**Antiinflammatory Bioactivities of Green Tea Catechins at the Gut**
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## I. Objectives

The *objective of this application* is to establish evidence-based recommendations for green tea caatechins, based on improvements in endotoxemia and restored gut barrier function, that promote optimal health in humans. The *central hypothesis* is that green tea extract (GTE) catechins function to limit metabolic endotoxemia by ameliorating microbial dysbiosis-mediated inflammation that otherwise provokes intestinal permeability. 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) translate intestinal-level benefits of GTE, and 2) define antiinflammatory catechin-microbiota interactions. Upon completing these studies, we expect to substantially advance a critically needed strategy to manage disease-promoting, systemic inflammatory responses that are driven by endotoxemia.

## II. Background and Rationale

***Limiting intestinal endotoxin translocation is an antiinflammatory strategy.*** Metabolic endotoxemia is defined by 2-3 times higher circulating endotoxin compared with healthy individuals, but is significantly less than that of levels observed in sepsis patients.[1] Endotoxin is a component of Gram-negative bacteria.[2] Considerable evidence supports the premise that hepatic NFκB activation is mediated, at least in part, by the translocation of gut-derived endotoxin (e.g. LPS) to the portal circulation, and its ligation to hepatic Toll-like receptor 4 (TLR4). Indeed, nonalcoholic steatohepatitis (NASH) has well-recognized etiology mediated along the gut-liver axis.[3] In particular, dietary patterns (e.g. HF diet) that provoke insulin resistance prevent the lowering of intestinal pH otherwise resulting from bacterial fermentation of carbohydrates.[4] Consequently, the more neutral pH of the gut lumen favors the pathogenic bacteria overgrowth and inhibits the growth of beneficial bacteria (e.g. butyrogenic *Bifidobacterium*). Short chain fatty acids (SCFAs), especially butyrate, that are synthesized by resident microbes in the gut enhance barrier function.[5] Indeed, butyrate-producing *Clostridium butyricum* mitigates NASH in rats (including NFκB p65 expression).[6] These changes correlated with increased expression of intestinal tight junction proteins (TJPs), specifically zonula occluden (ZO) and occludin (OCC), and lowered circulating endotoxin. Thus, treating gut dysbiosis limits barrier permeability, and reduces hepatic exposure to gut-derived, and inflammation-inducing, bacterial products (i.e. LPS).[3] This is consistent with NASH patients having intestinal bacteria overgrowth that correlates with circulating endotoxin and expression of hepatic TLR4, but not TLR2.[7] Further, our evidence[8] and that of others[9] show that mice lacking TLR4/MyD88 signaling are protected from NASH despite increased portal vein endotoxin levels.[9] Antibiotics also attenuate steatosis and endotoxemia in rodents,[10] and probiotics in obese mice lower NFκB inflammation.[11]

***GTE Lowers NFκB Activation In A TLR4-Dependent Manner.*** NFκB centrally regulates inflammation and is activated in livers of patients[12] and rodents with NASH.[13-15] Despite pharmacokinetic studies[16] showing that green tea catechins have limited bioavailability, short half-lives, and rapid biotransformation through phase II xenobiotic metabolism, our studies in a high-fat (HF)-induced NASH model show that catechin-rich GTE lowers hepatic NFκB activation by decreasing IκBα phosphorylation.[17,18] Consistent with our goal to translate dietary GTE as an antiinflammatory strategy, our research has focused on upstream targets by which GTE limits NFκB activation. Prominent targets to limit NFκB activation in NASH include those mediated intracellularly by reactive oxygen species (ROS) and extracellularly by signaling from TNF receptor-1 and TLR4.[17]

Further study in Nrf2-KO mice demonstrated that GTE decreased hepatic TLR4 mRNA.[17] This supported the alternative hypothesis that its antiinflammatory activities are mediated through a lowering of TLR4/NFκB signaling.[17] This was corroborated in WT mice fed an HF diet prior to GTE treatment.[19] We reported that GTE ameliorates NASH while lowering hepatic mRNA of TLR4, its co-receptors

CD14 and MD-2, and adapter protein MyD88.[19] GTE also decreased circulating endotoxin that was otherwise increased in HF control mice.[19] In our recent report,[8] GTE in WT mice fed an HF diet inhibited NASH and hepatic NFκB activation to levels not different from those of loss-of-function TLR4 mutant mice fed an HF diet. GTE also lowered serum endotoxin independent of genotype. Thus, GTE protects against NASH consistent with a mechanism of inhibiting TLR4-mediated NFκB activation.

