Protocol with Statistical Analysis Plan Cover Page:

Official Title:	Genomics, Environmental Factors and Social Determinants of Cardiovascular Disease in African-Americans Study (GENE-FORECAST**)**: Sodium Intervention Trial (SIT)/**GENE-FORECAST SIT**
NCT number:	NCT04717336
Document Type:	Protocol with Statistical Analysis Plan (SAP)
Document Date:	29JUL2024

Abbreviated Title: GENE-FORECAST SIT

NIH IRB #: 000070

Version Date: 29JUL2024

Title: Genomics, Environmental Factors and Social Determinants of Cardiovascular Disease in African-Americans Study (GENE-FORECAST**)**: Sodium Intervention Trial (SIT)/**GENE-FORECAST SIT**

NIH Principal Investigator: Georg Aue, MD, PhD, MBA (E)
Office of the Clinical Director
National Heart, Lung, and Blood Institute
Building 10-CRC, Rm 6-3142
9000 Rockville Pike
Bethesda, MD 20892
Phone: 301-451-7141
E-mail: aueg@nhlbi.nih.gov

Investigational Agents:

Drug Name:	Sodium Chloride
IND Number:	Not applicable
Sponsor:	Not applicable
Manufacturer:	Pine Pharmaceuticals

TABLE OF CONTENTS

STATEMENT OF COMPLIANCE

The 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

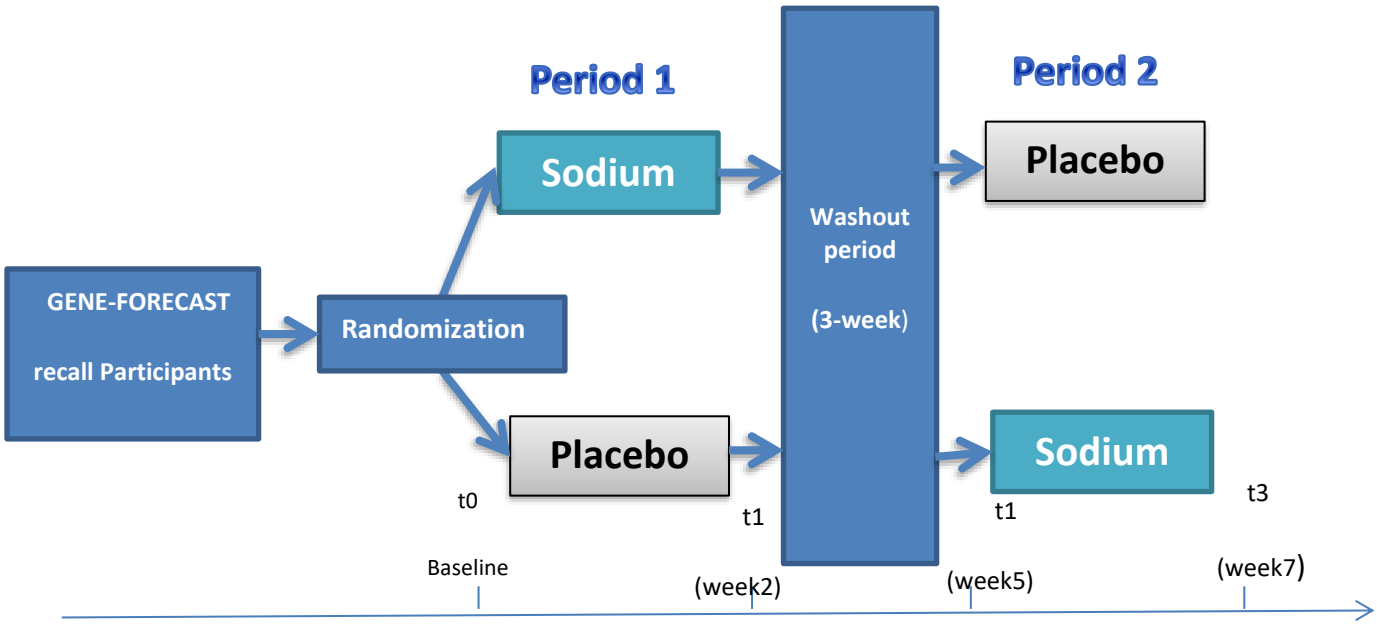
1.1 SYNOPSIS

Title:	Genomics, Environmental Factors and Social Determinants of Cardiovascular Disease in African Americans Study (GENE-FORECAST): Sodium Intervention Trial (SIT)/**GENE-FORECAST SIT**
Study Description:	The objective of this study is to implement a sodium intervention investigation to assess the effect of increased dietary sodium intake on changes in blood pressure, vascular function, microbiome, whole blood epigenome, whole blood and urine transcriptome as outcome measures. The study design will include a double-blind, cross-over treatment/placebo trial among 40 former African Americans GENE-FORECAST participants with normal blood pressure and will last 7weeks. It is hypothesized that exposure to increased dietary sodium will affect blood pressure, whole blood epigenome, whole blood and urine transcriptome, vascular function, microbiome and blood pressure.
Objectives:	**Primary Objective:** Define the effect of increased dietary sodium intake on microbiome and vascular function. **Secondary Objective:** 1) Determine if increased sodium affects blood pressure. 2) Determine if increased sodium affects whole blood epigenome. 3) Determine if increased sodium affects whole blood and urine transcriptome.
Endpoints:	**Primary:** Vascular function Microbiome **Secondary:** Blood pressure Whole blood epigenome Whole blood and urine transcriptome
