Effect of Gut Butyrate Delivery on Blood Pressure in African Americans with Hypertension

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STATEMENT OF COMPLIANCE

This Phase I trial will be carried out in accordance with International Conference on Harmonisation Good Clinical Practice (ICH GCP) and the following:

- United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46, 21 CFR Part 50, 21 CFR Part 56, 21 CFR Part 312, and/or 21 CFR Part 812)

National Institutes of Health (NIH)-funded investigators and clinical trial site staff who are responsible for the conduct, management, or oversight of NIH-funded clinical trials have completed Human Subjects Protection and ICH GCP Training.

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the Institutional Review Board (IRB) for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. In addition, all changes to the consent form will be IRB-approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

1 PROTOCOL SUMMARY

No text is to be entered in this section; rather it should be included under the relevant subheadings below.

1.1 SYNOPSIS

Title:	**Effect of Gut Butyrate Delivery on Blood Pressure in African Americans with Hypertension**
Study Description:	African Americans (AA) have the greatest burden of hypertension. Recently, gut microbial dysbiosis (a term that describes a poorly diverse gut microbial profile and lower short chain fatty acid (SCFA) production) has been linked to hypertension and may be involved in the pathogenesis of hypertension in African Americans. African Americans have been reported to have lower gut SCFA and SCFA can reduce blood pressure. This is a proof of concept pilot study to determine the relationship between gut SCFA and blood pressure (BP). Delivery of butyrate to the gut will be via enema.
Objectives:	The objectives of this research are to 1. Identify gut microbial taxa (SCFA butyrate-producing microbes) and circulating butyrate levels associated with hypertension via a cross-sectional design in AA without and with hypertension. 2. Quantify the relationship between SCFA (butyrate) absorption into the blood stream and subsequent changes in blood pressure in a 24-hour period after delivering butyrate into the gut via enema.

Endpoints:	Primary: If gut butyrate is related to BP, we expect to observe a reduction in BP (measured by 24-hr Ambulatory Blood Pressure monitoring) after the butyrate enema but no change in BP after the control saline enema.
Rationale:	In humans, butyrate producing microbial species are associated with blood pressure. Butyrate is absorbed in the colon, and once in the blood may exert beneficial effects on blood pressure. However, it is unclear which specific microbes are associated with blood pressure in African Americans or how much butyrate is absorbed into the blood which may affect BP.
Study Population:	Self-reported African Americans males and females ages of 30-50 without hypertension (normal BP/healthy control; systolic BP: 90-120/ diastolic BP: 60-89mmHg) and with hypertension (systolic BP: 130-159 mmHg/ diastolic BP: 80-99 mmHg) that do not take anti-hypertension medication will be recruited.
Phase:	**Phase I**
Description of Sites/Facilities Enrolling Participants:	All data collection from human subjects will be conducted at North Carolina A&T State University (1 site). The studies will occur in the School of Nursing (Noble Hall) laboratory. The space is equipped for blood draws and private bathrooms for subjects to self-administer enemas. Nursing research staff will support the study by being present during each experiment, draw blood, and monitor participants. We will recruit subjects via posted fliers on campus, in the community, at our collaborating physicians primary care clinical hypertension practice (Veita Bland, MD.) and other local family practice clinics, and on a weekly radio health broadcast ("Its a Matter of Health". Veita Bland, MD. Wednesdays 530pm). Dr. Veita Bland provide clearance and see participants if they do not have a primary care physician. She has a clinical research staff that coordinates the practice participation in other Phase II & III Clinical Research Studies. She will also review clinical data (blood pressures) and monitor any adverse reactions reported by the study staff and research participants.
Description of Study Intervention:	There are 2 groups; control (without hypertension; BP: 90-129/60-89mmHg; 5 male/5 female, n=10) and experimental (with hypertension; BP 130-159/80-99 mmHg; 5 male/5 female, n=10). Normotensive participants will be age and sex-matched to hypertensive group participants. In ***Aim 1:*** AA individuals with and without hypertension (5 male/5 female) will provide stool and blood samples, and 24-hour (hr) ambulatory blood pressure (ABP) monitoring, for comparison of fecal butyrate-producing microbes and circulating butyrate between AA with and without hypertension. Baseline comparisons of circulating butyrate, fecal butyrate produces, and 24-hr ABP measures will be made between the normal and hypertension groups. Individuals with normal BP will only participate in donating stool and blood samples and wearing a 24-hr ABP monitor. In ***Aim 2:*** In the crossover blinded randomized controlled pilot study, the 10 hypertensive AA subjects (5 male/5 female) will be randomized to self-administer a sodium butyrate (80mM butyrate in 0.9% saline, 60 ml total) or control saline (0.9%, 60 ml total) enema 1 week apart (7 days) in this crossover research design. Subjects will provide a stool sample before each study day (2 total), have their BP manually measured, be fitted with their ABP monitor, submit to a

blood draws (pre-enema), self-administer the randomized enema, submit to a 30 min & 60 min post-enema blood draw, and wear a 24-hour ABP monitor for the remainder of the day. Subjects will be provided with written and verbal instructions on how to self-administer the enema. The dietary supplement (sodium butyrate) will be compounded by a local pharmacy (Custom Care Pharmacy - 109 Pisgah Church Rd. Greensboro, NC) into an enema at the concentrations listed above.

Study Duration: The timeline for study completion includes a recruitment plan to enroll the 20 subjects (10 control normotensive and 10 stage-1 hypertensive subjects) in the 1st 3-4 months (~5-7 subjects per month) with the expectation of sample testing in months 5-8, data analyses and publication submission in months 9-12.

Study Team: **Ian Carroll PhD, (Co-PI) Molecular Microbiologist.** Dr. Carroll is an Assistant Professor at the UNC Chapel Hill Department of Nutrition. He will process, analyze and interpret microbiome data. **Marc Cook PhD, (PI) Molecular Exercise Immunologist.** Dr. Cook will facilitate every aspect of the intervention at NC A&T, collect data and samples, perform blood biomarker assays and analyze BP, blood, and microbiome data with Dr. Carroll. **Veita Bland MD, (Collaborator) Primary Care Physician & Clinical Hypertension Specialist**. Dr. Bland has her own private practice, affiliated with Cone Health Hospital system, and is experience in running Phase II and Phase III clinical trials concerning hypertension. She is the lead clinical contact, will lend her expertise in subject recruitment, analyses and interpretation of relevant clinical data collected (BP).

Description of Schema: Samples and Measures for each group. After consent, control subjects (without hypertension) will complete questionnaires, including 5-day dietary recall, which will be immediately followed by self-collection of a fecal sample (within a 24-hour period before their lab visit). ***experimental visit 1***, control subjects will be fitted for their 24-h ambulatory BP monitor and have one (1) blood draw (time 1) and will go about their day. They will return the ABP monitor the next day. This will complete their study.

After consent, subjects with hypertension will complete questionnaires, including 3-day dietary recall, which will be immediately followed by self-collection of a fecal sample (within a 24-hour period before their lab visit). At ***experimental visit 1***, control subjects will have their BP manually measured and will be fitted for their 24-h ambulatory BP monitor, they will submit to a blood draw (pre) and then self-administer an enema (double blinded randomization to either a control or butyrate enema). After completing the enema, they will have subsequent blood draws at 30 minutes (post-30) and 60 minutes (post-60) post enema. They will continue to wear the ABP monitor and will go about their day. They will return the ABP monitor the next day. Seven (7) days, but not longer than 21 days after the ***experimental visit 1***, they will repeat the steps with the remaining enema ***experimental visit 2***.

In Aim 1 (cross-sectional design), between-group (control and hypertension) analyses will be performed from subject data collected at ***experimental visit 1***. The analyses will include quantifying differences in

mean hourly (day and night-time) BP measures, circulating butyrate levels and additional measures in section 8.2 (time 1), SCFA producing microbial biomass in stool samples, and dietary macro- and micro-nutrient intake.
In Aim 2 (double-blinded crossover design), within-group comparisons will be analyzed between circulating butyrate and additional blood variables (section 8.2), mean hourly (day and night-time) BP measures, and fecal SCFA producing microbes.

1.2 SCHEMA

Visit 1 Screening

- Total n=20 (10 normotensive and 10 hypertensive; 5 male and 5 female each group). *A telephone screening for meeting inclusion criteria will be performed prior to Visit 1. Subjects with hypertension will need PCP clearance*
- Review inclusion and exclusion criteria, receive medical clearance documentation for hypertension subjects, and obtain informed consent. Urine pregnancy test for women of childbearing age.
- Obtain history and complete questionnaires: Demographic, physical activity.
- Obtain Body Composition measures (weight, height, fat & fat-free mass) via bioelectrical impedence scale.

Visit 2 Sample Collection and Randomization

- Normotensive (Control, n=10): Submit Stool sample, submit to 1 blood draw, wear 24-hr ABP monitor
- Hypertensive Group (n=10): Randomized to either enema (randomization.com) will be performed by only one member of our research team. Self-administer enema, blood draws, wear 24-hr ABP monitor.

Visit 3 Follow-up assessments of study endpoints and safety

- Normotensive: Return ABP monitor and End of Study.
- Hypertensive: Return ABP monitor. Will return for Visit 4.
- Review of adverse reactions or events.

Visit 4

- Hypertension Group (n=10): Repeat Visit 2 with remaining enema

Visit 5 Follow-up assessments of study endpoints and safety

- Hypertensive (n=10) Return ABP monitor and End of Study.
- Review of adverse reactions or events.

Follow-up Telephone Call 7 days and 30 days after participation and Review of Data: 12 month

2 INTRODUCTION

2.1 STUDY RATIONALE

AA are grossly underrepresented in current and prospective studies of hypertension, although this group exhibits a significantly greater prevalence of endothelial dysfunction and hypertension. Hypertension is associated with genetic and environmental factors, such as cellular programming, diet, stress, and exercise. However, the gut microbiome has been linked to hypertension, and specific microbial profiles, characterized by low SCFA production, are associated with vascular disease. SCFAs may reduce BP by mitigating endothelial dysfunction. We are focusing on butyrate because research reports that gut butyrate is related to BP in humans and animals.

2.2 BACKGROUND

Large studies have demonstrated that genetic variation accounts for <2% of population variation in BP, and that daily activities and environmental factors play a significant role in BP regulation. BMI has a significant impact on cardiovascular disease risk and BP, but these relationships are also variable. Regardless of these associations, recent attention has been focused on gut microbial composition, SCFA production (such as butyrate), and BP. Butyrate is only produced in the gut, via fermentation in the colon, and absorbed into the circulation where it activates receptors in blood vessels can reduces BP. In the first study performed in humans, Yang et al observed that hypertensive subjects exhibited reduced gut SCFA production that was related to hypertension. However, this lone human study did not address race/ethnic demographics, or stage of hypertension.

