% power to detect an interaction between treatment and health status (α=0.05). Thus, we plan to recruit 20 obese and 20 healthy adults for our study. Data from this investigation are expected to be normally distributed or achieved through appropriate transformation. Most data will be analyzed by repeated measures ANCOVA to consider effects due to health status, gender, GTE treatment, and their interactions. Multivariate regression analysis will be performed to define pairwise correlations between study variables in the presence or absence of potential study covariates. Statistical significance for all analyses will be set at $P < 0.05$.

C. Measurement/Instrumentation

Anthropometric Parameters and Blood Pressure. At screening, participants will rest for 15 minutes prior to determining blood pressure using an automated cuff. BMI will be calculated from height determined from a wall-mounted stadiometer and weight from a calibrated scale. Waist circumference will be assessed at the level of the umbilicus using a nonflexible measuring tape.

Blood/Urine Chemistries. Fasting plasma triglyceride, total cholesterol, and glucose will be measured by clinical assay (Pointe Scientific) at screening and throughout the study. Fasting insulin will be measured by ELISA (ALPCO) during the 4-wk study. Serum alanine aminotransferase (ALT) and urinary creatinine will also be measured throughout the study (Pointe Scientific).

Diet Assessment. Participants will be instructed to maintain a low-polyphenol diet throughout the study. Participants will record any consumption of polyphenol-rich foods throughout the study (a record sheet will be provided, see *Polyphenol Intake Record* attachment). Participants will be instructed to abstain from polyphenol-rich foods including tea of any kind, coffee, fruits, fruit juices, chocolate, nuts, legumes, and wine (a list will be provided, see *List of Polyphenol-Rich Foods* attachment). Food records (3-d) will be evaluated at wk 0, 2, and 4 using NDSR software and the NCC Flavonoid and Proanthocyanin database to assess energy, nutrient, and flavonoid intakes as we described (19, 71).

Plasma and Urine Catechin and Catechin-metabolites. To assess the absorption, metabolism, and excretion of green tea catechins during the pharmacokinetics trial (wk 0), plasma and urine catechin and catechin-metabolites will be measured by liquid chromatography-mass spectrometry (LC-MS). Green tea catechins (EGCG, ECG, EGC, EC) and their metabolites (e.g. valerolactones) will be measured using an adapted LC-MS method (72).

Metabolic Endotoxemia. Serum endotoxin will be measured at wk 0, 2, and 4 using a commercially available fluorometric assay that assesses endotoxin-mediated activation of recombinant Factor C and subsequent cleavage of a flurogenic substrate (PyroGene rFC; Lonza) as we detailed (43).

Gastrointestinal (GI) Permeability. A 4-sugar probe procedure appropriate for human clinical study will be performed as described (73). In brief, at wk 0 and 4, participants in the fasted state will ingest a 500-mL solution of sucrose (40 g), lactulose (5 g), mannitol (1 g) and sucralose (1 g) dissolved in water. Urine will be collected over a 24-hour period to assess GI permeability. Standardized sucrose-free meals (i.e. plain bagel, cream cheese, scrambled eggs, potato chips) will

be provided during the urine collection duration. Urinary sugars will be measured by LC-MS and permeability calculated as urinary excretion (%) and excretion ratios for sugars from 0-5 hours and 6-24 hours to reflect upper and lower GI permeability, respectively (74-77).

Inflammation. During the 4-wk intervention, we will define systemic and intestinal inflammation and the effects of GTE consumption. Circulating C-reactive protein and myeloperoxidase will provide information as to systemic inflammation, and fecal calprotectin will provide information regarding intestinal inflammation (78). These inflammatory markers will be measured using commercially available ELISA kits as we described (70). Additionally, we plan to perform polymerase chain reaction experiments to assess pro-inflammatory gene expression of white blood cells.

Microbiota Composition. Microbiota composition will be assessed from feces collected from 3 days during week 0 and 4 using previously described procedures (79-83). Total bacterial abundance will be determined by qPCR (84). Microbiota diversity and community structure will be determined for each sample by sequencing 16S rRNA genes using MiSeq. Bacteria will be identified by comparing sequences representing each operation taxonomic unit (OTU) with databases. Multivariate analysis techniques will be used to determine differences in obese and healthy individuals the effects of GTE.