Study Population:	Study population will include 40 former African American normotensive men and women ages 21-65 residing in the DC, Maryland, Virginia regions who previously participated in the GENE-FORECAST protocol.
Phase:	<0.
Description of Sites/Facilities Enrolling Participants:	All protocol activities will take place at the NIH Clinical Center.
Description of Study Intervention:	Participants will be enrolled in a double-blind cross-over sodium chloride/placebo intervention trial. Intervention product will be taken in capsule form (1 gram per capsule) 3 times per day.

Study Duration:	Participants will be enrolled concurrently along with ongoing GENE-FORECAST protocol. It is anticipated that it will take 36 months from enrollment to completion of data analyses.
Participant Duration:	It will take 7 weeks for each participant to complete 4 visits to the Clinical Center during the trial.

1.2 SCHEMA

Figure 1: Randomized double-blind crossover treatment/control flowchart



Participants will be randomized to a placebo or salt treatment arm for two weeks (Period 1), followed by a wash-out period for three-weeks, ending with a cross-over to the alternative treatment arm for two weeks (Period 2). The total length of participation is 7 weeks (Figure 1). Participants will be randomly assigned by a computer-generated code.

1.3 SCHEDULE OF ACTIVITIES (SOA)

It is estimated that participants will spend approximately 5 hours at the Clinical Center during baseline and approximately 3 hours during each of the subsequent visit. Obtaining and explaining the informed consent will take about 20-30 minutes. Administering the baseline questionnaires will take about 1.45 hours and 75 minutes for each subsequent visit. The clinic visit assessment, plus vascular function test, will take about 1.30 hours.

Table 1: Estimated length of time for study procedures

NIH Clinic Visit	Estimated time 4 hours	Total Participants	Duration of Study
a) Informed consent (baseline only)	20-30 minutes	40	7 wks
b) Baseline Questionnaires	1.75 hours	40	Baseline
c) Phlebotomy	30 minutes	40	7 wks
d) Physical examination	20 minutes	40	7 wks
e) 24 diet recall	30 minutes	40	7wks
f) Microbiome	15 minutes	40	7 wks
g) Psychophysical Taste Task: Sucrose and Salt Detection Thresholds	30 minutes	40	7 wks
h Psychophysical Taste Task: Sucrose and Salt Preference	20 minutes	40	7 wks
i) Vascular Function	1.0 hrs.	40	7 wks