As SCFAs are products of gut microbial metabolism, the results above suggest that the gut plays a role in BP control in humans. Oral administration of SCFAs reduced BP in mice, but this effect was attenuated in mice deficient in SCFA receptors Olfr78 and GPR41, which are present in EC and blood vessels. Further, prolonged (14d) butyrate infusion suppressed Angiotensin-II-induced hypertension. Recent studies show that butyrate-producing microbes, and gut butyrate concentrations, were lower in AA compared to native Africans and related to lower dietary fiber intake in AA. The result of low fiber intake is likely related to the prevalence of diseases, such as colon cancer incidence, glucose intolerance, and vitamin deficiency in the AA population. Decreased fecal butyrate content could indicate that more is absorbed by the gut. However, the above studies report that SCFA-producing microbes are less prevalent, suggesting a corresponding decrease in gut SCFA availability.

The use of enemas for delivering drugs has been considered in clinical care. High doses of butyrate can disrupt intestinal epithelial cell barrier function in animals. Here, we propose to use a low dose (80 mM) butyrate enema that has been reported safe in healthy volunteers and clinical populations. In respect to hypertension, gut SCFA concentration has been associated with BP control, but no clear mechanisms have been identified. In humans, we do not know whether SCFAs act outside of the colon (i.e., in the blood) to affect vascular function or work inside the colon, as butyrate has been shown to stimulate colon serotonin release and serotonin has some effect on BP. The latter mechanism is also poorly understood in humans. Thus, interventions to increase colonic butyrate are needed to determine its impact on vascular health. This proof of concept study will quantify the association between gut butyrate, circulating levels, and BP. Butyrate enemas have been used in healthy (*Vanhoutvin SA et. al. 2009)* and clinical populations of intestinal dysfunction (Steinhart, A.H. et al. 1996, Hamer et al. 2010) and have shown that delivery of butyrate, via enema, is safe and is not associated with any side effects. Further, there have been no reported side effects of oral ingestion of butyrate (Krokowicz et al. 2014).

Innovation: Direct delivery of butyrate into the gut has been shown to be safe and novel approach to quantify gut butyrate absorption and its effect on BP. Ultimately, data generated from this study will

be used to identify specific microbial butyrate producing taxa related to hypertension for the development of clinical prebiotic and probiotic interventions to increase SCFA production in the gut.

2.3 RISK/BENEFIT ASSESSMENT

2.3.1 KNOWN POTENTIAL RISKS

Potential Risks: Potential Risks: We assess the potential risks in our studies to be the following: 1) Enema: risk of psychological discomfort with performing the enema is possible and frequent bowel movement after the enema is expected. 2) Blood drawing: risk of bruising and infection is minimal with blood draw. Minor discomfort with venipuncture is also common; 3) Risk associated with 24 hour ABP monitoring: Infrequent sleep disturbances have been reported when the cuff inflates; 4) Risk associated with collecting fecal samples: Risk includes psychological discomfort, potential embarrassment in collecting this sample, and awkwardness of collecting the sample; 5) Failure to maintain participant confidentiality risk: collection of private health information always poses a risk to patient confidentiality as we will be routinely collecting data concerning BP, fecal bacteria, and clinical blood measures.

Protection against Risk: Concerning: 1). Enema: There are no known procedures for minimizing the risk of psychological discomfort in self-administering the enema. Subjects will have free access to clean and private restrooms to self-administer the enema and have subsequent bowel movements 2). Blood drawing: To minimize risks of the blood draw, a certified phlebotomist will use aseptic techniques to perform all blood draws. 3) 24-hour ABP monitoring: There are no procedures for minimizing the risk of infrequent sleep disturbances related to nighttime BP monitoring. BP will be taken every 30 minutes during wake hours and every hour during sleep hours 4). Risk associated with collecting fecal samples: To minimize risks, all personal protection equipment will be provided for the subject (gloves, collection containers). There are no known procedures for minimizing the risk of psychological discomfort in collecting the sample. 5). Failure to maintain subject confidentiality: To minimize risks, all information will be de-identified and handled in accordance with HIPPA guidelines.

No Social, economic, or legal consequences have been identified as potential risks.

2.3.2 KNOWN POTENTIAL BENEFITS

Potential Benefits: Research participants may not receive a direct clinical benefit from this research study, particularly healthy control subjects. Subjects will have 24h ABP results, which that they would not normally have. Each participant will have a dietary assessment and be informed about which food choices would benefit their BP. Further, the subject's primary physicians may have access to additional health-related information (24hr ABP results), at the subject's consent. The benefits to society will be the identification of strategies and novel therapeutic targets to reduce the risk of and attenuate hypertension in African American men and women in the North Carolina Triad area. Further, we will be able to inform the public about the benefits of gut health on clinical health outcomes. The risks to subjects are reasonable and minimal in relation to the anticipated benefits to participants and others because the potential benefits are primed to have a powerful impact on current and future health status of African Americans with hypertension.

2.3.3 ASSESSMENT OF POTENTIAL RISKS AND BENEFITS

Psychological:
Risk associated with the questionnaires: It is likely (10-25%) that subjects may feel a little uncomfortable, embarrassed, or incur emotional distress while sharing your personal health information or completing the questionnaires to assess stress or depression. To minimize these risks, subjects are free to skip any questions they do not wish to answer.

Emotional distress or Embarrassment: It is common (>25%) that subjects may experience embarrassment while collecting stool samples and/or self-administering an enema because of the personal nature of the sampling and administration of the enema rectally. In this study, subjects will be instructed (verbally and written) on how to perform the stool collection at home by themselves (provided all personal protection equipment) and return the sample to the laboratory. Also, subjects will be instructed (verbally and written) on how to properly self-administer the enema. Subject will also be given access to a private bathroom to perform the enema. There will also be an option for the subject to request the enema be administered by a nurse (collaborator). This will reduce the likelihood of embarrassment when participating in collecting the fecal sample (home) and enema self-administration (lab).

Consequences of breach of confidentiality: This study involves the collection of private health information (health history, measurement of biological markers in blood, blood pressure, and fecal microbial samples) and poses a risk to subjects' confidentiality. As a control for this risk, subject data will be de-identified by assigning each subject a study identification number. Files with subject information will be kept in locked office space within a locked storage cabinet. Electronic records will be kept on a laboratory computer (password protected) in an electronic file (e.g., RedCap, and/or Microsoft excel & Microsoft Word) that is also password protected.

Physical:

Medication (enema) side effects: An enema is primarily used to provide relief for occasional constipation. We expect that it will be very common (>50%) that subjects may have a bowel movement with 10 min of self-administering each enema (control (saline) or experimental (butyrate)). Side effects of a saline enema are reported to be abdominal pain, diarrhea, nausea, and vomiting. These side effects are often related to the severity of constipation individuals are hoping to alleviate. Since we are requiring a stool sample be collected within 24 hours of their data collection, we do not expect any subjects to be constipated. If they are, we will postpone their testing until the constipation issue is resolved. Enemas have also been used, clinically and experimentally, for drug delivery. Each active butyrate enema will consist of 60 mL (2.02 oz or 4 tablespoons) of an 80 mmol/L sodium butyrate solution titrated to a pH of 7.0 with sodium chloride added to maintain iso-osmolality. This concentration was not shown to "prompt any adverse effects in the treatment group and there were no withdrawals from drug toxicity". (Reference: Steinhart, A.H. et al. Aliment Pharmacol Ther 1996; 10: 729-736 and Hamer et al. Clinical Nutrition 2010 29: 738-744).

Pain: Blood drawing: risk of bruising is common (>25%) and infection is rare (<1%) with blood draws. Minor discomfort with venipuncture is also common (>25%). Blood will be drawn by a nurse or phlebotomist who is skilled to minimize pain and discomfort during the procedure.

Discomfort: The risk associated with 24-hour ambulatory BP monitoring has been reported to cause infrequent (1-10%) sleep disturbances when the cuff inflates. Because participants become familiar with the ABP monitor recordings during the day time, it is thought that participants are more familiar with it which reduces the occurrence of sleep disturbances.

Stool collection: There is no reported data on the risk of infection with self-fecal collection. Mishandling the sample can lead to infections (1-10%). This would be extremely rare in fairly healthy people who do not have existing opportunistic gut bacterial infection (e.g., Clostridium difficile (C. diff)). To reduce the risk of infection, subjects will be provided with personal protective equipment (gloves, collection and storage materials) and written step-by-step instructions on how to collect the samples. They will also be educated on safe hand washing techniques to reduce this risk to only rare cases (<1%). **Importantly,** samples will be collected via the clean catch method and the swab method. Subjects will be given written instructions (and equipment-gloves and toilet hat) to safely catch the bowel movement and store in a container with a screw top lid. Also, subjects will use a sterile swab and apply it to used toilet paper after having a bowel movement and store. They will handle their stool with the appropriate provided protective equipment to obtain the sample, which will also reduce the risk infection. They will store the samples in

their respective containers, place them in a bag, and place it in their freezer until they transport the sample (in a container with freezer packs) to the lab the next morning for their study.

No Social, economic, or legal consequences have been identified as potential risks.

3 OBJECTIVES AND ENDPOINTS

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Primary		
The primary objective is to demonstrate that the gut butyrate concentration is indirectly proportional to BP (greater gut butyrate leads to reduction in BP).	*In this proof of concept pilot study, the primary clinical endpoint is to:* 1. *Quantify the effect of butyrate into the gut (via enema) on BP measured over a 24-hour period.* *We hypothesize there will be a significant reduction in mean hourly BP, some time during the daytime and/or nighttime (dipping) 24-hour ABP monitoring period, ONLY after the butyrate enema. Also, we will quantify the effect of butyrate on blood biomarkers (inflammation, oxidative stress, and nitric oxide levels) that may correspond with lower BP in Hypertensive subjects.* *Data does not exist for estimating the effect size of the proposed treatment (cross-sectional or randomized double blinded cross-over study), which itself will be used to determine appropriate ranges of effect sizes for subsequent studies. We consulted Dr. Michael Love (UNC-CH NC TraCS Biostatistician).*	*Delivering the SCFA butyrate into the gut (via enema) and quantifying its absorption into the blood stream (where it is normally produced and absorbed), will aid in determining the role of gut SCFA production in the regulation of BP, and circulating biomarkers related to BP. We expect a reduction in BP within 24 hours of delivery of gut butyrate only. We also expect a reduction in blood biomarkers related to BP after the butyrate experiment only.*