Hepatic TLR4 signaling is mediated through the adaptor proteins MyD88 and TRIF, which regulate "early" and "late" NFκB activation, respectively.[20] MyD88-dependent signaling occurs immediately after cell surface binding of LPS.[21] In contrast, TRIF-dependent signaling first requires endocytosis of LPS,[21] which facilitates endotoxin clearance.[22] Neither GTE nor TLR4$^{mut}$ genotype affected TRIF,[8] whereas GTE lowered hepatic MyD88 and circulating endotoxin regardless of genotype. This suggests that GTE attenuates NFκB activation in a TLR4/MyD88-dependent manner and lowers endotoxemia, likely without affecting LPS clearance. These findings strongly support examining GTE to target endotoxin to mitigate TLR4 inflammation.

***GTE Improves Gut Health.*** Two mechanisms, acting independently or additively, likely explain GTE-mediated decreases in endotoxemia. GTE potentially improves microbiota composition to decrease LPS-derived Gram-negative bacteria (e.g. *E. coli*) and/or it improves gut barrier function. Epigallocatechin gallate (EGCG), the predominant catechin in GTE prevents IFN-γ-induced increases in epithelial permeability *in vitro.*[23] However, there are no reports in humans examining GTE on gut permeability. This is surprising considering that catechin levels in the intestinal lumen can easily reach *2000 μM*[24] despite their low oral bioavailability (<5 μM).[16] Thus, catechins potentially exert direct benefit at the intestine to improve gut barrier integrity.

Our report supports that GTE alleviates endotoxemia by decreasing gut permeability.[19] GTE treatment following HF-induced NASH resulted in decreases of circulating endotoxin and site-specific improvements of intestinal TJP mRNA levels. Although TJPs were unaffected in the jejunum, GTE increased duodenal OCC and ZO and ileal expression of OCC and claudin (CLDN). In WT and TLR4$^{mut}$ mice fed an HF diet, TJP mRNA was unaffected by genotype, whereas GTE increased duodenal and jejunal CLDN mRNA regardless of genotype.[8] Because mRNA levels do not necessarily reflect a functional benefit in gut integrity,[25] we assessed gut permeability in WT mice fed an HF diet containing GTE. Following oral gavage of FITC-dextran, GTE attenuated its serum levels that were otherwise increased in HF controls. Corroborating evidence also shows that GTE decreased endotoxin in the portal vein that was otherwise increased in HF mice. Serum FITC-dextran also correlated with portal vein endotoxin levels ($r= 0.49$; $P<0.01$). Further, GTE decreased ileal TNFα mRNA that was otherwise higher in HF mice. Thus, GTE alleviates endotoxemia, at least in part, by decreasing gut-derived endotoxin translocation, consistent with an antiinflammatory mechanism that enhances barrier function by improving intestinal TJP expression.

A complementary line of evidence supports that catechins mitigate endotoxemia by improving microbiota composition. Microbial fermented green tea (FGT) attenuates HF-induced obesity in mice.[26] This is consistent with our works examining GTE in obese models.[18,27,28] FGT decreased the ratios of *Firmicutes*/*Bacteroidetes* and *Bacteroides*/*Prevotella*. The *Firmicutes* phylum predominates in obese mice.[29] Thus, obesity susceptibility is a potential consequence of increased energy absorption resulting from microbial fermentation. Conversely, decreased *Firmicutes* by FGT potentially explains the observed reduction in adiposity without affecting energy intake, which is similar to our works in mice fed GTE.[17,18,27,28] Lowered *Bacteroides*/*Prevotella* also suggests a shift towards carbohydrate fermentation. This would enhance gut integrity by increasing SCFA availability to the host. Thus, GTE potentially alleviates dysbiosis by reducing Gram-negative bacteria (e.g. *Bacteroidetes, Proteobacteria)* that drive endotoxin-mediated inflammation while increasing SCFA from populations (e.g. *Prevotella*) to improve gut integrity.[30] Indeed, similar to others,[31] our underway studies indicate that GTE increases microbial functions relating to butanoate and propanoate metabolism. SCFA, both

straight chain and branched chain, will be assessed directly in our studies in humans and *in vitro*. Straight chain SCFA promote commensal bacteria growth and inhibit certain Gram-negative bacteria.[32] In contrast, branched chain SCFA, which are generated from branched chain amino acids by microbial metabolism, are associated with metabolic disorders involving insulin resistance.[33,34]