Visit to the NIH Clinical Center:
Once potential participant arrives at the Clinical Center, they will report to admissions and be greeted by the GENE-FORECAST-SIT staff who will escort him/her to a private room to administer informed consent and baseline questionnaires. GENE-FORECAST-SIT staff (Research Nurse/Physician Assistant) will subsequently escort potential participant to phlebotomy followed by the collection of microbiome specimens from the GENE-FORECAST-SIT staff. A physical examination by the Physician Assistant will be performed on the participant, they will then have a standard physical exam, including blood pressure measurement and vitals concluding with administration of vascular function tests using Endo-PAT and SphygmoCor by the Physician Assistant. Two blood pressure measurements will be obtained from the participant by the Clinical Center Nursing Staff and an average recorded. The blood pressure will be measured using a Dinamap and will be obtained from the left arm after 5 minutes of rest with the participant in a sitting position with legs uncrossed. A 24-hour diet recall will be administered by staff from the Clinical Center Nutrition Department. Taste assessment will be administered by an Associate Investigator on the study.

Table 2: Specific activities by visit

Activity	Visit 1 (baseline)	Visit 2 (after 2-week treatment)	Visit 3 (after 3-week washout)	Visit 4 (after second 2-week treatment)
24-Hour nutrient recall	x	x	x	x

Psychophysical Taste Task: Sucrose and Salt Detection Thresholds	x	x	x	x
Psychophysical Taste Task: Sucrose and Salt Preference	x	x	x	x
History and physical exam with medication history	x	x	x	x
Baeke's physical activity questionnaire	x			
Pittsburg sleep questionnaire	x			
Stool/skin/mouth swabs for microbiome analysis	x	x	x	x
Urine sample	x	x	x	x
Blood sample	x	x	x	x
Vascular function	x	x	x	x
24-hour blood pressure monitor given to participant	x		x	
24-hour blood pressure monitor returned by participant		x		x

2 INTRODUCTION

2.1 STUDY RATIONALE

Little is known about the relationship between dietary sodium and gut microbiome ecosystem in humans; or how it might be altered by incremental changes in sodium intake. The GENE-FORECAST-SIT study will be the first to test the hypothesis that changes in dietary sodium intake are sufficient to influence the composition of the gut microbiome in association with sodium-induced changes in vascular function, epigenome, transcriptome and blood pressure in African Americans.

2.2 BACKGROUND

There is an abundant body of evidence from longitudinal population studies that have established a striking relationship between certain dietary patterns and the risk of cardiovascular disease. Diets rich in fruits, vegetables, whole grains, fish, nuts and fats such as olive oil have been associated with reduced risk of cardiovascular disease and adverse outcomes [1]. Similarly, controlled dietary intervention trials with the DASH diet have demonstrated that a low-sodium diet that is rich in fruits, vegetables, whole grains and low-fat dairy products is an effective approach to reducing blood pressure in hypertension patients [2]. It is noteworthy that African Americans appeared to have the greatest benefit from this dietary intervention. However, the molecular mechanisms that mediate the effect of diet on blood pressure and the development of cardiovascular disease remain to be further elucidated.

It is well established that increased dietary sodium can predispose to an increase in blood pressure; particularly in 'salt-sensitive' individuals [3]. In most individuals there are a powerful series of homeostatic systems (renal, neurohumoral, vascular) that function to maintain the normal set-point blood pressure across a very broad range of dietary sodium intakes. Yet, a sub-set of 'salt-sensitive' individuals exhibit changes in vascular function and modest elevations in blood pressure in response to relatively modest changes in dietary sodium intake [4]. It is noteworthy that several studies have suggested an apparent increased prevalence of 'salt-sensitive' blood pressure among African Americans [5].
Commonly used animal models of hypertension often employ high dietary sodium intake as a means of inducing sustained hypertension. The Dahl Salt-Sensitive Rat is a genetic model of hypertension that becomes hypertensive upon the administration of a high-sodium diet; whereas the genetically related Dahl Salt-Resistant Rat remains normotensive despite the high dietary sodium intake. Although there are multiple factors that appear to mediate this genetic predisposition to salt-sensitive hypertension, an intriguing recent report has noted a striking difference between the gut microbiome in Dahl salt-sensitive vs salt-resistant animals [6]. In addition, recent studies have demonstrated a relationship between increased dietary sodium intake and modulation of the immune system in animal models [7,8]. The proposed project seeks to further extend the working hypothesis that the effect of dietary sodium on vascular function and blood pressure may be modulated by the gut microbiome and its influence on the immune system. The selected dosage of sodium chloride is based on prior research.[8-10]
It is becoming increasingly clear that human biology and pathobiology is influenced in part by the trillions of microbes that co-exist within our bodies in a mutually beneficial manner. The microbe ecosystems that live within the human gastrointestinal tract are shaped by our dietary intake that supports the predominance of certain flora relative to others. Conversely, the microbe composition of each individual's gut can influence the digestion and generation of certain metabolites from certain foods. The various patterns of gut microbiome composition have been implicated in the regulation of the immune system as well as the pathogenesis of obesity, autoimmune disorders and cardiovascular disease [11-13]. For example, Stan Hazen's team has shown striking differences in the human microbiome between vegetarians and those who are frequent 'meat-eaters'. This dietary difference is associated with differential generation of a microbe metabolite, trimethylamine-oxidase (TMAO). Increased generation of the microbe metabolite, TMAO, is associated with increased risk of cardiovascular disease. Moreover, it has been shown that pharmacologic inhibition of the generation of this gut microbe metabolite attenuates atherogenesis in animal models. Taken together, these clinical and animal model studies suggest a causal link between dietary patterns, the gut microbiome and cardiovascular disease.