4 STUDY DESIGN

4.1 OVERALL DESIGN

- In this proof of concept pilot study, our **central hypothesis** is that low gut butyrate availability is related to hypertension in African Americans (AA), and that acutely increasing gut butyrate (via a one-time butyrate enema) will translate into greater circulating butyrate and an acute and measurable reduction in BP. There are 2 groups; normotensive – control group (n=10; 5 men & 5 women) and hypertensive – experimental group (n=10; 5 men & 5 women).
- This is a Phase I clinical trial that will utilize a one-time dose dietary supplement (butyrate) compounded in an enema to determine the effect of gut butyrate delivery on absorption and BP in AA men and women with mild to moderate hypertension.
- In Aim 1, we will complete a cross-sectional study to quantify the differences in fecal SCFA microbes, circulating butyrate, and define associations between butyrate producing microbes, circulating butyrate, and BP between normotensive and hypertensive participants. Normotensive participants (control group) will submit a fecal sample with dietary records, have their BP measured, donate a blood sample, and wear a 24-hour ambulatory BP monitor for the remainder of the day. In Aim 2, we will perform a randomized double blinded placebo controlled cross-over study to determine the effect of a butyrate enema on BP in participants with mild-to-moderate hypertension. Participants with hypertension will perform the experiment by submitting a stool sample and dietary records, then have their resting BP measured manually before on their non-dominate arm. After the manual BP measurement, subjects will have the ambulatory BP (ABP) monitor placed on their non-dominant arm and undergo a blood draw. Subjects will then self-administer an enema (random control or butyrate), have their blood drawn 2 additional times within 1 hours (at 30 min and 60 min post enema) and wear a 24-hour ABP monitor for the remainder of the day. The monitor will measure BP every 30 minutes during waking hours (until 10pm) and every 1 hour during sleep hours (10pm-7am). The BP monitor will be removed when the subject returns the monitor the next morning. Seven (7) days, but no longer than 21 days, after performing the 1st experiment, participants will repeat the study with the remaining enema.
- Measurements in blood and stool: In each blood sample collected, we will measure plasma butyrate levels (mmol/L; performed in the Biomarker Mass Spectrometry Core in the Gillings School of Global Public Health at UNC-CH). In Dr. Cook laboratory, we will measure plasma markers related to BP ((IL-1β, CRP), oxidative stress (8-Isoprostane), nitric oxide (quantifying total nitrate/nitrite), and serotonin) via Elisa. Rationale for the additional blood biomarkers are that 1. Hypertension is joined with elevated inflammation, greater oxidative stress, and lower nitric oxide availability (endothelial dysfunction) and 2. butyrate is anti-inflammatory and may increase nitric oxide availability (improve endothelial function) and subsequently be followed by lower BP. We expect hypertensive subjects to have greater inflammation, oxidative stress, and lower nitric oxide levels compared to control subjects. Serotonin has been shown to be a byproduct of microbial metabolism and may be related to hypertension. We expect lower levels of IL-1 β and CRP, oxidative stress, and increased nitric oxide after the butyrate enema and no change after the control enema. The stool samples collected will be delivered to and processed by Dr. Carroll and analyzed in the UNC-CH Microbiome Core. Dr. Carroll will perform bioinformatic microbiome analyses of the raw sequence data.
- Dietary data analyses: Dietary intake will be assessed by three 24-h dietary recalls, collected with dietary recall sheets and either in person or by telephone using Nutrition Data System for Research (NDSR, University of Minnesota) software, using a multiple pass system. This method has been previously validated against doubly labeled water and has been proven to be accurate for assessing dietary intake in groups (Blanton et al 2006).

- To minimize bias in the methodology: 1. The PI's of this project will not consent any participant, but other research study personnel will obtain consent. 2. Randomization of experimental enemas (control and butyrate) will be performed by the compounding pharmacy and the key will be provided to the PI's at the completion of the study; 3: Sample testing (fecal microbiome and SCFA in blood) will be completed by core facilities at UNC-CH. RANDOMIZATION PROCESS
- The experimentation will occur at a single site (NC A&T) but sample testing and data analyses will occur at 2 sites (NC A&T and UNC-Chapel Hill).

4.2 SCIENTIFIC RATIONALE FOR STUDY DESIGN

Gut microbial imbalance and low SCFA production is implicated in hypertension and other diseases in AA, which are characterized by decreased gut SCFA production. This is a randomized crossover design where participants with hypertension will be their own control to quantify the effect of gut butyrate on BP. Subjects with hypertension will perform both enema treatments (control saline enema or experimental butyrate enema) no sooner than 7 days, but no later than 21 days, apart. We expect to quantify measurable increases in circulating butyrate in the blood that will correlate with measurable decreases in BP after the butyrate enema and not the control saline enema. In Aim 1, comparisons between normotensive and hypertensive participants will be made concerning fecal microbial characteristics and SCFA producing bacterial biomass, circulating (blood) butyrate levels, and 24-hour BP (averaged every hour during waking and nighttime hours). The control group will be age (±1 year) and sex matched to the hypertension group participants. ***Rationale for Length of Washout Period***: We propose no less than a 7 days between self-administration of the enemas to be a sufficient washout period because the laxative effects of the enema are normally immediate (within 30 minutes if no bowel obstruction is present which will not be likely as subjects will need to have a bowel movement the day before or morning of the experiment), transient (not lasting longer than a 24 hour period if no significant constipation), and absorption and metabolism of gut butyrate is expected to be fairly quick (as colonocytes will absorb). Also, we expect circulating butyrate levels to exert their effect (if any) on BP within a 24-hour period. Participation in the study will be halted if subjects have any issues with constipation and cannot provide a stool sample before the experiment.

4.3 JUSTIFICATION FOR DOSE

Enemas have also been used, clinically and experimentally, for drug delivery. Each active butyrate enema will consist of 60 mL (2.02 oz. or 4 tablespoons) of an 80 mmol/L sodium butyrate solution titrated to a pH of 7.0 with sodium chloride added to maintain iso-osmolality. This concentration was not shown to "prompt any adverse effects in the treatment group and there were no withdrawals from drug toxicity". (Reference: Steinhart, A.H. et al. Aliment Pharmacol Ther 1996; 10: 729-736 and Hamer et al. Clinical Nutrition 2010 29: 738-744). Participants with hypertension will only perform 1 control enema and 1 butyrate enema seven days apart.

4.4 END OF STUDY DEFINITION

Normotensive participants (control) will be considered to have completed the study after he or she has completed Visit 3 (shown in Schedule of Activities section 1.2).

Hypertension participants will be considered to have completed the study after he or she has completed all phases of the study at Visit 5 (shown in Schedule of Activities section 1.2)

5 STUDY POPULATION

5.1 INCLUSION CRITERIA

Normotensive (control subjects **without hypertension**): In order to be eligible to participate in this study, an individual must meet all of the following criteria (which will be assessed after an initial telephone interview and at Visit 1 (screening and consent visit):

1. Provision of signed and dated informed consent form
2. Be an African American adult (man or woman) between 30 - 50 years of age with normal blood pressure (*never diagnosed with hypertension*) (systolic: 90-120 and diastolic: 60-89 mmHg).
3. Body Mass Index of 18.5-30 kg/m^2.
4. Not have any other diagnosed cardiovascular disease
5. Not exercise regularly (Participate in less than 60 minutes of exercise/week)
6. Not be pregnant or be lactating
7. Be free of active diseases that affect your intestines (i.e., chronic constipation, diarrhea, Crohn's disease, ulcerative colitis, irritable bowel syndrome, diverticulosis, stomach or duodenal ulcers, diabetes, hepatitis, HIV, and cancer)
8. Have not taken antibiotics in the past 3 months
9. Have not been regularly taking medications that impact intestinal function (i.e., laxatives, enemas, anti-diarrheal agents, narcotics, antacids, antispasmodics, antidepressants, anticonvulsants, antibiotics, herbals, homeopathy, and home remedies) or fiber supplements.
10. Have no plans of travel out of town during the study periods.
11. Agreement to adhere to Lifestyle Considerations (see section 5.3) throughout study duration

Hypertension subjects not on anti-hypertension medication (intervention group). *The pool of subjects that do not take hypertension medication may be limited. If we encounter a heavily medicated subject population, we may include subjects taking diuretics only*: In order to be eligible to participate in this study, an individual must meet all of the following criteria (which will be assessed after an initial telephone interview and at Visit 1 (screening and consent visit):

1. Provision of signed and dated informed consent form. *Letter of clearance or signature of PCP on informed consent.*
2. Be an African American adult (man or woman) between 30 - 50 years of age *with stage-1 to stage-2 hypertension* (systolic: 130-159 and diastolic: 80-99 mmHg).
3. Not taking any anti-hypertension medications (although we may enroll individuals only taking a diuretic where resting BP levels are within the range of stage-1 hypertension: systolic BP 130-140 mmHg. Subjects can resume taking the diuretic after they remove the monitor).
4. Body Mass Index of 18.5-30 kg/m^2
5. Not have any other diagnosed cardiovascular disease
6. Not exercise regularly (Participate in less than 60 minutes of exercise/week)
7. Not be pregnant or be lactating
8. Be free of active diseases that affect your intestines (i.e., chronic constipation, diarrhea, Crohn's disease, ulcerative colitis, irritable bowel syndrome, diverticulosis, stomach or duodenal ulcers, diabetes, hepatitis, HIV, and cancer)
9. Have not taken antibiotics in the past 3 months
10. Have not been regularly taking medications that impact intestinal function (i.e., laxatives, enemas, anti-diarrheal agents, narcotics, antacids, antispasmodics, antidepressants, diuretics, anticonvulsants, antibiotics, herbals, homeopathy, and home remedies) or fiber supplements.

11. Have no plans of travel out of town during the study periods.
12. Agreement to adhere to Lifestyle Considerations (see section 5.3) throughout study duration

5.2 EXCLUSION CRITERIA

An individual who meets any of the following criteria will be excluded from participation in this study:

1. Exercise more than 60 minutes per week for more than 4 consecutive weeks.
2. Diagnosed with stroke, history of myocardial infarction (heart attack); liver, lung, or kidney diseases; peripheral vascular disease or cancer within the last 6 months.
3. Presence of metabolic disease (diabetes mellitus), inflammatory diseases (e.g., inflammatory bowel diseases, rheumatoid arthritis, and systemic lupus erythematosus); kidney stones or gallbladder problems; diagnosed liver, lung or kidney diseases;
4. Pregnancy, lactation, or actively trying to conceive.
5. Taking anti-hypertension medications (i.e., calcium channel blockers, ACE inhibitors, angiotensin- receptor blockers, β-blockers, vasodilators, etc.) other than diuretics (e.g., hydroclorothiazide, chlorothiazide, furosemide, etc) or medications known to affect inflammation or metabolic function (anti-inflammatories, statins, thyroid medication) in the past 1 month. If diuretics are used, subjects may able to participate if they agree to refrain from taking their diuretic the day of the experiment. In this instance, resting systolic BP while on their medication will still need to be greater than 130 mmHg.
6. Current smoker or tobacco use within the last 10 years

5.3 LIFESTYLE CONSIDERATIONS

During this study, participants are asked to:

- Refrain from consuming alcohol once their testing period starts (3 days before you collect your fecal sample and your Visit 2.)
- Not change their eating habits.
- Participants must not change their exercise habits (start to exercise or exercise more than 60 min per week during the study).
- Participants taking diuretics may be asked to refrain from taking their medication on the day of the experiment.

If the participant cannot adhere to these lifestyle considerations, they may be withdrawn from the study.

5.4 SCREEN FAILURES

Screening Failures: We will account for the total number of potential subjects contacted, and those actually enrolled in the study. Individuals who do not meet the inclusion/exclusion criteria for participation in this trial (screen failure) will be excluded. Individuals who do not agree to perform every aspect of the study, refuse to adhere to the lifestyle considerations during the study, or take longer than 7 days to sign and return the informed consent (after participating in the initial telephone screening and/or Visit 1 informed consent, respectively), may be rescreened if they decided to participate later. Rescreened participants will be assigned the same participant number as for the initial screening.