D. Detailed Study Procedures

Overview of Study Procedures. Potential participants who call the study center in an anonymous manner for more information as well as those identified through ResearchMatch will be given a brief description about the study and asked a few questions to determine their eligibility (*see Phone Script* attachment). If they meet the eligible criteria, they will be invited to the study center for a screening meeting. During the meeting, the Informed Consent (see *Informed Consent* attachment) will be explained and provided for them to review. The participant will then be given the opportunity to review the Informed Consent form. If he/she chooses to participate in the study, they will then be asked to provide written consent. Women choosing to participate will also be asked to self-administer a urinary pregnancy test. If women self-report that the pregnancy test is negative, they will continue with the screening process. After receiving informed consent, the participants' height, weight, waist circumference, and blood pressure will be measured. Additionally, a small fasting blood sample will be collected to blood chemistry analysis. If they are not fasted at least 10 hours), they will be asked to come back in the fasted state at time of mutual convenience. These blood results in combination with anthropometric parameters will determine the participant's eligibility.

Eligible participants who agree to proceed with the study will be invited to meet with a registered dietitian or trained research personnel who will instruct them on how to complete a dietary food record, explain the details regarding following a low-polyphenol diet throughout the intervention, and provide instruction of foods to avoid prior to the pharmacokinetic trial at wk 0. Prior to the initial pharmacokinetics trial, participants will be asked to collect 3 days of fecal samples and return to the study center to participate in a sugar probe test. After completing the sugar probe test, participants will return to the study center at least 3 days after to complete a 12-h pharmacokinetics trial. Afterwards, participants will be provided a 2-wk supply of GTE-rich or placebo confections. After 2 wk, fasting blood will be collected from the participants and the other 2-week supply of confections will be provided for the remainder of the study. Upon completing the 4-week intervention, participants will complete a second sugar probe test. We estimate that completion of all study procedures will take 6-8 wk per participant. Each step of the study procedure will be discussed in detail below. See *Study Design* attachment for an overview of the study.

Screening Meeting. Potential participants who have met the initial criteria of the study (based on the telephone interview) will be invited to the study center at a mutually convenient time. During this time, the participant and a member of the research team will meet in a private, quiet conference room or office. The individual will be provided the informed consent form, and its contents will be described to the potential participant. The participant will then have the opportunity to review it, and if they choose to participate in the study, they will be asked to provide written consent. Although the participant will be asked to sign the informed consent, the participant will be told that they will not be asked to participate if their body measurements, blood pressure, or plasma chemistries (see *Enrollment Criteria*) do not meet the study criteria. All female participants will be asked to undergo a self-administered pregnancy test using a pregnancy urine test strip to ensure that only non-pregnant women are included in the study. If the participant has provided consent, we will then measure the participant's height, weight, waist circumference, and blood pressure. Next, if the participant is fasted for at least 10 hours, we will ask if a trained individual can draw a small blood sample (10 mL; 1 tube) so that we may measure blood chemistries (glucose, cholesterol, triglyceride). All samples will be coded to maintain participant anonymity. If the participant's anthropometrics, blood pressure, or plasma chemistries do not meet the study criteria, they will be told that do not meet the study criteria.

Potential participants who meet the criteria, will be contacted within a few days after their screening meeting to provide them with their blood and body measurement results and inform them of their eligibility to participate in the study. Consistent with our CLIA exemption, blood results will be provided in a categorical manner (i.e. "normal", "marginally high", "high") rather than providing actual blood concentrations of lab values (see *Subjects Results Sheet*). Potential participants having any blood values outside of the "normal range" will be directed/encouraged to follow-up with their own physician. Those having body measurements, blood pressure, and blood values within acceptable limits (see Inclusion/Exclusion criteria), will be invited to participate in the study. Subjects will be included or excluded based on a best fit of the inclusion and exclusion criteria (an example of best fit would be if a potential subject's BMI is 27 kg/m^2, which is outside the specified BMI inclusion range for overweight/obese participants they might still be included in the study if they meet all other inclusion and exclusion criteria more closely than other potential subjects). Participants will be read one phone script if they qualify and another phone script if they do not qualify (see *Participant Eligibility Phone Script* attachment). Potential participants who qualify for the study will be communicated a message as follows: I have your screening results. "Congratulations, you meet the eligibility criteria for our clinical trial. Your body measurements, blood pressure, and fasting blood chemistries of glucose, triglyceride, and cholesterol were all within the specified range. Your glucose falls in the [category], triglyceride falls in the [category], and cholesterol falls in the [category]. A summary of your results has been prepared for you, which you will receive when you come in for your trials. We look forward to and are thankful for your participation." Potential participants who do not qualify will be provided the following message: "I have your screening results. Unfortunately, you do not meet the eligibility criteria for our clinical trial. Your body measurements, blood pressure, glucose, triglyceride, and/or cholesterol were not within the eligible range. Your glucose falls in the [category], triglyceride falls in the [category], and cholesterol falls in the [category]. A summary of your results has been prepared for you. Please let me know if you would like to drop by the study center to collect a hard copy of your information or if you would like them emailed to you. Thank you so much for your time and effort." All individuals regardless of study eligibility will be encouraged to consider sharing these results with their physician.