2.3 RISK/BENEFIT ASSESSMENT

2.3.1 Known Potential Risks

Risk of participating in the study is minimal given the benefit to the scientific community derived from findings related to the effect of salt on vascular, blood pressure and microbiome among African Americans. The salt capsules are generally safe and well tolerated but side effects can occur -including discomfort while swallowing, increased thirst, indigestion, nausea, vomiting, headaches, mild elevations in blood pressure, mild changes in blood chemicals (electrolytes), lightheadedness, tiredness, mild swelling of arms/legs, and/or diarrhea. Although it would be very rare to have an allergic reaction to the capsules, any time a new medication is

introduced there is a small chance of an allergic reaction which would most commonly manifest itself as a rash.

See Section 2.3.3 for detailed description of risks

.

2.3.2 Known Potential Benefits

There is no direct benefit to the participant for participating in the study. However, findings generated from the study will add to the scientific body of knowledge regarding the effect of salt among African Americans who have a disproportionately higher prevalence of cardiovascular disease

2.3.3 Assessment of Potential Risks and Benefits

The risks are very minor and include:

Vascular function testing and 24-hour blood pressure monitor: Subject may experience brief discomfort, pain or tingling sensations when the blood pressure cuff is inflated to reduce blood flow to the hand for a few minutes during the vascular function tests and/or 24-hour blood pressure monitor.

Phlebotomy: Transient discomfort and minor bruising may occur when blood is drawn.

Microbiome collection:: Collection will comprise of skin, saliva and stool samples utilizaing Norgen kits. These tests carry very minor risks, with the transient mild skin irration at the sites where your skin is swabbed although this is very unlikely.

Salt capsules: Sodium chloride is approved by the Food and Drug Administration (FDA) for a variety of clinical uses and is available over the counter (OTC). The capsules given as part of this study contain sodium chloride (salt) in powder form or placebo. The amount of salt added to subject's usual daily diet as part of this study is equivalent to about half a teaspoon of salt or a packet of Ramen chicken noodle soup. The salt capsules are generally safe and well tolerated but side effects can occur including discomfort while swallowing, increased thirst, indigestion, nausea, vomiting, headaches, mild elevations in blood pressure, mild changes in blood chemicals (electrolytes), lightheadedness, tiredness, mild swelling of arms/legs, and/or diarrhea. Although it would be very rare to have an allergic reaction to the capsules in this study, any time a new medication is taken there is a small chance (less than 1 person out of 1000 treated) that you could have an allergic reaction which would most commonly manifest itself as a rash.

The GENE-FORECAST-SIT staff will monitor subjects during study participation to detect any potential serious adverse reactions to the salt capsules.

24 Hour food intake recall: Subjects may be asked a series of questions by staff from the Clinical Center Nutrition Department to recall any foods or beverages they may have eaten or drank over the past 24 hours. There may be a minor psychological discomfort of not being able to recall any food or beverage item questions over the past 24 hours.