Despite their similar metabolic benefits, and both being derived from the *Camellia sinensis* plant, GTE and FGT differ substantially in their compositions. GTE contains 30% total catechins that is highly enriched in EGCG (48%), and relatively lesser amounts of epigallocatechin (EGC) (31%), epicatechin gallate (ECG) (13%), and epicatechin (EC) (8%).[27] In contrast, FGT has only 14% total catechins. It is comprised of mainly catechin (56%) and small proportions of EGCG (11%), EGC (15%), ECG (3%), EC (5%), and other low-level metabolites. That GTE and FGT elicit hepatoprotection, despite their unique compositions, suggests independent or additive benefits of catechins, or their metabolites arising from microbial metabolism, to ameliorate inflammation-inducing responses along the gut-liver axis. This concept is consistent with our preliminary studies in mice fed an HF diet formulated with GTE (2%), EGCG (0.3%; equivalent to the EGCG level in GTE), or catechin (CAT; 0.3%). EGCG and CAT similarly attenuated serum endotoxin and fecal calprotectin, a biomarker of intestinal neutrophil infiltration,[35] to levels no different from the levels lowered by GTE. Also, MS-metabolomics studies detected >50 metabolites in fecal samples. Of those, there were only two metabolites common to the treatments with GTE, EGCG, and CAT that were not detected in the LF or HF control mice. These were 3',4'-dihydroxyphenyl-γ-valerolactone and its microbial catabolite 3-hydroxyphenylacetic acid. Thus, while catechins provide direct antiinflammatory benefit to preserve barrier integrity, at least *in vitro*,[23] these data support independent and/or additive bioactivities of microbial catechin metabolites.

Further, a short-term study in adults shows that green tea improved microbiota composition (e.g. increased α-diversity, increased genera of SCFA producers) in association with reducing microbial functions relating to LPS biosynthesis.[31] We recognize that our hypothesis contrasts with the findings of a clinical study[36] showing that gut microbiota composition was unaffected in adults receiving EGCG-enriched GTE (560 mg/d; 12 wk). However, critical study limitations likely precluded an observable benefit on gut health. Participants were normal weight (BMI = 23 kg/m$^2$), and unlikely to have gut dysbiosis that occurs in obese or MetS persons.[37-40] Dietary control consisted of encouraging participants to maintain their usual diets, which was not assessed rigorously nor were total polyphenol intakes considered. Indeed, diet accounts for >50% of microbiota changes and the responsiveness of the microbiota to shift rapidly (i.e. hours to days)[41] in relation to its composition and functions. Compliance was evaluated based on pill counts without any biomarker measures. The parallel study design also limits statistical power for detecting a treatment effect. Finally, an intergenic spacer length polymorphism was used to profile and compare the microbiota among the groups. That technique is not based on DNA sequencing but based on comparing the length of the intergenic spacers among samples. Because different bacteria can and do have a similar length of the intergenic spacer, it cannot enable the detection or quantification of specific taxa (e.g. species, genera) of bacteria. In other words, even if some genera or species of bacteria were altered by EGCG, that technique would be unlikely to detect or quantify the changes. Actually, that technique is rarely used in recent years after next-generation DNA sequencing became available, which will be used in the proposed study. This will better identify and estimate the relative abundance of most bacteria, and treatment effects on gut microbiota (e.g. species composition and structure such as richness, evenness, change in relative abundance). Thus, the lack of prebiotic effect by EGCG in that study[36] is not surprising, and contrasts evidence that tea phenolics inhibit pathogenic bacteria growth (e.g. *Clostridium difficile;*[42] *Staphylococcus spp*[43]) while increasing the proportion of beneficial commensal bacteria (e.g. *Bifidobacterium spp*).[43-45]

In ***conclusion***, evidence shows that 1) limiting gut-derived endotoxin is an antiinflammatory strategy, 2) GTE lowers TLR4/NFκB-dependent inflammation, and 3) GTE improves gut health through improving gut barrier function and alleviating gut dysbiosis. Our team has the expertise and experience to safely perform controlled crossover interventions to assess metabolic parameters, gut barrier

permeability, and gut microbiota composition. Thus, after completing these studies we expect to substantially advance a critically needed strategy to manage disease-promoting, systemic inflammatory responses that are driven by endotoxemia.

**Anticipated Results**

Preclinical evidence supports the gut-level benefits of catechin-rich GTE to limit metabolic endotoxemia.[8,19] We expect these preclinical findings to be translated to similar gut-level improvements in MetS persons utilizing a novel, catechin-rich GTE confection (i.e. snack food). We expect to achieve >95% compliance (based on returned confections and circulating catechins) without any adverse effects or changes in body mass or energy intakes. Based on our mouse studies,[8,19] GTE is expected to decrease serum endotoxin in a time-dependent manner, and most substantially among MetS persons due to their endotoxemia. These benefits are expected to occur through an antiinflammatory mechanism that attenuates gut-derived endotoxin translocation. This would be consistent with GTE reducing endotoxin translocation to the portal vein by increasing intestinal expression of TJPs[8,19] Thus, GTE will decrease small intestinal and colonic permeability, based on decreased urinary excretion of lactulose/mannitol (0-5 h) and sucralose (0-24 h), respectively.[46]