In the event that a participant is telephoned and is unavailable, a message will be left requesting a callback at a convenient time or that at a member of the study team will try calling again at a later

time. No confidential or sensitive information will be shared with third parties or left on answering machines.

Dietary Meeting. Eligible participants who meet the study inclusion criteria, a dietary meeting will be scheduled with a registered dietitian or trained individual (i.e. nutritional sciences graduate student) from the research team. During the dietary meeting, the dietitian/trained individual will show the participant how to properly complete the 3-d diet record (see *Diet Record Form* attachment). Participants are expected to follow a low-polyphenol diet throughout the intervention to avoid potential confounding effects on the study outcomes. The dietitian/trained individual will clearly identify polyphenol-rich foods/drinks participants should avoid during the study and inform them to record if they accidently consume any polyphenol-rich foods/drinks during the study (see *List of Polyphenol-Rich Foods* and *Polyphenol Intake Record* attachments). The dietitian/trained individual will inform the participant of key foods to avoid, which includes any type of tea, coffee, fruits, fruit juices, chocolate, nuts, legumes, and wine. These foods are known to be rich in polyphenols, and consumption may confound the interpretation of the pharmacokinetics trial and intervention. In addition to dietary advice, the participant will be informed of the proper way to collect their urine and feces using the provided sterile collection containers. Fecal samples will be collected by the participant from 3 days prior to the pharmacokinetics trial. Participants will be provided a cooler with ice to store their fecal samples for up to 24 hours. The participant will then return their samples to the study center or coordinate with study personnel to meet at a public and mutual location and return their samples within 24 hours from collection.

The participant will be instructed to abstain from exercise for 24 hours prior to their sugar probe test and pharmacokinetics trial. For each visit to our study center, participants will be instructed to be fasted for 10-12 hours to ensure reliable blood sampling. Participants will be instructed to complete the dietary record for 3-d before the pharmacokinetics trial and second sugar probe test (wk 0 and 4) and their wk 2 study center visit.

Test Trials. Participants will be block randomized into two groups to receive either a GTE-rich or placebo (without GTE) confection. Participants will complete a pharmacokinetics trial prior to starting the intervention. Prior to (wk 0) and following 4-wk intervention, participants will collect 3 days of fecal samples and complete a sugar probe test. For this study, each participant will visit the study center a total of 6 times, including the screening and dietary meetings, to complete individual test trials. For each study center visit, participants will report in a fasted state (10-12 hours).

Prior to the first study visit, participants will complete a 3-d diet record. At the first study visit, participants will perform a sugar probe test. This test will consist of participants ingesting a test beverage of 40 g sucrose, 5 g lactulose, 1 g mannitol, and 1 g sucralose dissolved in water (500 mL). After ingestion, the participants will be provided a standardized sucrose-free meal within the first 5 hours and water throughout. Urine will be collected from 0-5 and 6-24 hours in provided containers.

The second study visit will be scheduled at least 3 d after the sugar probe test. The second study visit will consist of a 12-h pharmacokinetics trial. Participants will ingest one-half the daily GTE-rich confections (0.5 g GTE). Blood will then be collected at the following hourly time points: 0 (before confection ingestion), 0.25, 0.5, 1, 2, 3, 5, 8, 10, 12 h. Urine will be collected between 0-4, 4-8, 8-12, and 12-24 h post-ingestion into provided urinary collection containers. During the pharmacokinetics trial, participants will be allowed free access to water and provided a standardized-polyphenol free meal.

Upon completing the pharmacokinetics trial, the intervention phase will begin. Participants will be provided a 2-week supply of GTE-rich or placebo confections. They will be advised to consume 6 confections/ every day (2 confections/meal; 1 g GTE/d) for the following 4-wk. After 2 wk of the intervention phase, participants will complete a 3-day diet record and return for their third study visit. At this visit, fasting blood will be collected. Participants will then receive their final 2-wk supply of confections. After 4 wk, participants will complete a final 3-d diet record and perform the sugar probe test once again as described above. During each study visit, participants will have access to drinking water and use of the restroom as needed.