Mouth: At each clinical center visit, saliva samples from subjects mouth will be collected by passive drool or spit using a sterile collection tube. Providing study samples of these specimens that the body normally excretes will not cause any additional pain or discomfort.

Stool: Subjects will be provided with a stool specimen collection kit prior to their visit. There may be some physical discomfort of inability to obtain sample (e.g., constipation). Providing study samples of these specimens that the body normally excretes will not cause any additional pain or discomfort.

Urine: Subjects will be asked to give a urine sample in a sterile cup provided at the NIH Clinical Center to test for urine chemistries (e.g., potassium, chloride, sodium). There may be physical limitations in inability to provide sample (e.g. dehydration). Providing study samples of these specimens that the body normally excretes will not cause any additional pain or discomfort.

Psychophysical Taste Task with sucrose and salt detection thresholds and preference**:** Sucrose and salt detection thresholds and preference will be assessed to get subject responses to sweetness or saltiness. There may be a discomfort of tasting solutions and repeated spitting action to discard in waste receptacle.

Physical activity and sleep questionnaire: Subjects will be asked a series of questions related to their physical activity and sleep. They may experience minor psychological discomfort of answering personal questions regarding their physical activity and sleeping habits.

GENE-FORECAST-SIT staff will closely monitor the study participant in order to detect any potential severe adverse reactions to study medication. Specifically, GENE-FORECAST-SIT staff will contact individual study participant +/-2 days a week to anticipate missed calls and occasional inability to reach participant during the treatment phase of the trial to inquire about any severe adverse reactions. Participant will also be given the direct mobile telephone number of GENE-FORECAST-SIT staff to discuss any adverse reactions.

The risk of minimal potential risks outweighs the benefit of scientific knowledge gained from the effects of sodium on cardiovascular health, taste genotype and microbiome in African Americans.

3 OBJECTIVES AND ENDPOINTS

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Primary		
What effect does increase dietary sodium intake have on vascular function and microbiome?	Vascular function Microbiome	A sub-set of ‘salt-sensitive’ individuals exhibit changes in vascular function and modest elevations in blood pressure in response to relatively modest changes in

		dietary sodium intake. Studies have suggested an apparent increased prevalence of 'salt-sensitive' blood pressure among African Americans The various patterns of gut microbiome composition have been implicated in the regulation of the immune system as well as the pathogenesis of obesity, autoimmune disorders and cardiovascular disease.
Secondary		
What effect does increase dietary sodium have on blood pressure? What effect does increase dietary sodium have on whole blood epigenome? What effect does increase dietary sodium have on whole blood and urine transcriptome	Blood pressure DNA methylation RNAseq	It is well established that increased dietary sodium can predispose to an increase in blood pressure. DNA methylation is a well-established measure of the epigenome. RNAseq is a well-established measure of genetic transcriptome.

4 STUDY DESIGN

4.1 OVERALL DESIGN

This study involves a call-back and recruitment of 40 normotensive participants from the GENE-FORECAST protocol. Those in the upper tertile of vascular stiffness (as assessed by pulse wave velocity measures) will be oversampled. Participants will be randomized to a placebo or salt treatment arm for two weeks (Period 1), followed by a wash-out period for three-weeks, ending with a cross-over to the alternative treatment arm for two weeks (Period 2). The total length of participation is 7 weeks (Figure 1).

Study Site:
All study activities will take place at the NIH Clinical Center. The NIH Clinical Center Pharmacy will dispense the treatment and placebo capsules.

Statement of hypotheses:
***** Based on population history, it is postulated that the genomic architecture of individuals of African ancestry has been shaped by environmental pressures and natural selection in the context of high ambient temperatures in salt-poor conditions that may predispose to an increased sensitivity and avidity for sodium retention in African Americans.

* Increased dietary sodium intake is associated with vascular dysfunction and increased blood pressure in certain sub-sets of normotensive African Americans; and this effect is augmented in individuals with baseline evidence of vascular dysfunction.
* Increased dietary sodium intake is associated with changes in the whole blood epigenome as assessed by DNA methylation.
* Increased dietary sodium intake is associated with changes in the whole blood and urine transcriptome as assessed by RNA-seq.
* Increased dietary sodium intake is associated with changes in the skin, oral and gut microbiome.
* Increased dietary sodium take is associated with whole blood epigenome/transcriptome molecular signature that predisposes to a chronic "pro-inflammatory state."