Similar to our studies, GTE also will alleviate gut dysbiosis that is known to occur in MetS persons (e.g. depleted populations of SCFA-producing bacteria[47]). Selective antimicrobial activities of GTE will decrease LPS-derived Gram-negative bacteria (e.g. *Bacteroides*).[42,44] GTE will also increase commensal bacteria, including *Clostridia* (Cluster XIV and IV) and other members of the Gram-positive Phyla *Firmicutes* that generate SCFAs.[48,49] This will be corroborated by greater fecal butyrate and other straight chain SCFAs. Thus, these findings would suggest a mechanism, at least in part, that the alleviation of gut dysbiosis restores butyrate generation.

We have also considered antiinflammatory activities of GTE attributed to its catechins[23] and/or catechin metabolites (e.g. γ-valerolactones).[50,51] [52] Indeed, fecal calprotectin (**Fig 5**) and myeloperoxidase, and systemic TLR4/NFκB pro-inflammatory genes in PMBCs and circulating proteins (i.e. p65, TLR4, MyD88, IL-6 and -10) are expected to be lowered by GTE.[7,53] Measures of catechins/metabolites in feces and blood will be used to establish correlations with intestinal and systemic inflammatory biomarkers to suggest direct and/or indirect benefits of catechins/metabolites. Finally, GTE is not anticipated to affect liver function tests (i.e. ALT, AST), and their lack of change (and other clinical chemistries) would support the safe application of the GTE-rich confection to manage MetS risk. Thus, these translational studies will establish GTE to alleviate gut dysbiosis that otherwise provokes barrier dysfunction and host LPS-TLR4-NFκB inflammation to drive MetS risk.

**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**

The prevalence of MetS is at epidemic proportions in the US where it affects ~35% of all Americans and 50% of those >60 years in association with the high rates of obesity.[54] Because MetS symptoms are often subclinical, most individuals are left unmanaged other than to encourage lifestyle modification. It is therefore not surprising that many MetS persons progress to more advanced disorders (e.g. NASH) and premature mortality.[55,56] This is consistent with NASH being recognized as the hepatic manifestation of MetS.[57,58] Indeed, NASH is the most prevalent liver disorder in the Western world where it affects ~20% of adults[59] including 80-100 million Americans.[60] Although MetS development and progression can be averted by improved diet,[61,62] these recommendations have had little impact.[63] Correlative evidence supports that poor diet influences endotoxemia, gut barrier dysfunction, and glucose tolerance in humans.[64-66] By contrast, green tea lowers risks for cardiovascular and liver

diseases.[67,68] Evidence from our preclinical models also clearly show that GTE alleviates endotoxemia and TLR4/NFκB inflammation by improving gut health. However, no studies have targeted gut health with GTE to manage endotoxemia and alleviate MetS progression. *Thus, our studies are expected to be of public health and scientific significance because they represent the first translational study in MetS persons examining GTE to manage disease-promoting, intestinal-level responses leading to endotoxemia and TLR4/NFκB inflammation. They will also establish independent benefits of catechins/catechin metabolites relative to altered microbial populations and functions. Thus, these studies are a critical step to advance a timely dietary recommendation to mitigate the growing burden of MetS, and by association NASH, by ameliorating endotoxin-TLR4 signaling.*

The potential impact of green tea to improve health is supported by data indicating that 4 out of 5 Americans drink tea, with ~50% considered habitual consumers.[69] The US is also agriculturally active in producing tea (*Camellia sinensis*) as part of a $17.6 billion global market, with annual sales of green tea outpacing black tea (11% vs 3.9%).[70] Green tea is safe when consumed as recommended,[71] but rigorous investigation is needed to establish evidence-based dietary recommendations that improve the health and well-being of Americans. In this regard, the status quo is that GTE decreases TLR4/NFκB inflammation and MetS criteria in preclinical models, in part, by limiting gut permeability to reduce endotoxemia.[8,19] Endotoxin-TLR4 signaling is also implicated to *initiate* insulin resistance.[1] However, no translational studies in MetS persons have examined GTE on gut barrier function despite it improving progressive pathologic responses of insulin resistance in humans, including NASH.[72] *In our opinion, the proposed research is innovative, because it is a substantial departure from the status quo by shifting the focus to establish antiinflammatory bioactivities of GTE acting at the intestinal-level to protect against an early insult mediating metabolic disorders. Our translational approach also will be the first to use a GTE-rich "healthy snack food"[73] to reduce metabolic endotoxemia. This approach recognizes consumers' shift in eating behaviors that show increased preference for convenient snack foods[74] and circumvents a barrier in that most Americans consume green tea limitedly in lieu of catechin-deplete black tea.[75-77]* Thus, the outcomes of our nutritional approach that integrates clinical, biochemical, metagenomics, and metabolomics aspects is expected to vertically advance an easily implementable dietary approach that will define GTE bioactivities while also establishing timely recommendations to manage MetS, and alleviate this serious problem of public health concern.