Sugar Probe Test. On the morning of testing at wk 0 and 4, participants will arrive at the study center after abstaining from food and only consuming water for 10-12 hours. A sugar probe test will be conducted as described (73). Upon arrival, participants will be asked to empty their urinary bladder. Subsequently, they will then be asked to ingest a drink containing 40 g sucrose, 5 g lactulose, 1 g mannitol, and 1 g sucralose dissolved in water (500 mL). Within 2-hours after ingesting the sugar test beverage, participants will be asked to consume an additional 500 mL of water. Additionally, participants will be provided with standardized sucrose-free meals during the 24-h period. All foods consumed during the 24-hour period will be free of artificial sweeteners. Urine will be collected from 0-5 and 6-24 hours in sterile containers. For each urinary collection period, participants will be provided 2-L urine collection bottles and a Styrofoam cooler with ice packs to store collected urine until they return their samples the next day.

Pharmacokinetics Trial. Within 3-d after completing the first sugar probe test, participants will return to the study center to perform a 12-h pharmacokinetics trial. At least 3-d prior to the pharmacokinetics trial, participants will abstain from polyphenol-rich foods and drinks, including any type of tea, coffee, fruits, fruit juices, any chocolate (dark, milk), nuts, legumes (soy), and wine. Participants will arrive to the study center after abstaining from food and only consuming water for 10-12 hours. Participants will be provided a 15-min rest period, prior to the first blood collection. During the rest period, the participants will sit in a phlebotomy chair and a flexible catheter will be inserted into a forearm vein by a trained phlebotomist. After a 15-min sitting rest period, a blood sample will be obtained from a 3-way stopcock connected to the end of the catheter. Blood will be collected with a syringe and transferred to appropriate tubes for processing and subsequent determination of biochemical measurements. Following the first blood collection, participants will consume 3 GTE-rich confections (0.5 g GTE). Blood samples will be collected at 0 (prior to ingestion), 0.25, 0.5, 1, 2, 3, 5, 8, 10, and 12 h. Additionally, urine will be collected between 0-4, 4-8, 8-12, and 12-24 h. During the 12 h trial period, participants will be able to freely move and walk around if they choose to, as long as they stay in close proximity to the study center. We will provide a dedicated office space for participants to sit down in between blood draws. If the participant chooses to move around, we will wrap their arm with a bandage to minimize any risks and ensure the catheter stays in place to prevent the need to insert a needle again. Additionally, a person of the research team will escort and monitor the participant if they choose to move around the study center. After each blood sample is obtained, the catheter will be flushed with saline to prevent the formation of clots and to minimize the likelihood of having to insert a needle again. At 4 hours, each participant will be provided with a standardized polyphenol-free meal. A trained phlebotomist will perform all cannula insertions, needle sticks, and blood collections. After the 12 h trial, participants will be provided with a light snack (e.g. granola bar) and prepared standardized polyphenol-free meals to consume for the remainder of the 24-h urine collection period. Participants will also be provided with a 2-wk supply of GTE-rich or placebo confections. Participants will be advised to consume 6 confections each day (2 per meal; 1 g GTE/d) for the entire 4-week intervention study. Participants will be contacted periodically by email, text, or phone, by research personnel to provide polite reminders to enhance study compliance.

Intervention Phase / Test Confections. After completing the pharmacokinetics trial, participants will partake in a 4 wk intervention. Participants will be block randomized to receive a GTE-rich or placebo (no GTE containing) confection. Confections will be low carbohydrate and low calorie. Each serving (6 g) provides <15 kcal, while delivering 1 g of decaffeinated GTE. The catechin profile (82% catechins (w/w); 63% EGCG, 18% EGC, 10% ECG, 9% EC) is similar to that of freshly brewed green teas. Sucrose will be the main carbohydrate source because sugar alcohols alter microbiota composition (66) and would confound interpretation. Decaffeinated GTE was chosen to minimize potential side effects to prevent excess caffeine consumption or caffeine/drug interactions that could result during comorbidity management of metabolic conditions. Placebo confections will be identical to GTE-rich confections, but without GTE. Confections will be packaged in single-serve, oxygen impermeable containers, and coded for study blinding. Participants will be asked to store the confections in the refrigerator to maintain quality and freshness.