5.5 STRATEGIES FOR RECRUITMENT AND RETENTION

We will recruit 10 normal-to-overweight (BMI 18.5-30 kg/m^2) African American (AA) adults (5 male/5 female, 30-50 y.o.) with normal BP (without hypertension) (90-120/60-89mmHg) and 10 age- and gender-matched normal-to-overweight AAs with stage-1 and stage 2 hypertension (130-159/80-99 mmHg). AA with hypertension will perform the intervention, while acting as their own control, to determine the effect of gut butyrate on BP. This is a single site study where participants will be recruited from the NC A&T community, surrounding area, and from the private practice of our collaborating physician Dr. Bland. Fliers will be posted on campus, in community centers, and at Dr. Bland's office. Dr. Bland will also recruit during her weekly radio show "It's a Matter of Health" on NC A&T radio station 90.1 FM. Subjects will respond to fliers and contact the research staff via email or phone. Our group is experienced in culturally sensitive methods of subject recruitment in AA, via strong community relationships and health education, and we expect no issues with recruitment. Also, the sample size is small (n=10) in the intervention portion and we do not expect that we will exhaust the subject pool that may complete the study. We will work to minimize non-compliance and provide monetary incentive in this short study. Incentives will occur in the form of $100 gift cards for completing each portion of the study. For control subjects, they can receive $100 for completing the study (at Visit 3). Hypertension subjects can receive up to $200 for completing the study ($100 after completing the enema, blood draws, submitting fecal sample, and returning the ABP monitor at Visit 3 and Visit 5).

6 STUDY INTERVENTION

6.1 STUDY INTERVENTION(S) ADMINISTRATION

6.1.1 STUDY INTERVENTION DESCRIPTION

Participants with hypertension will self-administer 2 enemas, 7 days apart. 1 enema will be a 0.9% control saline enema (60 ml) and the other enema (experimental) will contain sodium butyrate at a concentration of 80 mM in 60ml total volume. Both the subjects and the PI's will be blinded to the order the enemas are performed (double blinded crossover design). One member of the research staff will perform the blinding and will release the key at the conclusion of the study (all participants completed). Both enemas will be made/compounded at Custom Care Pharmacy (Greensboro, NC) for quality control and matched for sodium concentration.

Justification for IND exemption: According to the Guidance for Clinical Investigators, Sponsors, and IRBs IND Guidelines, the use of the sodium butyrate enema would be classified as exempt from the need for FDA review and regulation for this study. Sodium Butyrate is a short chain fatty acid that is commercially available as a dietary supplement. Delivery of butyrate, via enema, has been used and reported in published clinical studies. Two studies have used the butyrate enema to quantify its effect on gut inflammation (Hamer et al. Clin Nutr 2010, 29:738-44; Steinhart et al. Aliment Pharmacol Ther 1996, 10:729-36. In these studies, there were no reported adverse reactions or side effects in its use in the clinical populations.
Rationale: Although butyrate is commercially available in ingestible forms (powder and pills), we do not normally ingest butyrate. Certain bacteria in our gut (colon) produce it from fermenting food products (fiber). The level of butyrate producing bacteria has been associated with blood pressure (BP) and it is believed that greater butyrate production in the gut is associated with better cardiovascular health and

lower BP. The butyrate produced in the gut is absorbed by it into our blood stream, just as other nutrients. Our purpose in utilizing the butyrate enema is to deliver a measurable, and safe, amount of butyrate into the colon (where it is naturally produced). We are not using it to diagnose, treat, or cure high BP (hypertension). We are only using this delivery method to quantify how much is absorbed into the blood stream and measure if it has any effect on BP in individuals with moderately elevated BP.

The enemas will be compounded by a local pharmacy based on concentrations previously used in published studies. Our collaborating study physician will write a prescription for the placebo (0.9% saline, as found over-the-counter) and butyrate enema to be made. Each active butyrate enema will consist of 60 mL (2.02 oz or 4 tablespoons) of an 80 mmol/L sodium butyrate solution titrated to a pH of 7.0 with sodium chloride added to maintain iso-osmolality. The compounding pharmacy will label each enema with the following wording: "Caution: New Drug—Limited by Federal (or United States) law to investigational use," in conjunction with the regulations found in 21 CFR 312.6. The study is a double-blinded randomized controlled trial.

Randomization will be performed by 1 member of the research team at the beginning of the study. Enemas will be assigned a letter (e.g., A or B) and each letter will be placed in an envelope. Each time a subject completes consent, the research staff responsible for randomization will randomly choose an envelope which will coincide with which enema they will receive first at Visit 2 (A or B). They will receive the other enema at their Visit 4. That staff member will reveal the key upon the conclusion of the study.

6.1.2 DOSING AND ADMINISTRATION

Participants with hypertension will complete 2 studies, 1 with each enema (control saline or butyrate) in a random order. Dosing was based on previous studies that report no adverse events associated with the sodium butyrate enema below 80 mmol/L concentration. Enemas will be performed in the morning (before 9am). We will educate participants on how to efficiently perform the self-administered enema, to reduce variability, and measure remaining solution to calculate dose delivered. Nurses will be present to aid in administering enema's if participants are uncomfortable with doing this themselves. The PI and nurses will be present during the entire experiment time. BP will be monitored, via the 24-hour ABP monitor, for 1 ½ hours after the enema in the laboratory and then the subject will go home with an ABP monitor and will wear it until they return it the following day (see procedures in section 4.1, bullet 3).

6.2 PREPARATION/HANDLING/STORAGE/ACCOUNTABILITY

6.2.1 ACQUISITION AND ACCOUNTABILITY

Subjects with hypertension will need medical clearance from their primary care physician (PCP) before they can be included in the study (see Schema 1.2 – Visit 1 and Inclusion - Section 5.1). If they do not have a PCP, they will be referred to Dr. Bland, our collaborating and study physician to provide medical clearance. Dr. Bland will write the prescription for the enemas to be compounded by the pharmacy for all participants. The pharmacy will generate both enemas and research staff (not the PI's) will pick up the prescriptions. Randomization process is described in section 6.1.1.

6.2.2 FORMULATION, APPEARANCE, PACKAGING, AND LABELING

Both enemas (control and butyrate) will be in an enema applicator bottle (60ml total volume). Sodium butyrate is available for purchase as a dietary supplement. We will supply the pharmacy with order information for its purchase for them to use. The compounding pharmacy will label the package with Enema A or B. One will be the experimental enema with butyrate and one will be the control. Only the pharmacy and the research staff member responsible for the randomization will know which enema A and B are. The pharmacy will also label the enema with this statement: Caution: New Drug—Limited by Federal (or United States) law to investigational use" in accordance with the regulations found in 21 CFR 312.6.

6.2.3 PRODUCT STORAGE AND STABILITY

Both enemas will be stored at room temperature by the pharmacy and by the researchers until giving them to the participants on the testing day. Again, their randomization schedule will be completed once they complete informed consent (at Visit 1) and have a random envelope chosen for their treatment enema. They will receive that enema, either A or B, at Visit 2 and the other enema at Visit 4.

6.2.4 PREPARATION

Preparation of both enemas will be done by the compounding pharmacy. The study staff will not need to do any preparation to the products other than mixing (shaking well) before giving them to the subjects before self-administration.

6.3 MEASURES TO MINIMIZE BIAS: RANDOMIZATION AND BLINDING

Aim 2 is a double blinded randomized placebo-controlled crossover trial. Only the participants with hypertension will perform the enema in a randomized order. The subjects, nor the PI's, will know which enema the subjects are receiving. Randomization will be performed by 1 member of the research team at the beginning of the study whom is also not involved in any informed consent process. Enemas will be assigned a letter (e.g., A or B) and each letter will be placed in an envelope (blinded allocation concealment). Each time a subject completes consent, the subject will be presented with a stack of envelopes will choose an envelope which will contain a card coinciding with which enema they will receive first at Visit 2 (A or B). They will receive the first enema at Visit 2 and the remaining enema at their Visit 4. The research team member will reveal the key upon the conclusion of the study.

Since there has been no reported side effects of the butyrate enema, we do not anticipate being able to identify which enema was administered because of side effects. The only side effect for both would be a bowel movement. We expect all participants to have a movement within 10 minutes of the administration.

6.4 STUDY INTERVENTION COMPLIANCE

Adherence to the protocol will consist of participants completing the following:
Control subjects (without hypertension):

1. Completing the 3-day dietary recall and submitting a fecal sample immediately before (day prior) or on the testing day.
2. Submitting to a manual resting BP measurement and a (1) blood draw.

3. Wearing the 24-hour ambulatory blood pressure monitor after the blood draw and returning the monitor with activity diaries on the following day.

Hypertension subjects:

1. Completing the 3-day dietary recall and submitting a fecal sample immediately before (day prior) or on each experimental testing day (two in total).
2. Submit to a manual BP measurement and blood draw (time 0) then self-administer the enema and submitting to subsequent manual BP measurements and blood draws (time 30min & 60 min) after the enema.
3. Wearing the 24-hour ambulatory blood pressure monitor after the blood draws and returning the monitor with activity diaries on the following day.

Adherence to the protocol will be encouraged with a monetary payment after completion of the steps above and returning the monitor.

6.5 CONCOMITANT THERAPY

Not Applicable

6.5.1 RESCUE MEDICINE

Not Applicable

7 STUDY INTERVENTION DISCONTINUATION AND PARTICIPANT DISCONTINUATION/WITHDRAWAL

7.1 DISCONTINUATION OF STUDY INTERVENTION

The proposed project is a phase I clinical trial that does not have multiple sites. There is no inclusion of vulnerable populations and does not involve “high risk” procedures. This is an uncomplicated study that includes enemas as the sole experimental intervention. With this, the PI, study physician, and nursing collaborators will primarily be responsible for monitoring the safety of each participant. The PI will be responsible for reviewing overall study progress and minimizing research associated risk by monitoring activities of the research and updating records on a weekly basis with study team members. Dr. Cook, in consultation with the study physician (Dr. Bland), will report any adverse events to the IRB. Any sign, symptom, or abnormal assessment during the study will be immediately reported to Dr. Bland via phone, when she is not present, whom will assess the event at that time. Every effort will be made to determine the cause of any events that occur during the subjects’ participation in the study to determine if the adverse event was related to study activities. Under the direction of the study physician and IRB, we will suspend any activity that related to any consistent signs of initiating adverse events in our subject population. Whenever appropriate, we will amend informed consent and/or study protocol documents in response an adverse event.

7.2 PARTICIPANT DISCONTINUATION/WITHDRAWAL FROM THE STUDY

Participants are free to withdraw from participation in the study at any time upon request.

An investigator may discontinue or withdraw a participant from the study for the following reasons:

- Pregnancy
- Significant study intervention non-compliance

- If any clinical adverse event (AE), other medical condition, or situation occurs such that continued participation in the study would not be in the best interest of the participant
- Participant develops any infection that requires antibiotic prescription and usage
- Disease progression which requires discontinuation of the study intervention
- If the participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation
- Participant with Hypertension unable to complete the enemas within 21 days of each other.

The reason for participant discontinuation or withdrawal from the study will be recorded on their Case Report Form (CRF). Subjects who sign the informed consent form and are randomized but do not receive the study intervention may be replaced. Subjects who sign the informed consent form, and are randomized and receive the study intervention, and subsequently withdraw, or are withdrawn or discontinued from the study, will be replaced.