## III. Procedures

### A. Research Design

We will conduct a *randomized double-blind, placebo-controlled crossover trial* in metabolic syndrome (MetS) and healthy individuals. We will enroll adults with (n = 20) and age- and gender-matched healthy (n = 20) men and women (18-65 y). 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, while instructed to abstain from all tea varieties and follow a low-polyphenol diet.[78] Blood will be collected at day 0, 14, and 28 for metabolic assessments and safety monitoring by assessing a serum chemistry panel [e.g. liver function (aminotransferases), renal function (creatinine, BUN), CBC, glucose, insulin, insulin resistance[79]]. At the same time points, blood pressure and anthropometrics will be assessed, and dietary nutrient and flavonoid intakes will be evaluated from 3-d food records using NDSR dietary analysis software and the NCC Flavonoid and Proanthocyanidin database as we described.[73,80] On day 28, participants will provide a 3-d fecal sample. Following blood collection, they will complete a gut permeability test and collect urine through day 29 (described below). Upon completing these procedures, participants will undergo ~1-mo wash-out before repeating the study identically, but with crossover to the alternate treatment.

***GTE-rich Confections.*** The Bruno and Vodovotz labs have previously developed a starch-based GTE-rich confection[73] 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 8 calories. Thus, 6 confections will provide about 51 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[73] is similar to about 5 servings/day of green tea. 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 [81]. 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.*** Male and female adults with MetS (18-65 y) having no history of liver, cardiovascular disease, or cancer will be enrolled. They are expected to meet ≥3 established criteria for MetS,[82] and we will enroll those fulfilling these specific ***inclusion criteria*** to improve participant homogeneity: fasting glucose (100-126 mg/dL), waist circumference (>89 cm or 102 cm for F/M), HDL-C (<50 or <40 mg/dL in F/M), triglyceride >150 mg/dL. Although elevated blood pressure is a symptom/criteria for MetS, only those individuals having blood pressure <140/90 mmHg will be enrolled to avoid those having with Stage 2 hypertension.[83] Age- and gender-matched healthy adults (19-25 $kg/m^2$) will also be enrolled. They will be normoglycemic (glucose <100 mg/dL), normotensive (<120/80 mmHg) and normolipidemic (HDL-C >50 mg/dL or >40 mg/dL in F/M; triglyceride <150 mg/dL). Major ***exclusion criteria*** for all participants include: concurrent tea consumption (Camellia sinensis or herbal varieties); vegetarian; user of dietary supplements, prebiotics, or probiotics within past month; hemochromatosis; any gastrointestinal disorders or surgeries; chronic diarrhea; Parkinson's disease; smoker; alcohol consumption (>2 drinks/d); recent use of antibiotics or antiinflammatory agents. We will also not enroll any participants using any medications to manage diabetes, hypertension, or hyperlipidemia (e.g. statins, metformin, ACE inhibitors), any medications known to be contraindicated for use with green tea ingestion (e.g. antipsychotic medications [Clozapine, lithium, Diazepam], blood thinning medications [Warfarin], high blood pressure medications [nadolol], and monoamine oxidase inhibitors [selegiline]), women who are pregnant or lactating, or have initiated or changed birth control in the past 3 months.

***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 social media (e.g. Facebook). The posted advertisements will instruct interested participants to complete an online eligibility survey or 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).

***Power Calculation and Data Analysis.*** We performed a power analysis using serum endotoxin (day 28) as the primary outcome. Mean serum endotoxin of MetS and healthy adults was used to predict a conservative 30% improvement by GTE in MetS persons; this corresponds to a 10 EU/mL decrease in endotoxin in MetS. Simulations were used to estimate power based on 20 healthy and 20 MetS adults randomized to treatment vs control in a 50/50 ratio, respectively. Based on a linear model with treatment and health status, and within-subject measures, we will have >90% power ($\alpha$=0.05) to detect the primary contrast of interest (GTE vs placebo in MetS adults) and additionally, >85% power ($\alpha$=0.05) to detect an interaction between treatment and health status. Most data will be analyzed by linear mixed effects models (LMMs) with random effect for subjects (to account for repeated measures) and fixed effects for health status, gender, GTE treatment, time period, and their interactions. Multivariate regression analysis will define correlations between study variables with consideration of potential covariates. Statistical significance for all analyses will be set at $P \leq 0.05$.