Type/design of trial:
A randomized, double-blind, placebo-controlled, cross-over design will be implemented.

Method to minimize bias:
Participants will be randomly assigned by a computer-generated code.

Number of study arms and intervention duration:
Participants will be randomized to a placebo or salt treatment arm for two weeks (Period 1), followed by a wash-out period for three-weeks, ending with a cross-over to the alternative treatment arm for two weeks (Period 2). The total length of participation is 7 weeks (Figure 1).

Name of study intervention:
Sodium Chloride

4.2 SCIENTIFIC RATIONALE FOR STUDY DESIGN

The study is testing the hypothesis that a modest increase in dietary sodium intake is sufficient to change the gut microbiome and trigger alterations in the blood transcriptome that may mediate the resultant in the well described salt-sensitive changes in vascular function, epigenome, transcriptome and blood pressure observed in African Americans. The study design as a randomized, double-blind, placebo-control cross-over design enables each participant to serve as her/his own control in response to the dietary salt intervention. The cross-over design allows for an intervening 'wash-out' period to ensure that the participant's diet and sodium homeostasis returns to baseline. The design's use of each participant as her/his own control reduces the impact of inter-individual variability in the basal dietary habits and microbiome; and optimizes statistical power to detect differences in baseline blood transcriptome gene expression profiles and gut microbiome in response to the dietary intervention. Our previous studies of the human transcriptome indicate that the current design is very robust to detect changes in gene expression by DNA methylation, RNA-seq and vascular function. The randomization ensures that the sequence order of the intervention (placebo vs salt tablets) is due to chance and avoids a systematic 'contamination' or carryover effect of the active treatment. The double-blind administration of tasteless capsules ensures that neither the participant or research staff are aware that there could be an increased ingestion of dietary salt. The dose of sodium chloride in the capsule has previously been shown to affect biomarkers sensitive to salt balance such as measures of the renin-angiotensin-aldosterone system and blood pressure. The daily dose of sodium chloride tablets given in the protocol falls well within the wide variance of 'normal',

safe, intake in various cultures around the world. The increment in salt intake beyond the participant's usual diet is equivalent to eating 1-2 bowls of soup and is a dose commonly administered to treat orthostatic hypotension in patients who suffer from this disorder. It should be noted that the protocol focuses on normotensive volunteers and excludes patients with hypertension or heart failure for whom a transient increase in dietary sodium could pose a risk. We will assess, gut, skin and saliva microbiome. The oral cavity is an excellent source of easy-to-access biological material for studies of oral microbiome [26]. This is due to the quick, non-invasive and low-cost collection [27]. The most used source of oral samples is saliva (collected by passive drool or spit). The collection of the skin microbiome samples is commonly performed using the swabbing method [28]. The sampling for skin microbiome was guided by the aim to consider two of the three skin microenvironments types [29]: Sebaceous and moist microenvironments. For the first, the Retroarticular Crease was chosen and for the latter, the Antecubital Fossa was selected. These sites provide also the advantage of being relatively less exposed than dry sites such as the forearm and hence less likely to have the external environment microbiome over-represented.
The oral samples are not collected to investigate gut microbiome but rather to evaluate changes in oral microbiome [26] with respect to changes in sodium intake. The aim is to find out if changes in sodium intake are correlated with microbiome changes in the oral cavity, in the gut and on the skin. Each site is considered separately first and then patterns of changes between sites are considered.

4.3 JUSTIFICATION FOR DOSE

Sodium chloride (1gm/capsule) and placebo will be dispensed by the NIH Pharmacy. Participant would be instructed to take 3 pills per day for a total of 3 grams. The dosing of three grams of sodium chloride per day was selected because it is sufficiently higher than normal dietary intake necessary to affect the outcome parameters and doesn't pose any health dangers. This dosage is the standard amount used in such protocol.[8-10]

5 STUDY POPULATION

5.1 INCLUSION CRITERIA

African American men and women who are former GENE-FORECAST participants between 21 and 65 years of age. I This criterion is inclusive of self-identified AA of both Hispanic, Latino and non-Hispanic, Latino ethnicities. Normotensive participants with systolic blood pressure (SBP) <140 mm Hg and diastolic blood pressure (DBP) <90 mm Hg and the absence of a history of prior diagnosis of hypertension.

