Protocol for NCT04497727

1. **Project Title:** Gut Inflammation and Gut-Gut Microbiome Interactions in the Pathogenesis of Hypertension.

2. **Investigators:** Drs. Carl J. Pepine, Chris Forsmark, Angela Pham, Eyad Alakrad and Eileen Handberg.

3. **Abstract:** Dysbiosis of the gut microbiome has been associated with a variety of disorders and disease conditions ranging from mental health diseases to obesity. One such disease is hypertension. The initial point of contact between the gut microbiome and the host is their gut epithelium. The gut epithelium and the gut microbiota have evolved together to exist in balance during health. The epithelium has barrier, immune and metabolic functions that support and contain the microbiota. The microbiota in turn makes products that support the health of the gut epithelium, such as short chain fatty acids. There is emerging evidence to suggest that the gut itself, as well as the gut microbiome, is disturbed during hypertension. Little is known about this from animal models of hypertension except in studies by our group, and even less is known about the health of the gut in people with hypertension. We aim to examine the gut epithelium of the colon from colonoscopy biopsies, and we will derive gut organoids from this tissue. We will test the hypothesis that gut epithelium is distinct in terms of basic growth and differentiation and gene expression, in people with hypertension compared to those without cardiovascular disease (CVD) and normal blood pressure (e.g. reference subjects)

4. **Background:** It is estimated that almost half of the adults in the United States have high blood pressure[1], a leading modifiable risk factor for CVD, stroke and dementia. Approximately 20% of these people are termed treatment-resistant hypertensives because they do not achieve goal blood pressures even taking three or more antihypertensive medications of different classes [1].
   The gut and its microbiome have been shown to be associated with hypertension in both animal models and patients[2-4]. The gut microbiome is distinct in people and animals with hypertension from those with normal blood pressure. Both the bacterial populations of the gut microbiome and the functions of those populations differ[5, 6]. Most strikingly, the transfer of fecal matter from hypertensive people or animals to animals with normal blood pressure increases their blood pressure[6-8]. In animal models of hypertension, gut pathology such as decreased villus length and goblet cell number and increased fibrosis and muscularis layer are also observed[2]. Additionally, the gut barrier has reduced strength in hypertensive animals allowing large molecules normally retained in the gut to enter ("leak") into the blood circulation[5]. In humans with hypertension there is also evidence of gut leakiness as there are increased markers of barrier dysfunction and substances normally well contained behind the gut barrier appear in the circulation[5]. Furthermore, there are more immune cells with surface markers targeting them to the gut in the circulation of hypertensive subjects, another sign that there is a potential problem in the gut[5]. We also have unpublished data in experimental models that gut epithelial cells express different proteins when the animal is hypertensive. Altogether, this suggests that the gut and its microbiome may be a novel target for blood pressure control.
   Gut pathology has not been explored in human hypertension, except by the above-mentioned circulating markers. This has prompted us to hypothesize that the interaction

between the gut and its microbiota is faulty in hypertension, particularly in the light of observations of a good balance between the two in health.
We propose to investigate these interactions in human gut by utilizing colonoscopy biopsies for two main purposes. Firstly, to examine the gene expression profile of the gut epithelium from individuals with and without hypertension. Secondly, we will use the crypts of the gut to culture gut organoids from the stem cells present in the crypts. These are three dimensional cultures that grow and differentiate to form an organ that is structurally similar to the gut. They have a lumen, a polarized epithelium, good barrier function and contain all of the cell types in the correct proportions that are found in the gut epithelium *in vivo*. We will use these to test whether there is a fundamental difference in the epithelium of the gut in hypertension compared to normotension. We will also test gut organoid growth and differentiation in direct comparison to those from normotensive individuals grown under identical culture conditions. In these, we hypothesize that there will be no difference between those from people with hypertension compared to those without hypertension in terms of cellular composition and gene expression patterns.

5. Specific Aims:

We will compare basic properties of gut epithelia of hypertensive and normotensive reference subjects. We will determine if there are fundamental differences in the gut epithelium in hypertension compared to normotension. We propose the following aims:

Aim 1: To determine if gene expression patterns are distinct in gut epithelium derived from hypertensive compared to normotensive reference individuals.
Aim 2: To determine if gut organoids prepared from gut biopsies have different growth rates and patterns, including the proportions of cell populations in the organoids, when derived from hypertensive versus normotensive reference subjects.

6. Research Plan:

These studies will include a controlled sampling of human hypertension (n=60, 30 hypertensive subjects and 30 normotensive reference subjects, equal numbers of males and females) recruited at UF.

Aim 1: From one part of the biopsy, the gut epithelium will be isolated, and immediately frozen (-80 degrees) until RNA isolation for RNA sequencing and examination of gene expression within the epithelium.
Aim 2: The remainder of the biopsy will be immediately (within 30 minutes of collection) used to prepare gut organoid cultures using methods essentially as developed by Stemcell™ Technologies. The growth parameters and the proportions of each cell type, for example, enterocyte, Paneth, goblet, enteroendocrine, making up the organoid will be determined by immunohistochemistry or fluorescence activated cell sorting (FACS) analysis.