**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 Chemistries.*** At screening, fasting plasma glucose, triglyceride, and HDL-cholesterol will be measured by spectrophotometry utilizing validated clinical assays (Pointe Scientific). At day 0, 14, and 28 of the interventions, collected blood will be sent to The Ohio State University Medical Center for metabolic evaluations by assessing a serum chemistry panel [e.g. [e.g. liver function (aminotransferases), renal function (creatinine, BUN), CBC, glucose, insulin, insulin resistance[79]].

***Diet Assessment/Control.*** To improve diet homogeneity and limit potential confounding effects of polyphenol intakes on study outcomes, participants will follow a low-polyphenol diet during each study phase, as described by Dr. Vodovotz.[78] In brief, participants will receive weekly dietary education under the auspice of a registered dietitian (PI Bruno) to omit or limit polyphenol-rich foods. Participants will be instructed to abstain from foods containing >50 mg/serving of total polyphenols and limit those having 10-50 mg/serving of polyphenols to a maximum of 2 servings/d. Foods containing <10 mg/serving will be unrestricted. This results in a decrease of dietary polyphenols by 45% compared to usual intakes (1568 mg/d), and with 95% adherence.[78] Participants will be provided a list of common polyphenol-rich foods to avoid (see *List of Polyphenol-Rich Foods* attachment) as well as a form to record any consumption of these foods/drinks. Fiber intakes with this dietary approach are also similar to the low intakes of the typical American diet (~15 g/d).[84] Food records (3-d) will be evaluated on three occasions during the intervention using NDSR software and the NCC Flavonoid and Proanthocyanin database will be used to assess energy, nutrient, and flavonoid intakes as we described.[73,80]

***Metabolic Endotoxemia.*** Serum endotoxin will be measured using a fluorometric kit from fasting blood samples at day 0, 14, and 28 as we described.[19] Briefly, the fluorometric assay assesses endotoxin-mediated activation of recombinant Factor C and subsequent cleavage of a fluorogenic substrate (PyroGene rFC; Lonza). In our hands, inter- and intra-assay CV is 4.2-6.1%. To facilitate data interpretation, the following complementary markers of metabolic endotoxemia will be measured in plasma collected at day 0, 14, and 28: trimethylamine N-oxide (TMAO), soluble cluster of differentiation 14 (sCD14), and lipopolysaccharide-binding protein (LBP). Trimethylamine N-oxide is produced by certain gut bacteria and is reflective of gut dysbiosis. Soluble CD14 and LBP guide bacterial toxins to immune cells for recognition and are reflective of increased gut barrier permeability.

Trimethylamine N-oxide will be quantified by LC/MS whereas LBP and sCD14 will be quantified by ELISA using commercial kits.

***Gut Permeability.*** At wk 4 (d 28), fasted participants will ingest a sugar probe solution [sucrose (40 g), lactulose (5 g), mannitol (1 g), sucralose (1 g), and erythritol (1 g)] as described.[85] Sucrose-free meals will be provided for 24 h (through d 29). Urinary sugars will be assessed using our established LC-MS method.[85] Sugar excretion (%), and mannitol-normalized excretion ratios of sugars from 0-5 h and 6-24 h will be calculated to reflect upper and lower GI permeability.[86-89] This approach is based on lactulose being absorbed in the small intestine, but not the colon, whereas sucralose is absorbed in the colon; sucrose is used to reflect gastric permeability.[46] Thus, these studies will define site-specific gut permeability, and benefit of GTE treatment.

***Microbiota Composition and Function.*** Microbiota composition and the predicted functional metagenome will be assessed from fecal samples collected on 3 days at wk 4 (d 25-27), as Dr. Yu (co-PD) described.[90-94] Effects of GTE and MetS status on total bacterial abundance will be determined by qPCR.[95] Microbiota will be characterized for diversity and community structure by sequencing 16S rRNA genes using MiSeq. Briefly, one amplicon library (targeting the V3-V4 hypervariable region using bacteria-specific primers) will be prepared by pooling PCR products from three replicate PCR reactions for each sample. Amplicon libraries will be sequenced using the 2x300 pair-ended sequencing reactions. Sequencing reads (≥50,000/sample) will be subjected to quality control to filter out artificial sequences prior to bioinformatics analysis using Qiime.[96] Bacteria will be identified by comparing sequences representing each operation taxonomic unit (OTU) with RDP and Silva databases. [97,98] α-Diversity (OTU richness, Shannon-Wiener diversity index, evenness) will be calculated using Qiime. Comparisons of diversity among samples (β-diversity) will be performed using the Bray-Curtis dissimilarity,[99] followed by multivariate analyses (e.g. principle coordinate analysis, permutational multivariate analysis of variance, analysis of similarity, partial least square discriminant analysis). The functional metagenome will be predicted using PICRUSt[100] and PanFP[101] based on reference genome databases and the KEGG Orthology classification scheme to obtain a functional gene abundance matrix.