7.3 LOST TO FOLLOW-UP

A participant will be considered lost to follow-up if he or she fails to return for the data collection or experimental scheduled visits for 2 weeks and is unable to be contacted by the study site staff.

The following actions must be taken if a participant fails to return for a required study visit:

- The site will attempt to contact the participant and reschedule the missed visit within 1 week of contact and counsel the participant on the importance of maintaining the assigned visit schedule and ascertain if the participant wishes to and/or should continue in the study.
- Before a participant is deemed lost to follow-up, the investigator or designee will make every effort to regain contact with the participant (where possible, 3 telephone calls and, if necessary, a certified letter to the participant's last known mailing address or local equivalent methods). These contact attempts will be documented in the participant's study file.
- Should the participant continue to be unreachable, he or she will be considered to have withdrawn from the study with a primary reason of lost to follow-up.

8 STUDY ASSESSMENTS AND PROCEDURES

8.1 EFFICACY ASSESSMENTS

Telephone screening

1. Once subjects respond, via email or phone, to recruitment materials, we will assess if they meet the inclusion criteria for either group via phone (*see telephone screening questionnaire*). After completion of the questionnaire, the screener will be able to make the potential subject aware of their eligibility. The telephone screener will be able to let the. We will mail their informed consent or schedule a time for the participant to pick one up and schedule their Visit 1 at that time.

Visit 1: Screening visit – Informed consent and questionnaires

1. Medical history, physical activity, and dietary recall questionnaires: The PI (Dr. Marc Cook) will review the medical history questionnaire to ensure all the questions and their answers regarding

the participant's health, current medication use, medical history and exercise pattern are clear. The PI will also confirm that their PCP has agreed with their participation in the study. Participants will also fill out a physical activity readiness questionnaire (PAR-Q) to determine the amount of time they exercise currently. The PI will review these documents to ensure eligibility to participate in this study. Participants will be coached on how to complete their food and beverage intake for the 3 days following this initial screening. We will provide the paper forms to complete the records before they submit the stool samples. Pre-menopausal women will be asked to confirm they are not pregnant by taking an over-the-counter pregnancy test we will provide. This is the only time a participant will be asked to take a pregnancy test. A member of the research team will assess that the result is negative for them to proceed through the study.

2. Manual blood pressure will be recorded 3 times within 3 minutes, on the participants non-dominant arm, after sitting for 5 min. An average of the last 2 BP readings will be recorded as their resting BP. This BP measurement will also confirm that they are Normotensive (BP ≤129 mmHg systolic) or have Hypertensive (BP ≥ 130 mmHg systolic).
3. Body Composition: Height and body weight measured on a scale that will determine body weight, body fat mass, body muscle mass, and total body water. The scale requires standing on the scale barefoot and hold on to the machine. The test takes approximately 2 minutes to complete the measurement.

Visit 2: Measurements and Sample collection

1. Body Composition (both groups): Body weight will be measured on a scale that will determine body weight on the same scale as before. The test takes approximately 2 minutes to complete the measurement. This is to ensure no significant shifts in body composition (weight gain or loss ±2.2 lbs. or 1 kg) since their visit 1.
2. Stool sample collection (both groups): Participants will submit a stool sample collected at home within 24-hours of this visit (collected the day before or the morning of the experiment) and will submit the 3-day dietary recall. Stool samples will be collected by the clean catch method and the swab method. Participants will use the protective equipment given (gloves, toilet hat, spoon, and sterile container) to collect a spoonful stool sample for fecal butyrate analysis. Participants will also apply a sterile swab to used toilet paper to collect the sample for fecal microbiome analyses and place in the sterile container. If there is minimal residue on the toilet tissue after wiping, the subject will need to gently rub the tip of the sterile swab across their stool sample collected, via the clean catch method, and place in the sterile container.
3. Placement of 24-hour ABP monitor (both groups): Participants will be fitted for their ABP monitor, rest for 5 minutes, and have their resting BP measured 3 consecutive times. The cuff will remain on their non-dominant arm throughout the experiment and until they return the monitor the next day.
4. Blood sampling (both groups): Blood will be taken from the antecubital (forearm) vein by a phlebotomist or nurse. A small amount (about 3 tablespoons) of blood will be taken. Plasma butyrate will be measured by the UNC-CH Biomarker Mass Spectrometry Core in the UNC Gillings School of Global Public Health. Dr. Cook will measure biomarkers in plasma (IL-1β, CRP), oxidative stress (8-Isoprostane), nitric oxide (quantifying total nitrate/nitrite), and serotonin via Elisa (R&D-Abcam) in his laboratory.
    a. In hypertensive subjects only: Blood draw will occur right before they complete the enema. After the enema, participants will have less blood taken (about 2 tablespoons) at 30 minutes and 60 minutes (1 hour) after administering the enema.

5. In Hypertensive subjects only: Blood pressure will be measured manually and then after the placement of the ABP monitor on their non-dominant arm before the enema, manually before the 30 min blood draw and manually before the 60 min blood draw. The PI and nursing research staff will ensure that participants are not experiencing any symptoms related to the treatment, as well as make sure their BP is within the normal range for them. Participants will be monitored for longer if they exhibit a ≥ 20 mmHg change in their BP along with symptoms (presyncope). If Dr. Bland is not present, she will be called and alerted to the event immediately.
6. 24-hour blood pressure monitoring (both groups): will be obtained via Mobil-O-Graph® 24h PWA (IEM) ambulatory blood pressure (ABP) monitoring device. Subjects will be fitted with the non-invasive monitor (Mobil-O-Graph BP PWA) to measure BP and arterial stiffness for 24-hrs on the day they perform each enema. The 24-hr period will begin after completion of the enema and blood draws. The BP cuff will be fitted to participant's non-dominant arm with cuff size determined by upper arm circumference. BP measurements will be obtained at 30-min intervals during the day (700–2200 hours) and 60-min intervals at night (2200–700 hours). Participants will be instructed not to exercise before or during the monitoring period and to pause momentarily and maintain their body position during each BP measurement. They will also be given instructions (verbal and written) on how to keep a blood pressure diary, where they will record their activities at the time the monitor was recording the blood pressure.

Visit 3: Return the 24-hour BP monitor (both groups). Participants will receive compensation at this time. **This will complete the control (Normotensive subjects study)**

Visit 4: **Hypertension participants only**: Repeat Visit 2 by completing the other enema.

Visit 5: Return the 24-hour BP monitor. You will receive compensation at this time.

8.2 SAFETY AND OTHER ASSESSMENTS

- ***Assessment of Eligibility****. Preliminary review of eligibility will occur during their initial telephone screening performed by research staff. They can be excluded from the study then if then if they do not meet all the inclusion criteria. If it is appropriate to continue, they will be sent (or can pick up) an informed consent form prior to their official Visit 1 to review and have their primary care physician (PCP) review. Participants with hypertension will need to obtain approval from their PCP to ensure they are healthy enough to participate in this study. We expect most, if not all, of our participant pool to be recruited from Dr. Bland's practice. They will not be required to have a full physical.*
    - *If the participant has a PCP, the participant will be given their informed consent for their PCP to review the study protocols. The PCP will acknowledge that the patient has mild-to-moderate hypertension (within the ranges specified in the informed consent) and there are no contraindications to their participation. The PCP will provide the research team with a letter stating that they have reviewed the study information and they approve of their patient participating in the study.*
    - *If the participant **does not** have a PCP, they will be referred to Dr. Bland. She will review their history and provide an assessment their ability to participate by confirming they do have mild-to-moderate hypertension and there are no contraindications to their participation. This will be done free of charge to the participant. However, because they do not have a regular PCP, they may become a patient of Dr. Bland's (or referred to*

another physician) which they would pay for services. This is not prerequisite on them participating in the study.
    - *After this, the PI will assess if the participant continues to meet all the inclusion criteria to be admitted into the study.*
    - *Dr. Bland will review the provided medical history form of each participant and confirm their PCP has given consent for them to participate before writing the prescription for the enema's to be compounded by the Pharmacy.*
- ***Vital signs**. Blood pressure will be monitored every 30 minutes while they are in the lab during the experiment and for 24-hours after they self-administer the enema (every 30 minutes during waking hours and every hour while they are asleep). If the participant experiences any AE's during the experiment (in the lab), the research team will contact Dr. Bland immediately, via phone, if she is not present. If they experience any AE's after leaving, participants will be directed to immediately contact the research team (PI or nurses) to assess and we will contact Dr. Bland if needed. If they experience any SAE's they will be directed to seek emergency services and Dr. Bland will be notified immediately.*
- ***Biological specimen collection and laboratory evaluations**. We will collect stool samples and blood. Bioinformatics on gut microbial composition (diversity, richness, biomass) and identification of specific SCFA producing microbes will be performed and compared between AA with and without hypertension. Blood will be collected to measure circulating butyrate levels at rest and after each enema (in hypertension group only). Gut microbial bioinformatics and blood butyrate concentrations will be measured by Microbiome Core and Biomarker Mass Spectrometry Core laboratory facilities, respectively, at UNC-Chapel Hill. In Dr. Cook's laboratory, we will also measure biomarkers related to vascular health and blood pressure via enzyme linked immunosorbent assays (Elisa) (e.g., inflammatory cytokines (C-reactive protein & interleukin-1β), nitric oxide, endothelin-1, oxidative stress (8-isoprostane), and serotonin). Each of these measures has been identified to be related to elevated BP in humans. Butyrate, being anti-inflammatory, and the abundance of microbes that produce butyrate are likely related to BP. Each of these measures, along with body composition measures and macronutrient (carbohydrate – with soluble and insoluble fiber, fat, & protein) of 3-day dietary recall will be utilized to help explain gut microbial composition, circulating biomarkers, and the effect of butyrate enema on BP in the 24 hour period. Dr. Carroll will perform bioinformatic statistical analyses in his laboratory. Comparative statistical analyses will be performed to define the relationship between BP, gut microbial composition, circulating butyrate, and biomarkers of vascular health.*
- ***Counseling procedures, including any dietary or activity considerations**. At the screening visit (1), participants will be counseled on how to complete their dietary recall.*
- ***Administration of the enema**. Participants, at Visit 1 and 2 (right before administration), will be educated on how to self-administer the enema. They will be provided with a private bathroom space to administer it. Research staff will be close by to support the participant if needed. If the participant is uncomfortable with self-administering the enema, a research staff who is a nurse may administer it for them if the participant chooses. After the administration, the participant will recap the enema applicator bottle and return it to the research staff to measure how much they were able to deliver.*
- ***Assessment of study intervention adherence**.*
  Adherence to the protocol will consist of participants completing the following:
  <u>Control subjects (without hypertension)</u>:
    a. Completing the 3-day dietary recall and submitting a fecal sample before the testing day.
    b. Submitting to a blood draw.

c. Wearing the 24-hour ambulatory blood pressure monitor after the blood draws and returning the monitor with activity diaries on the following day.

Hypertension subjects:

a. Completing the 3-day dietary recall and submitting a fecal sample before each experimental testing day.
b. Self-administering the enema and submitting to a blood draws.
c. Wearing the 24-hour ambulatory blood pressure monitor after the blood draws and returning the monitor with activity diaries on the following day.

Adherence to the protocol will be encouraged with a monetary payment after completion of the steps above and returning the monitor.