- Willingness and ability to participate in study procedures.

5.2 EXCLUSION CRITERIA

- Individuals who are pregnant or breast-feeding.
- Individuals with high blood pressure or a history of hypertension.
- Individuals with a history of myocardial infarction, stroke, heart failure, diabetes, chronic liver or kidney diseases.
- Individuals who are taking antihypertensive, antidepressants, antidiabetic and antibiotic medications.
- Individuals currently participating in another NIH protocol.
- Individuals unable to provide informed consent.

5.3 INCLUSION OF VULNERABLE PARTICIPANTS

The study will not involve vulnerable groups other than NIH employees who volunteer for participation in study. NIH employees will be provided requisite information according to OHSRP Policy 404 prior to agreeing to participate in study.

5.4 LIFESTYLE CONSIDERATIONS

During the study, participants are asked to:

- Reframe from taking antibiotics. Taking such mediation will result in early withdrawal.

5.5 SCREEN FAILURES

All Participants will go through screening activites after the consent has been signed. Females will undergo a pregnancy test. Participants who consent to participate in the trial and subsequently have a positive pregnancy test prior to randomization will be considered a screen failure and not enrolled in the study. Participants who consent to participate but present with systolic blood pressure (SBP) >140 mm Hg and diastolic blood pressure (DBP) >90 mm Hg at physical examination will also be considered a screen failure.

Screen failures are defined as participants who consent to participate in the study but are not subsequently assigned to the study intervention or enter the study. A minimal set of screen failure information is required to ensure transparent reporting of screen failure participants to meet the Consolidated Standards of Reporting Trials (CONSORT), publishing requirements and respond to queries from regulatory authorities. Minimum information will include demography, screen failure details, eligibility criteria, and any serious adverse event (SAE).

5.6 STRATEGIES FOR RECRUITMENT AND RETENTION

- **Target study sample:**

Participants will be recruited from those who previously participated in the GENE-FORECAST protocol. A total of 40 normotensive African American men and 21-65 years old will comprise the total sample size. We anticipate screening 150 potential participants.

- **Anticipated accrual rate:**

40

- **Source of participants:**

Former GENE-FORECAST participants.

- **Recruitment venues:**

The GENE-FORECAST-SIT staff will contact individuals to determine interest in participating in GENE-FORECAST-SIT protocol and screen for eligibility over the telephone based on self-report information.

- **How potential participants will be identified:**

A listing of former normotensive GENE-FORECAST participants will be generated, including those in the upper tertile with vascular stiffness, by the data base manager that consented for re-contact via the GENE-FORECAST protocol.

-

5.6.1 Costs
There will be no out-of-pocket cost to participants.

5.6.2 Compensation
Participants will receive $50 per visit disbursed through the Research Volunteer System (RVS) for Visits 1,2, 3, and 4. A final payment for $300 will be mailed or sent via direct deposit to the participant by the RVS at the completion of the study and recovery of the study equipment (24hr BP monitor; Smart-Bottles). The total possible compensation for participating in the completed study will be $500. Participant will also receive a $25 voucher for lunch each visit and be afforded a one-way pre-paid taxi trip to the NIH Clinical Center.

6 STUDY INTERVENTION
6.1 STUDY INTERVENTIONS(S) ADMINISTRATION
6.1.1 Study Intervention Description
There are no unapproved drugs or devices being used in this study.
Sodium Chloride is Food and Drug Administration (FDA) approved for a variety of clinical conditions and is included in a wide variety of commonly consumed processed foods.