***Plasma and Fecal Catechins and Catechin-Metabolites.*** Using our targeted LC-MS/MS-based panel of >50 metabolites, we will quantify GTE catechins and metabolites in fecal samples (d 27) and plasma (d 0, 14, 28) following enzymatic hydrolysis with β-glucuronidase/sulfatase.[73] Analyte identification and quantification will be confirmed by comparing fragmentation patterns relative to purified standards or reference databases. In addition to defining the interactive effects of MetS status and GTE treatment on catechin metabolism, time-dependent plasma measures will objectively establish *subject compliance* to the dietary intervention.

***Fecal Short Chain Fatty Acids.*** Separate LC-MS/MS procedures that are established based on a published report,[33] will evaluate ten $C_2$-$C_6$ straight chain SCFAs (i.e. butyrate, acetate, propionate) and branched chain SCFAs (e.g. isobutyric acid, isovaleric acid). Straight chain SCFAs are predominantly from bacterial fermentation of fibers whereas branched chain SCFAs are derived mainly from bacteria metabolism of branched chain amino acids. The former are often depleted with gut dysbiosis whereas the latter are often increased. In brief, fecal SCFAs are derivatized with 3-nitrophenylhydrazine to increase their stability prior to injecting on the LC-MS/MS system operated in ESI mode and quantifying against area ratios of authentic standards relative to $^{13}C_6$-3NPH.

***Host and Intestinal Inflammation.*** At wk 4, we will define the effects of GTE on inflammatory responses. We will assess intestinal inflammation by measuring fecal calprotectin and myeloperoxidase (i.e. indices of neutrophil inflammatory responses) by ELISA as we described.[102] We will also measure systematic inflammatory responses in plasma by ELISA (CRP, IL-6 and -8, TNFα, MPO) and by RT-PCR analysis of peripheral blood mononuclear cells (PMBCs; isolated at blood collection) to assess the expression of genes involved in TLR4/NFκB signaling (TLR4, MyD88, p65, IL-6 and -8, TNFα, MPO, MCP-1).

**D. Detailed Study Procedures**

***Overview of Study Procedures.*** Potential participants who call the study center in an anonymous manner for more information 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 be asked to provide written consent. 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 for blood chemistry analysis. If they are not fasted at least 10 hours), they will be asked to come back in the fasted state at a 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 at wk 0 (d 0), who will instruct them on how to complete a dietary food record and explain the details regarding following a low-polyphenol diet throughout the intervention. At this first visit, a fasting blood sample will be collected and the participants will be provided 2-wk supply of confections. They will be instructed to consume 6 confections per day. After 2 weeks, the participant will come in for a mid-intervention visit (d 14). A fasting blood sample will be collected and the participant will be provided another 2-wk supply of confections to complete the 4-wk intervention. Prior to the participants' last study visit, they will collect 3-d stool samples and return the samples or meet with study personnel to return their stool samples upon collection. At the end of the intervention (d 28), participants will visit the study and a fasting blood sample will be collected. Following this, participants will perform a gut permeability test. After completing the gut permeability test, participants will then follow about a 1-month wash-out period. Following the wash-out period, participants will repeat these procedures, except receiving the alternative study treatment (GTE, placebo) than what they previously received. We estimate that completion of all study procedures (2 4-wk interventions and 1-month wash-out period) will take 10-12 weeks per participant. Each step of the study procedures 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. 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 (15 mL; 1.5 tubes) so that we may measure blood chemistries (glucose, HDL, 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 male subject's waist circumference is 101 cm, which is outside the specified waist circumference inclusion range for MetS 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 fall 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 next visit. 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 fall 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 e-mailed 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.

***Intervention Timeline.*** Each participant will complete a 2-phase (GTE vs placebo) randomized, crossover study. Each study intervention will consist of 3 study visits (Day 0, 14, 28) over a 4-wk period. After completing the first intervention, participants will undergo a wash-out period (1 month), and afterwards, begin the alternative intervention.