- *Same as Study Intervention Compliance, section 6.4*

- ***Administration of questionnaires or other instruments****. Participants will keep a dietary record for 3 days before they submit any stool sample. They will also be provided with a paper diary to record their activities when their blood pressure was being recorded by the ambulatory BP monitor. Subjects will also complete a "perceived stress" questionnaire in effort to assess the current levels of perceived psychological stress. Heightened stress has been associated with higher blood pressure (Hamer M. 2012 Psychosom. Med. Nov-Dec;74(9):896-903) and reduced gut microbial diversity (Moloney RD. et al. 2014 Feb;25(1-2):49-74).*
- ***Assessment of adverse events (AE's).*** *Research staff (the PI and Nursing Faculty at NC A&T) will be present during the experiment, will draw blood, and will assess any adverse events witnessed or reported by the participant (specifically unfavorable changes in BP, although this phenomenon and others have not been previously reported). Dr. Bland will be notified, via phone of she is not present, immediately. We will also see the participants the following day when they return their ABP monitor. At that time, we will collect their BP diary and follow up with any issues reported by the participant. Participants will be instructed to discontinue the use of the ABP monitor if any significant skin irritation occurs that is not bearable.*
    - ***Follow-up.*** Study staff (Dr. Cook and Nurses) will record all reportable events with start dates occurring any time after informed consent is obtained until 7 (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation. We will instruct participants to inform us (phone call or email) if they feel they are experiencing symptoms that may be related to their participation in this study. We will also contact them, via their preferred contact method (phone or email) at days 7 and 30 post study to inquire about them experiencing AE or SAEs. At each study visit, we will inquire about the occurrence of AE/SAEs since the last visit. Events will be followed for outcome information until resolution or stabilization.

8.3 ADVERSE EVENTS AND SERIOUS ADVERSE EVENTS

8.3.1 DEFINITION OF ADVERSE EVENTS (AE)

In this study, an Adverse Event means any untoward medical occurrence associated with the use of the study specific intervention tools (enema, sample collections, BP monitor) in the participants, or whether or not an event occurs while the participant is actively enrolled in the study and requires assessment that the AE could be intervention-related.

8.3.2 DEFINITION OF SERIOUS ADVERSE EVENTS (SAE)

An Adverse Event or suspected adverse reaction is considered "serious" if, in the view of either the investigator or funding agency, it results in any of the following outcomes: death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

8.3.3 CLASSIFICATION OF AN ADVERSE EVENT

8.3.3.1 SEVERITY OF EVENT

For adverse events (AEs) not included in the protocol defined grading system, the following guidelines will be used to describe severity.

- **Mild** – Events require minimal or no treatment and do not interfere with the participant's daily activities.
- **Moderate** – Events result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.
- **Severe** – Events interrupt a participant's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually potentially life-threatening or incapacitating. Of note, the term "severe" does not necessarily equate to "serious".

8.3.3.2 RELATIONSHIP TO STUDY INTERVENTION

All adverse events (AEs) will have their relationship to study intervention assessed by the clinician (Dr. Bland) who may examine and evaluate the participant based on temporal relationship and his/her clinical judgment. The degree of certainty about causality will be graded using the categories below. In a clinical trial, the study product will always be suspect.

- **Related** – The AE is known to occur with the study intervention, there is a reasonable possibility that the study intervention caused the AE, or there is a temporal relationship between the study intervention and event. Reasonable possibility means that there is evidence to suggest a causal relationship between the study intervention and the AE.
- **Not Related** – There is not a reasonable possibility that the administration of the study intervention caused the event, there is no temporal relationship between the study intervention and event onset, or an alternate etiology has been established.

8.3.3.3 EXPECTEDNESS

Dr. Veita Bland, MD and the PI Dr. Marc Cook, will be responsible for determining whether an adverse event (AE) is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the study intervention. Dr. Cook and clinical research nursing staff will monitor participants during and immediately after the experiments.

8.3.4 TIME PERIOD AND FREQUENCY FOR EVENT ASSESSMENT AND FOLLOW-UP

The occurrence of an adverse event (AE) or serious adverse event (SAE) may come to the attention of study personnel during study visits and interviews of a study participant or upon review by the research staff.

All AEs including local and systemic reactions not meeting the criteria for SAEs will be captured on the appropriate case report form (CRF). Information to be collected includes event description, time of onset, clinician's assessment of severity, relationship to study product (assessed only by those with the training and authority to make a diagnosis), and time of resolution/stabilization of the event. All AEs occurring while on study must be documented appropriately regardless of relationship. All AEs will be followed to adequate resolution.

Hypertension (>130/80mmHg), which will be present at the time that the participant is screened will be considered as baseline and not reported as an AE. However, if the study participant's condition deteriorates at any time during the study (increase in BP to greater than or equal to 180/100 mmHg), it will be recorded as an AE.

Changes in the severity of an AE will be documented to allow an assessment of the duration of the event at each level of severity to be performed. AEs characterized as intermittent will be documented at onset and duration of each episode.

Study staff (Dr. Cook and Nurses) will record all reportable events with start dates occurring any time after informed consent is obtained until 7 (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation. We will instruct participants to inform us (phone call or email) if they feel they are experiencing symptoms that may be related to their participation in this study. We will also contact them, via their preferred contact method (phone or email) at days 7 and 30 post study to inquire about them experiencing AE or SAEs. At each study visit, we will inquire about the occurrence of AE/SAEs since the last visit. Events will be followed for outcome information until resolution or stabilization.

8.3.5 ADVERSE EVENT REPORTING

Dr. Cook, Dr. Bland (study physician), and nursing collaborators present will primarily be responsible for monitoring the safety of each participant. The PI will be responsible for reviewing overall study progress and minimizing research associated risk by monitoring activities of the research and updating records on a weekly basis with study team members. Dr. Cook, in consultation with the study physician, will report any adverse events to the IRB and Funding agency. Any sign, symptom, or abnormal assessment during the study will be immediately reported to subject and their primary physician for follow-up assessment. Every effort will be made to determine the cause of any events that occur during the subjects' participation in the study to determine if the adverse event was related to study activities. Under the

direction of the study physician and IRB, we will suspend any activity that related to any consistent signs of initiating adverse events in our subject population. Whenever appropriate, under the direction of the IRB, we will amend informed consent and/or study protocol documents in response an adverse event. Participants will have hypertension, but BP's will be measured every 30 (wake hours) to 1 hour (sleeping hours). We will review the BP immediately upon the return of the ABP monitor.

8.3.6 SERIOUS ADVERSE EVENT REPORTING

The PI and/or study physician (Dr. Bland) will immediately report any serious adverse event to the IRB and funding agency, whether or not considered study intervention related, including those listed in the protocol or investigator brochure and must include an assessment of whether there is a reasonable possibility that the study intervention caused the event. Study endpoints that are serious adverse events (e.g., all-cause mortality) will be reported in accordance with the protocol unless there is evidence suggesting a causal relationship between the study intervention and the event (e.g., death from anaphylaxis). In that case, the investigator will immediately report the event to the IRB and funding agency.

All serious adverse events (SAEs) will be followed until satisfactory resolution or until the site investigator deems the event to be chronic or the participant is stable. Other supporting documentation of the event may be requested by the Data Coordinating Center (DCC)/study sponsor and should be provided as soon as possible.

The PI will be responsible for notifying the IRB (via phone call and email to the scientific review officer) of any unexpected fatal or life-threatening suspected adverse reaction as soon as possible, but in no later than 7 calendar days after the IRB's initial receipt of the information.

8.3.7 REPORTING EVENTS TO PARTICIPANTS

Current and future participants will be notified of all unexpected events that occur by the research staff. For example, these may include frequent bowel movements or a measurable increase in BP or hypotensive event (BP measured below systolic BP 90 mmHg and/or diastolic BP 60 mmHg) after self-administration of the enema. These have not been previously reported but are possible. After consideration of any unexpected event by the IRB, we will follow their instruction on whether they determine it is in the best interest to notify current and future participants by updating the informed consent risks. This may require the current subjects (signed consent forms) to reconsent to the study to allow them the opportunity to decide if they still want to continue their participation.

8.3.8 EVENTS OF SPECIAL INTEREST

Not Applicable

8.3.9 REPORTING OF PREGNANCY

Not Applicable

8.4 UNANTICIPATED PROBLEMS

8.4.1 DEFINITION OF UNANTICIPATED PROBLEMS (UP)

The Office for Human Research Protections (OHRP) considers unanticipated problems involving risks to participants or others to include, in general, any incident, experience, or outcome that meets **all** of the following criteria:

- Unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol-related documents, such as the Institutional Review Board (IRB)-approved research protocol and informed consent document; and (b) the characteristics of the participant population being studied;
- Related or possibly related to participation in the research ("possibly related" means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- Suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

If any UP arises, we will report problems to the IRB and grant agency and follow their instructions to addressing these UPs, which may include but are not limited to: Modifying the inclusion/exclusion criteria and informed consent to mitigate the newly identified risks, provide additional verbal and written information concerning the new identified risks, and suspend the enrollment of new participants or halting the study procedures for enrolled participants, if necessary.

8.4.2 UNANTICIPATED PROBLEM REPORTING

The principal investigators and/or study physician will report unanticipated problems (UPs) to the reviewing Institutional Review Board (IRB) and grant funding agency. The UP report will include the following information:

- Protocol identifying information: protocol title and number, PI's name, and the IRB project number;
- A detailed description of the event, incident, experience, or outcome;
- An explanation of the basis for determining that the event, incident, experience, or outcome represents an UP;
- A description of any changes to the protocol or other corrective actions that have been taken or are proposed in response to the UP.

To satisfy the requirement for prompt reporting, UPs will be reported using the following timeline:

- UPs that are serious adverse events (SAEs) will be reported to the IRB and funding agency within 3 days of the investigator becoming aware of the event.
- Any other UP will be reported to the IRB and study sponsor within 7 days of the investigator becoming aware of the problem.
- All UPs will be reported to appropriate institutional officials (as required by an institution's written reporting procedures), the supporting agency head (or designee), and the Office for Human Research Protections (OHRP) within 7 days of the IRB's receipt of the report of the problem from the investigator.

8.4.3 REPORTING UNANTICIPATED PROBLEMS TO PARTICIPANTS

Participants will be informed about UPs within 24 hours. We expect participants experiencing UPs to inform us as soon as possible. At that time, we determine if that is an unanticipated problem within 24 hours. If it significantly affects their activities of daily living during or within 14 days of their participation, we will notify current and future participants in writing, and by updating the informed consent, to make them aware of this new UP. We will also immediately follow-up with subjects who completed their participation to inquire whether they have experienced the UP at any time during and up to 14 days after their participation.

9 STATISTICAL CONSIDERATIONS

9.1 STATISTICAL HYPOTHESES

Our central hypothesis is that low gut butyrate availability is related to hypertension in AA, and that acutely increasing gut butyrate will translate into greater circulating butyrate and an acute and measurable reduction in BP.

1. We expect AA with hypertension will have significantly ($p< 0.05$) lower SCFA (butyrate) producing microbes in their stool samples, compared to their normotensive age and sex matched peers. We expect that AA with hypertension will have greater circulation of inflammatory biomarkers, oxidative stress, and lower nitric oxide, compared to the control group.