Recruitment: University of Florida (UF) will recruit participants from the UF GI clinic (with and without hypertension) who are scheduled for routine “surveillance” colonoscopy. Most patients undergoing screening colonoscopy have polyps noted (at UF, our adenoma detection rate is around 50%). These folks would have biopsies of other sites (polyps) already, and would get additional biopsies for study. The other 50% without polyps would only have biopsies as part of study. During screening of upcoming “surveillance” colonoscopies potential

participants will be asked by their gastroenterologist about their interest in taking part in this study. If the patient agrees to participate or desires more information about the study, the study coordinators in Cardiology Clinical Trials will contact the patient via phone, email, or an in-person meeting and provide more details and answer any questions. If they wish to take part in the trial, informed consent will be obtained prior to the colonoscopy procedure..

In order to address our hypotheses, colonoscopy biopsies from subjects diagnosed with hypertension as well as a reference group will be collected. After which, a member of Dr. Raizada or Dr. Pepine's group will immediately transport biopsy samples to Dr. Mohan Raizada's lab in the McKnight Brain Institute Building L4-135.

A. Specific Procedures: Inclusion/exclusion criteria for hypertension participants

Inclusion criteria:

- Ages: 18-80 years old with a weight ≥110 lbs.
- Diagnosis of hypertension or without hypertension for the reference cohort. 2017 ACC/AHA definition for hypertension (systolic blood pressure ≥130 mmHg and/or diastolic BP ≥80 mmHg) will be used.
- Scheduled elective colonoscopy.

Exclusion criteria:

- Hypertensive or reference individuals with history of autoimmune disease or other chronic inflammatory conditions.
- Pregnant or have been pregnant in the last six months.
- Antibiotic treatment within two months of study enrollment
- Currently taking a medication(s) (e.g. antibiotic, anti-inflammatory agents, glucocorticoids, other immune modulating medications, antacids or proton pump inhibitor drugs like Prilosec) known to modify gut microbiota or currently taking a medication(s) and stopping the medication(s) to prepare for the colonoscopy then restarting after procedure is completed.
- The use of probiotics
- History of intestinal surgery, inflammatory bowel disease, celiac disease, lactose intolerance, chronic pancreatitis or other malabsorption disorder.
- History of blood transfusion within 4 weeks.
- Subjects who, in the opinion of the investigator, will be uncooperative or unable to comply with study procedures

Subjects will participate until the biopsy procedure is completed.

Specific Procedures:

- Methods/Statistical Analyses: Each study participant will provide, in addition to body weight, BMI, BP, PR, and CVD risk factors, a colonoscopy biopsy.
- Analysis of gene expression in the epithelium of a portion of the biopsy will be examined and gut organoids cultured from the remainder.
- The subjects' medical records will be reviewed and summarized for the study. All collected data will be stored in an encrypted computer data base kept behind UF's firewall. These data will be coded and connected to the biopsy samples by code, not identifiers. Biopsy samples will be processed immediately and portions either frozen for storage or the crypts isolated for gut organoid culture. The experiments will be performed in Dr. Raizada's laboratory in the McKnight Brain Institute and remaining samples stored until the end of the study. Experiments examining gene expression in the colonic epithelium of normotensive and hypertensive rats using the same methodology intended for use here, revealed significant differences between groups with a sample size of n=4 per group. Hence, we expect a sample size of n=60 to provide sufficient power to observe differences in gene expression. Multiple gut organoids can be generated from a single biopsy. This allows mean characteristics for each individuals' organoids to be determined, reducing variability. We have no previous data upon which to base a formal power analysis. However, we believe these culture conditions, the ability to have multiple repetitions of the experiment in each biopsy sample, and 30 subjects per group will be adequate to determine whether there are differences worth pursuing in a larger cohort of hypertensive and normotensive subjects in a future study.

Prepped colonoscopy biopsies: Dr. Christopher Forsmark, Dr. Eyad Alakrad, and Dr. Angela Pham in the UF GI Division will perform all colonoscopy procedures on subjects (hypertensive or healthy reference subjects) to obtain colon biopsies. All subjects will have up to 4 additional biopsies collected for research. Biopsy and blood samples will be coded as quickly as possible and securely stored with only a subject number assigned for this study.

Medical Record Abstraction. For participants in this study, their pertinent medical history will be abstracted from their records and entered into a RedCap database. These data will be correlated with the research results to determine any relationships or mechanisms in hypertension. We will correlate the research results with the type of hypertension (i.e. controlled, uncontrolled, treatment-resistant).

Subject Confidentiality. Clinical information will not be released without written permission of the subject. The site Investigator must assure that the privacy of subjects, including their personal identity and personal medical information, will be maintained at all times. UF site has additional privacy obligations to study subjects under the Health Insurance Portability and Accountability Act (HIPAA). After a subject has signed an informed consent form, the coordinator will review the signed informed consent(s) and the PI will review that portion of the subject's medical record that is directly related to the study including electronic medical records. This shall include all study relevant documentation including subject medical history to verify eligibility, laboratory test result reports, admission/discharge summaries for hospital admissions occurring while the subject is in the study, and autopsy reports for deaths occurring during the study (when available). All study files will be kept on site in locked drawers. The only personnel with access to this data are the PI and study staff. All data and samples will be coded with a study ID.

7. **Possible Discomforts and Risks:** Colonoscopy procedures are only those performed as part of a clinically indicated procedure. Possible risks for colonoscopy biopsies include

persistent bleeding after biopsy or perforation of the wall of the colon which might require surgery. The chance of a serious complication from colonoscopy biopsy is less than 1 in 10,000.

This study will collect personal data and PHI. There is slight risk of personal information becoming public, but measures are in place to prevent that unlikely event.

8. **Possible Benefits.** There are no direct benefits of participating in this study.

9. **Conflict of Interest.** Study investigators and staff have no conflicts to report.

10. **References**

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