On Day 0, participants will come to the study center in a fasted state and provide a blood sample (45 mL) to measure endotoxin, TMAO, LBP, sCD14, a panel of clinical chemistries, and catechins. Following the blood collection, participants will be instructed on how to complete a 3-d diet record as well as be informed about maintaining a low-polyphenol diet throughout the duration of the intervention. Participants will then be provided a 2-wk supply of confections, for which they will be instructed to consume 6 confections each day (2 confections/meal; 1 g GTE/d).

On Day 14, participants will return to the study center in the fasted state and return any uneaten confections. Another blood sample (45 mL) will be collected for measurement of biomarkers as described above. Participants will then be asked to complete another 3-d diet record and be reminded to keep maintaining a low-polyphenol diet.

Prior to the final visit on Day 28, participants will collect a stool sample during the preceding 3 days. Participants will collect their stool samples in the comfort of their home and will be asked to return the samples on ice or meet with study personnel to return the samples to the study center within 24 h upon

collection. On Day 28, participants will come to the study center in the fasted state. A fasting blood sample (60 mL) will be collected to measure the biomarkers mentioned above along with analysis of white blood cell inflammatory genes. Following blood collection, participants will perform a gut permeability test. Participants will consume a test beverage that contains sucrose (40 g), lactulose (5 g), mannitol (1 g), sucralose (1 g), and erythritol (1 g) dissolved in water (250 mL). After ingestion of the beverage, participants will be instructed to collect their urine from 0-5 and 6-24 hours in the provided containers.

After completing the first intervention, participants will partake in a wash-out period (1 month) and then repeat the same study procedures (i.e. Day 0, Day 14, Day 28), but will receive the alternative confection (i.e. GTE vs placebo). During each study visit, participants will have access to drinking water and use of the restroom as needed. Details about each study visit and associated test trials that will occur is explained below:

***Intervention Phase / Test Confections.*** During the 4-wk intervention, participants will receive a GTE-rich or placebo (no GTE containing) confection. Confections will be low-carbohydrate and low-calorie. Each serving (6 g) provides <8 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[81] and would confound interpretation. Decaffeinated GTE was chosen to minimize potential side effects such as excess caffeine consumption or caffeine/drug interactions that could result from 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. At the first visit, participants will provide a fasting blood sample and then receive a 2-wk supply of confections. Halfway through the intervention (d 14), 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 (d 28), participants will perform a gut permeability test and provide another fasting blood sample. Compliance will be evaluated based on the number of confections returned throughout the study as well as objectively by measuring catechin/catechin-metabolites in the plasma. To ensure randomization, a random number generator will be used to randomly assign the sequence by which the participant will complete each of the interventions.

***Gut Permeability Test.*** On the morning of testing at d 28 of the intervention, participants will arrive at the study center after abstaining from food and only consuming water for 10-12 hours. A gut permeability test will be conducted as described[85]. 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, 1 g sucralose, and 1 g erythritol dissolved in water (250 mL). Within 2-hours after ingesting the sugar test beverage, participants will be asked to consume an additional 500 mL of water. Participants will be instructed to abstain from foods that contain artificial sweeteners during the 24-hour period. Urine will be collected from 0-5 and 6-24 hours in sterile containers with 10% thymol (inhibit bacterial growth). For each urinary collection period, participants will be provided 2-L urine collection bottles and a cooler with ice packs to store collected urine until they return their samples the next day.

***Sampling Handling.*** For the entire study (~3 months), including the screening phase and completion of both interventions (GTE vs placebo), we plan to collect a total of 315 mL or 1.3 cups of blood. At screening, 15 mL of blood will be obtained to ensure adequate sample to perform biochemical measures of eligibility. During each 4-wk intervention, we will collect a total of 150 mL or 0.6 cups. At day 0 and 14 we will collect 45 mL (0.2 cups) and at day 28 we will collect 60 mL (0.25 cups). All blood will be collected into evacuated blood collection tubes. The greater volume of blood collected at day 28 is necessary to ensure that there is adequate blood to perform the same analyses as for day 0 and 14 as well as for additional analyses (e.g. white blood cell inflammatory markers). 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.

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 stool samples for up to 24 h after collection. We will then have the participants return their stool 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.[103]

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 triglyceride, HDL, liver function (aminotransferases), renal function (creatinine, BUN), CBC, glucose, insulin, pro-inflammatory gene expression, catechins (EGCG, ECG, EGC, EC), catechin-metabolites (e.g. valerolactones, catechin-derivatives), serum endotoxin, TMAO, LBP, and sCD14. Urine will be stored at -80ºC until analysis can be completed. Analysis from urine will include sucrose, mannitol, lactulose, sucralose, and erythritol. Fecal samples will be stored at -80ºC until analysis can be completed. Feces analysis will include microbiota composition, myeloperoxidase, calprotectin, and SCFAs. 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, which 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 be 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 of 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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