2. We expect circulating butyrate to be significantly lower in AA with hypertension which will be inversely related to BP levels (low circulating butyrate = higher BP).

3. In the hypertension group, we expect the butyrate enema to increase circulating butyrate levels and elicit an acute reduction in BP within 24 hours of self-administration of the butyrate enema. We expect no change in circulating butyrate levels or BP after the control (saline) enema.

9.2 SAMPLE SIZE DETERMINATION

Power Analysis and Sample size estimate: This is a proof of concept pilot study. Data does not exist for estimating the effect size of the proposed treatment (cross-sectional or cross-over study), which itself will be used to determine appropriate ranges of effect sizes for subsequent studies. We consulted Dr. Michael Love (UNC-CH NC TraCS Biostatistician). Sample size for this study was influenced by cost of study (subject renumeration and sample analyses) and the ability to feasibly recruit and retain subjects within the grant timeline and complete data analyses the study in the 1-year grant period. Further, VANHOUTVIN SA et al. (reference in reference section) recruited 11 subjects (10 completed) in their study on the effects of butyrate enema on visceral perception and safety.

9.3 POPULATIONS FOR ANALYSES

Aim 1: 10 normal-to-overweight (BMI 18.5-30 kg/m^2) AA adults (5 male/5 female, 30-50 y.o.) with normal BP (90-129/60-89mmHg) and 10 age- and gender-matched normal-to-overweight AAs with stage-1 hypertension (130-159/80-99 mmHg) will provide fecal and blood samples to quantify differences in the abundance of fecal butyrate-producing microbes and circulating butyrate between AA with- and without hypertension. *We hypothesize* that participants with normal BP, when compared to individuals with

hypertension will have a greater abundance of butyrate producing microbes in their stool and greater plasma butyrate concentrations.

Aim 2: In this crossover blinded randomized controlled trial, over two visits that are 1 week apart, the same 10 hypertensive AAs will be randomized to self-administer a sodium butyrate (80mM butyrate in saline) or control saline enema in this paired research design. Butyrate enemas are well tolerated in human studies. BP will be manually monitored immediately after the enema, and a 30 min and 60 min post enema. At these times, post-enema blood will be collected to determine plasma butyrate concentrations. Also, participants will wear a 24h ABP monitor to measure oscillations in BP after the enema. *We hypothesize* that only the butyrate enema will elicit a reduction in BP within the 24-hour period after the enema. We expect to see no significant change in BP after the control enema.

9.4 STATISTICAL ANALYSES

9.4.1 GENERAL APPROACH

Statistical Analyses: **Aim 1**: In this age and sex-matched cross-sectional design study (differences between individuals with normal BP and hypertension), paired *t*-test will be used to determine differences circulating blood butyrate, blood biomarkers (inflammatory markers, oxidative stress, nitric oxide levels – section 8.2), the relative abundance of butyrate producing fecal microbes. ANOVA with covariate analyses (i.e., BMI, intake of dietary fiber (grams), mean difference in resting BP between groups) will be used to determine these variables relationship to blood butyrate, blood biomarkers, butyrate producing microbes, and BP between the control and hypertension groups. Stratification method will be utilized to analyze results stratified by age (30-40 yr old and 40-50 yr old) and BMI (normal to overweight).

Aim 2: Repeated measures ANOVA will be employed to determine the relationship between enema (butyrate and saline) treatment on circulating butyrate (0, 30, 60 min), biomarkers, and hourly mean (day-time and night-time) systolic and diastolic BP measured via 24-hr ABP monitoring. Utilizing a generalized linear regression model analyses, we will utilize variables (i.e., dietary carbohydrate, fat, protein, and fiber intake; blood biomarker values) to estimate their relationship with BP. Magnitude of change in blood butyrate after each enema will be calculated to perform analyses to quantify the relationship between blood butyrate changes and BP changes. 24-hr ABP data will be averaged for each hour. Analyses of these measures will be performed following Edwards & Simpson Blood Press Monit. 2014 June ; 19(3): 153–163. Stratification method will be used to quantify any effect of "stage of hypertension (systolic: 130-139 and 140-149, 150-159)" on magnitude of change in BP after the treatments. Sensitivity analyses (including correlation, regression, and subjective) will be performed to explore extreme or questionable results in changes in BP utilizing appropriate variables and covariates (e.g., change in circulating butyrate, dietary fiber intake).

Overall: Data will be considered statistically significant at a $p \leq 0.05$ and all statistical methods will be tabulated with their corresponding confidence intervals. Hypothesis testing with a $p \geq 0.05$ will be considered not statistically significant and will be reported as inconclusive. Pearson r and non-parametric Spearman Rho correlation analysis will be used to determine relationships between enema, circulating butyrate, and difference/change values of BP.

Microbiome Analyses. We will perform bioinformatic microbiome analyses of the raw sequence data. PICRUSt will be employed to identify differentially abundant genes in samples. Predictive functional

profiles will be used to identify and quantify SCFA and butyrate producers. Comparisons will be made between normotensive and hypertensive subjects using Fishers exact tests. With this, we will identify the relative abundances of specific butyrate-producing microbial taxa to quantify their association with BP. Relative abundance values of these taxa will be input in the statistical models explained in Aims 1 & 2.

Missing Data: Depending on the missing variable we will consider utilizing regression analyses, pairwise deletion analyses, or multiple data imputation methods.

9.4.2 ANALYSIS OF THE PRIMARY EFFICACY ENDPOINT(S)

Please see section 9.4.1.

9.4.3 ANALYSIS OF THE SECONDARY ENDPOINT(S)

N/A

9.4.4 SAFETY ANALYSES

N/A

9.4.5 BASELINE DESCRIPTIVE STATISTICS

Descriptive statistics (mean ± SEM) will be reported for all subject characteristics (besides gender) and outcome variables (BP, blood butyrate and biomarkers). Correlative statistics (Pearson *r*, Spearman *Rho,* Fisher Exact Tests) will be used to determine the relationship between circulating butyrate, microbial SCFA producing biomass, and resting BP, as well as average hourly BP measured with the ABP monitor.

9.4.6 PLANNED INTERIM ANALYSES

This intervention is not being used to treat or cure hypertension. Therefore, we will not be performing any interim analyses of efficacy or appropriateness of treatment using this experimental design.

9.4.7 SUB-GROUP ANALYSES

African Americans are the sole population being studied in this proposal. We do not intend to perform sub-group analyses (e.g., sex) in this small pilot study. The participants will be sex, age, and BMI matched. These data will be used to perform power analyses for subsequent studies.

9.4.8 TABULATION OF INDIVIDUAL PARTICIPANT DATA

Group means, not individual participant data, of all variables will be reported.

9.4.9 EXPLORATORY ANALYSES

Not Applicable

10 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

10.1 REGULATORY, ETHICAL, AND STUDY OVERSIGHT CONSIDERATIONS

10.1.1 INFORMED CONSENT PROCESS

10.1.1.1 CONSENT/ASSENT AND OTHER INFORMATIONAL DOCUMENTS PROVIDED TO PARTICIPANTS

Consent forms describing in detail the study intervention, study procedures and assessments, risks and benefits, compensation and cost to participants, and confidentiality will be provided to the participant before starting any procedures or collecting any data. If the participant has hypertension, they will be provided with a consent form to discuss with their physician. Informed consent will be completed at Visit 1 (consent visit), which is required prior to starting intervention/administering study intervention.

The following consent materials are submitted with this protocol: 1. Control subject (normotensive) and Hypertension subject informed consents. Also included are questionnaires (health history, physical activity, demographic, telephone), diaries (3-day dietary recall, 24-hour ambulatory blood pressure diary), recruitment materials (email blast, radio advertisement, and flyer), and informational forms (swab method, ambulatory blood pressure monitor booklet).

10.1.1.2 CONSENT PROCEDURES AND DOCUMENTATION

Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continues throughout the individual's study participation. Consent forms will be Institutional Review Board (IRB)-approved and the participant will be asked to read and review the document. The investigator/research staff will explain the research study to the participant and answer any questions that may arise. A verbal explanation will be provided in terms suited to the participant's comprehension of the purposes, procedures, and potential risks of the study and of their rights as research participants. Participants will have the opportunity to carefully review the written consent form and ask questions prior to signing. The participants will have the opportunity to discuss the study with their family or surrogates or think about it prior to agreeing to participate. The participant will sign the informed consent document prior to any procedures being done specifically for the study. Participants will be informed that participation is voluntary and that they may withdraw from the study at any time, without prejudice. A voluntary participation/withdrawal statement is included in the informed consent. A copy of the informed consent document will be given to the participants for their records. The informed consent process will be conducted and documented in the source document (including the date), and the form signed, before the participant undergoes any study-specific procedures. The rights and welfare of the participants will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

10.1.2 STUDY DISCONTINUATION AND CLOSURE

This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. ***Study oversight***: The research study staff will have weekly meetings to assess the study progress and review, as a group, any issues that arise (including adverse events). Under the counsel of Dr. Bland (after she comprehensively reviews the AE's or SAE's) and the IRB, we will act accordingly to halt any procedures scheduled to be performed. If the study is prematurely terminated or suspended, the PI (Dr. Cook) will promptly inform study participants, the Institutional Review Board (IRB), and sponsor and will provide the reason(s) for the termination or suspension. Written notification, documenting the reason for study suspension or termination, will be provided to all study participants, funding agency and sponsor, and any regulatory authorities. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule.

Circumstances that may warrant termination or suspension will be assessed by Dr. Bland and include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Insufficient compliance to protocol requirements
- Data that are not sufficiently complete and/or evaluable
- Determination that the primary endpoint has been met
- Determination of futility

Study may resume once concerns about safety, protocol compliance, and data quality are addressed, and satisfy the funding agency, IRB, and/or any other regulatory authorities.

10.1.3 CONFIDENTIALITY AND PRIVACY

This study is funded through NC TraCS, which is funded through the NIH CTSA award. Therefore, this pilot project will automatically have a Certificate of Confidentiality.

Participant confidentiality and privacy is extremely important and will be upheld by the research team. All information and data collected generated will be held in strict confidence. Confidentiality will be extended to cover testing of biological samples in addition to the clinical measures (blood pressures) by assigning subject ID numbers. No information concerning the study or the data will be released to any unauthorized third party (e.g., personal PCP) without prior written approval to the research staff.

All research activities will be conducted in as private a setting as possible.

Study staff, authorized representatives of the funding agency, and representatives of the Institutional Review Board (IRB) and/or regulatory agencies may inspect all documents and records required to be maintained by the investigator, for the participants in this study. We will comply with requests for access to such records when necessary.

The study participant's contact information will be securely stored for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IRB, Institutional policies, or funding agency requirements (no less than 4 years after the study ends).

Study participant research data, which is for purposes of statistical analysis and scientific reporting, will be transmitted to and stored in a password protected software (RedCap) and statistical analyses files (Graphpad, SPSS) on a password protected computer. This will not include the participant's contact or identifying information. Rather, individual participants and their research data will be identified by a unique study identification number. At the end of the study, all study databases will remain de-identified and stored indefinitely.