Participants will be asked to consume 6 confections each day (2 with each meal) for 4 weeks. Consumption of 6 GTE-rich confections is equivalent to delivering 1 g GTE/d. Participants will receive a 2-wk supply of confections after completing the pharmacokinetics trial. Halfway through the intervention (wk 2), participants will visit the study center in a fasted state (10-12 h). Participants will return any leftover confections. After a 5 min stabilization period, a single fasting blood sample will be collected following procedures stated above. After collecting blood, the participants will be provided the final 2-wk supply of confections. At the conclusion of the intervention phase (wk 4), participants will perform a second sugar probe test. Compliance will be evaluated based on the number of confections returned throughout the study.

Sampling Handling. During the pharmacokinetics trial, at each time point, a blood sample (20 mL; 1.4 tablespoons x 10 time points = 200 mL or 0.85 cups) will be collected into evacuated blood collection tubes. Collecting a total of 200 mL or 0.85 cups at the pharmacokinetics trial is necessary to ensure adequate amount of plasma for each time point to accurately analyze the green tea catechin metabolites over the 12-hour period. During blood collection, participants may feel an initial pain when inserting the needle, bruising around the insertion area, lightheadedness, or fainting, which are common when donating blood. However, we do not foresee any additional significant risks for collecting this amount of blood over 12 hours, other than the risks stated previously.

Throughout the span of the study (~6-8 weeks in duration depending on participant/investigator availability) which consists of one acute ingestion of GTE-rich confections, a blood collection at wk 2 and 4, and 1 screening day, we will be collecting a total of ~255 mL or ~1.08 cups) of blood. Urine will be collected in provided containers (VWR) containing 10% thymol to inhibit bacterial growth. Feces will be collected using a commercial commode specimen collection system (Fisher Scientific). Briefly, the collection kit consists of the necessary materials (e.g. disposable spatula, gloves, waste bag) for participants to easily and hygienically collect their stool without contaminating the sample or themselves. We will provide participants with coolers with ice to store their fecal samples for up to 24-h after collection. We will then have the participants return their fecal samples to the study center or coordinate with study personnel to meet at a public and mutual location to return their samples within 24-h after collection. Volumes of urine will be recorded and fecal mass and observations will be recorded based on the Bristol Stool Chart (85).

During each blood collection, plasma and white blood cells will be obtained by centrifugation, and then transferred to cryogenic storage tubes. Serum samples will be obtained by allowing the blood to clot, followed by centrifugation and transfer to cryogenic storage tubes. Tubes will be

stored at -80° C until analysis can be completed. Analyses will include plasma glucose, insulin, triglyceride, total cholesterol, catechins (EGCG, ECG, EGC, EC), catechin-metabolites (e.g. valerolactones, catechin-derivatives), C-reactive protein, myeloperoxidase, pro-inflammatory gene expression and serum endotoxin and ALT. Urine will be stored at -80ºC until analysis can be completed. Analysis from urine will include catechins, catechin-metabolites, sucrose, mannitol, lactulose, sucralose, and creatinine. Fecal samples will be stored at -80ºC until analysis can be completed. Feces analysis will include microbiota composition and calprotectin. Remaining plasma, serum, urine, and fecal samples not used for these analyses will be archived for 5 years at -80 C in the event we decide to measure additional inflammatory, antioxidant, or microbiota related markers. Appropriate notation has been made in the informed consent to alert participants that we will be archiving specimens and that they have the right to refuse our use of these specimens for future analyses. Lastly, approval from OSU IRB will be sought via a protocol amendment prior to the analysis of any additional biomarkers not specified herein.

Privacy/Confidentiality. For all data and records that are a part of this study, a number (i.e. code) will be assigned to each participant, and will only be available to research personnel. Any records containing the names of participants will be stored in a locked filing cabinet or on a password protected computer in the PI's laboratory or office. Research personnel under the supervision of the PI and the PI himself will be the only individuals that have access to this information. The names of participants will not be used for publication in any form. The records will be maintained until the data are published, up to a maximum of five years. All archived samples will be coded, but the key linking the code to each participant's identifiable information will have been destroyed. In addition, participants will be instructed that their participation in this study is voluntary and that they may withdraw at any time without prejudice. Data (food records and biochemical values) obtained from this study will stored on a computer in the PI's laboratory. In addition, a backup of digital data will be stored on the PI's computer in his office. Both computers are password protected and both doors are locked when work areas are not in use.

E. Internal Validity

For all data and records that are a part of this study, a number (i.e. code) will be assigned to each participant. Test confections will be assigned a code as well. This will minimize measurement bias when performing analysis on dietary records, and biochemical markers because all samples/records will be coded. The codes will only be broken once data analysis has been completed and verified by the PI.

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