10.1.4 FUTURE USE OF STORED SPECIMENS AND DATA

De-identified data (labeled with the subjects unique ID) collected for this study will be analyzed and stored at North Carolina A&T State University and some biological samples (blood and fecal) at University of North Carolina – Chapel Hill Core Facilities. After the study is completed, de-identified results from the study will be transmitted to and stored in password protected files (RedCap) on a password protected

computer, for use by other researchers including those outside of the study. Participants will be notified of this in the informed consent.

With the participant's approval (via informed consent) and as approved by the IRBs, de-identified biological samples will be stored as defined above. These samples will be used to research the causes of hypertension, its association with cardiovascular disease risk, and related complications to improve treatment strategies.

When the study is completed, access to study gut microbial data and/or samples will be stored in Dataverse.org. All participants will receive their individual BP data at the conclusion of the study in a paper packet. BP data will consist of the average (every hour) awake and sleeping BPs recorded via ABP monitor. These data may help to inform themselves or their PCP's about potential treatment strategies or lifestyle changes (diet and exercise) that may benefit them.

10.1.5 KEY ROLES AND STUDY GOVERNANCE

Provide the name and contact information of the Principal Investigator and the Medical Monitor.

Principal Investigator	**Principal Investigator**	**Medical Monitor**
Marc Cook, PhD	Ian Carroll, PhD	Veita Bland, M.D.
North Carolina A&T SU	*UNC- Chapel Hill*	*Bland Clinic P.A.*
405 Benbow Rd. Corbett Sport Ctr *Greensboro, NC 27411*	*111 Mason Farm Road 7312C MBRB. Chapel Hill, NC 27599*	*1317 N Elm St # 7 Greensboro, NC. 27401*
336-285-3547	*(919) 843-4893*	*(336) 373-1557*
mdcook@ncat.edu	*ian_carroll@med.unc.edu*	*spice1nc@aol.com*

10.1.6 SAFETY OVERSIGHT

Safety oversight will be under the direction of the research study staff composed of individuals with the appropriate expertise, including Dr. Bland (M.D. Clinical Hypertension Specialist), PI's, and the research staff collaborators (nurses). Research staff will meet on a weekly basis for data collection review, assessment of protocol and procedures, and assessment of safety of the study.

10.1.7 CLINICAL MONITORING

Site monitoring will be conducted to ensure that the rights and well-being of trial participants are protected, that the reported trial data are accurate, complete, and verifiable, and that the conduct of the trial is in compliance with the currently approved protocol/amendment(s), with International Conference on Harmonisation Good Clinical Practice (ICH GCP), and with applicable regulatory requirement(s).

- Monitoring for this study will be performed by Dr. Marc Cook, PhD, nursing collaborators (Dr. Tiffany Morris, PhD. and Dr. Ruthie Rogers, DNP), and overseen by Dr. Veita Bland, M.D. Dr. Bland will review all clinical data collected, assess any reported AE's and determine their severity and potential relatedness to the study experiment (if there is more than 7 days after an experiment was completed). She will follow-up with participants to assess their health (via physical), if necessary.

- At this one site, the research team will meet every week to review data collected and transfer data into all password protected electronic storage forms (e.g., RedCap and statistical analyses data).
- Safety monitoring will be in real time (monitoring BP and symptoms for no less than 1.5 hours after enema administration) and follow-ups at least 2 times (no later than 7 and again at 30 days) after the completion of their study.
- Independent audits will not be conducted in this study as all research staff will meet on a weekly basis (and more frequently if needed) to review data, study progress, and any unexpected events.

10.1.8 QUALITY ASSURANCE AND QUALITY CONTROL

We will perform internal quality management of study conduct, data and biological specimen collection, documentation and completion. Research staff will meet weekly to review all data collected and oversee the data entry.

Quality control (QC) procedures will be implemented beginning with the data entry system and data QC checks that will be run on the database will be generated. Any missing data or data anomalies will be communicated between the data entry research staff and the PI/clinical research staff for clarification/resolution.

The investigational site will provide direct access to all source data/documents and reports for the purpose of monitoring and auditing by the funding agency, and inspection by local and regulatory authorities.

10.1.9 DATA HANDLING AND RECORD KEEPING

10.1.9.1 DATA COLLECTION AND MANAGEMENT RESPONSIBILITIES

Data collection is the responsibility of the research staff under the supervision of Dr. Marc Cook. The investigator will be responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported.

All source documents will be completed in a neat, legible manner to ensure accurate interpretation of data. Data will be transferred into a password protected data managing software (RedCap) no more than 7 days after data is collected. All data that is collected will be entered into RedCap on a weekly basis.

Hardcopies of the study visit worksheets will be provided for use as source document worksheets for recording data for each participant enrolled in the study. Data recorded in the electronic case report form (eCRF) derived from source documents will be consistent with the data recorded on the source documents.

Clinical data (including adverse events (AEs) and unanticipated problems) and clinical laboratory data will be entered into a password protected file (on a password protected computer). Clinical data will be entered directly from the source documents.

10.1.9.2 STUDY RECORDS RETENTION

Study paper documents with identifiers will be retained for a minimum of 4 years in a locked file cabinet in a locked office space. After 4 years, all paper documents will be shredded. De-identified data will be stored in a password protected file on a password protected computer indefinitely.

10.1.10 PROTOCOL DEVIATIONS

A protocol deviation is any noncompliance with the clinical trial protocol, International Conference on Harmonisation Good Clinical Practice (ICH GCP), or Manual of Procedures (MOP) requirements. The noncompliance may be either on the part of the participant, the investigator, or the study site staff. As a result of deviations, corrective actions will be developed and implemented promptly.

It is the responsibility of the PI (Dr. Marc Cook) to use continuous vigilance to identify and report deviations within 3 working days of identification of the protocol deviation, or within 3 working days of the scheduled protocol-required activity. All deviations will be addressed in study source documents, reported to UNC-CH and NC A&T Institutional Review Board (IRB)'s. Protocol deviations will be sent to the reviewing IRB per their policies. The principal investigator will be responsible for knowing and adhering to the reviewing IRB requirements.

10.1.11 PUBLICATION AND DATA SHARING POLICY

This study will be conducted in accordance with the following publication and data sharing policies and regulations:

National Institutes of Health (NIH) Public Access Policy, which ensures that the public has access to the published results of NIH funded research. We will submit final peer-reviewed journal manuscripts that arise from NIH funds to the digital archive PubMed Central upon acceptance for publication.

This study will comply with the NIH Data Sharing Policy and Policy on the Dissemination of NIH-Funded Clinical Trial Information and the Clinical Trials Registration and Results Information Submission rule. As such, this trial will be registered at ClinicalTrials.gov, and results information from this trial will be submitted to ClinicalTrials.gov. In addition, every attempt will be made to publish results in peer-reviewed journals. Data from this study may be requested from other researchers 5 years after the completion of the primary endpoint by contacting Dr. Marc Cook (*mdcook@ncat.edu*) at NC A&T State University.

In addition, this study will comply with the NIH Genomic Data Sharing Policy, which applies to all NIH-funded research that generates large-scale human or non-human genomic data, as well as the use of these data for subsequent research. These data will include fecal microbial genome sequence data.

10.1.12 CONFLICT OF INTEREST POLICY

[The independence of this study from any actual or perceived influence. The PI's, or identified research staff, do not have any conflicts of interest to report. Any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed by removing the research staff personnel with the conflict of interest. Furthermore, persons

who have a perceived conflict of interest will be required to have such conflicts managed in a way that is appropriate to their participation in the design and conduct of this trial (e.g., removing them from a portion of the study). The study leadership in conjunction with NIH/NC TRaCS has established policies and procedures for all study group members to disclose all conflicts of interest and will establish a mechanism for the management of all reported dualities of interest.

10.2 ADDITIONAL CONSIDERATIONS

Listed Below

1. Saline or sodium butyrate enema could raise BP in individuals with salt-sensitive hypertension. However, there has been no association between a one-time enema increasing BP. In fact, receiving an enema may elicit a vagal response to lower BP.

2. Both enemas will be purchased from Custom Care Pharmacy (Greensboro) for quality control and matched for sodium concentration.

3. Biobanking of blood and fecal microbial samples will allow for further analysis of factors that are not addressed in this study, such as measuring additional SCFA's (acetate, propionate)

4. We will educate participants on how to efficiently perform the self-administered enema, to reduce variability, and measure remaining solution to calculate dose delivered.

5. We will work to minimize non-compliance and provide monetary incentive in this short study.

6. Our group is experienced in culturally sensitive methods of subject recruitment in AA, via strong community relationships and health education, and we expect no issues with recruitment.

7. The pool of subjects that do not take hypertension medication may be limited. If we encounter a heavily medicated subject population, we may include subjects taking diuretics only.

10.3 ABBREVIATIONS

The list below includes abbreviations utilized in this template. However, this list should be customized for each protocol (i.e., abbreviations not used should be removed and new abbreviations used should be added to this list).

AA	African American
AE	Adverse Event
ABP	Ambulatory Blood Pressure
ANCOVA	Analysis of Covariance
CFR	Code of Federal Regulations
CLIA	Clinical Laboratory Improvement Amendments
CMP	Clinical Monitoring Plan
COC	Certificate of Confidentiality
CONSORT	Consolidated Standards of Reporting Trials
CRF	Case Report Form
DCC	Data Coordinating Center
DHHS	Department of Health and Human Services
DSMB	Data Safety Monitoring Board
DRE	Disease-Related Event
EC	Ethics Committee
eCRF	Electronic Case Report Forms
FDA	Food and Drug Administration
FDAAA	Food and Drug Administration Amendments Act of 2007
FFR	Federal Financial Report
GCP	Good Clinical Practice
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
GWAS	Genome-Wide Association Studies
HIPAA	Health Insurance Portability and Accountability Act
IB	Investigator's Brochure
ICH	International Conference on Harmonisation
ICMJE	International Committee of Medical Journal Editors
IDE	Investigational Device Exemption
IND	Investigational New Drug Application
IRB	Institutional Review Board
ISM	Independent Safety Monitor
ISO	International Organization for Standardization
ITT	Intention-To-Treat
LSMEANS	Least-squares Means
MedDRA	Medical Dictionary for Regulatory Activities
MOP	Manual of Procedures
MSDS	Material Safety Data Sheet
NCT	National Clinical Trial
NC TRaCS	North Carolina Translational and Clinical Sciences
NIH	National Institutes of Health
NIH IC	NIH Institute or Center
OHRP	Office for Human Research Protections
PCP	Primary Care Physician
PI	Principal Investigator
QA	Quality Assurance

QC	Quality Control
SAE	Serious Adverse Event
SCFA	Short Chain Fatty Acid
SAP	Statistical Analysis Plan
SMC	Safety Monitoring Committee
SOA	Schedule of Activities
SOC	System Organ Class
SOP	Standard Operating Procedure
UP	Unanticipated Problem
US	United States

10.4 PROTOCOL AMENDMENT HISTORY

The table below is intended to capture changes of IRB-approved versions of the protocol, including a description of the change and rationale. A Summary of Changes table for the current amendment is located in the Protocol Title Page.

Version	Date	Description of Change	Brief Rationale

11 REFERENCES

Journal Citations:

Butyrate in Humans

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