6.1.2 Dosing and Administration
Sodium Chloride and Placebo: Sodium Chloride (1gm/capsule) and corresponding placebo will be provided by the NIH Pharmacy. Participant will be instructed to take 3 pills per day for a total of 3 grams. The dosing of three grams of sodium chloride per day was selected because it is sufficiently higher than normal dietary intake necessary to affect the outcome parameters and doesn't pose any health dangers. This dosage is the standard amount used in such protocol.[8-10]

6.1.2.1 Dose Escalation
There will be no dose escalation. Participants will be instructed to take 3 assigned salt or placebo pills per day for a total of 3 grams.

6.1.2.2 Dose Limiting Toxicity
Not applicable

6.1.2.3 Dose Modifications
Not applicable

6.1.2.4 Drug Administration
Participant will be instructed to take 3 pills per day for a total of 3 grams. If one capsule is missed per day, participant should not make up the next day by taking an additional capsule.

6.2 PREPARATION/HANDLING/STORAGE/ACCOUNTABILITY
6.2.1 Acquisition and Accountability
The sodium chloride and placebo capsules will be manufactured by Pine Pharmaceuticals in Tonawanda, New York and sent directly to the NIH Clinical Center Investigational Drug & Research Section Outsourcing Unit (IDOU). The sodium chloride and placebo capsules will be subsequently sent to the NIH Clinical Center pharmacy who will be dispense directly to

participants based on randomized double-blind ID list previously provided by PI. Expired and unused product will be discarded by the IDOU and not sent back to the manufacturer.

6.2.2 Formulation, Appearance, Packaging, and Labeling

The sodium chloride and placebo capsules will be formulated by Pine Pharmaceuticals in Tonawanda, New York based on FDA guidelines. There will be no distinguishing characteristics between both capsules. The sodium chloride and placebo capsules will be put in a separate 'smart bottle' by Clinical Center pharmacy and labelled based on randomized double-blind participant ID list previously provided by PI with instruction to take two times a day. The smart bottle technology will electronically monitor adherence to daily treatment and is designed to remind participant to take treatment and to alert when treatment has been missed. Smart bottles will be purchased from a reputable vender with a history of collaboration in such trials.

6.2.3 Product Storage and Stability

The product will be stored in the Clinical Center pharmacy based on protocol designed to protect from light, temperature, and humidity. The smart bottle will also be designed to protect from such elements.

6.2.4 Preparation

The Clinical Center pharmacy will dispense the sodium chloride and placebo capsules in separate smart bottles. Capsules do not involve thawing, diluting, mixing and reconstitution/preparation.

6.3 Measures to Minimize Bias: Randomization and Blinding

A randomized, double-blind, placebo-controlled, cross-over design will be implemented (Figure 1). Participants will be randomly assigned by a computer-generated code based on study ID. All study staff will be blinded to randomization throughout the trial period. The randomized list will be provided to the Clinical Center pharmacy who will have the key to specific participant randomization.

6.4 Study Intervention Compliance

The sodium chloride and placebo capsules will be put in separate smart bottle by Clinical Center pharmacy and labelled based on randomized double-blind ID previously provided by PI instructing participant to take three times a day. The smart bottle technology will electronically monitor adherence to daily treatment and is designed to remind participant to take treatment and to alert when treatment has been missed. Smart bottles will be purchased from a reputable vender with a history of collaboration in such trials.

The smart bottle includes an electronic interface and database that allows clinical study staff to monitor, document, and calculate compliance. We will also collect spot urine test for sodium/potassium at baseline and at the end of each treatment test as an additional measure of adherence to the dietary sodium intervention.

6.5 Concomitant Therapy

Antibiotics are excluded from this trial. However, any other prescription medication is defined as a medication that can be prescribed by a properly authorized/licensed clinician is allowable.

Medications will be reported in the Case Report Form (CRF) as concomitant prescription medication, including over-the-counter medications and supplements.

7 STUDY INTERVENTION DISCONTINUATION AND PARTICIPANT DISCONTINUATION/WITHDRAWAL

7.1 DISCONTINUATION OF STUDY INTERVENTION

Participant will be discontinued from participation in study if:

- Completion of study trial
- If any clinical adverse event (AE), laboratory abnormality, or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the participant
- Positive pregnancy test
- Participant unable to receive study intervention for two consecutive days.

Any clinically relevant finding will be reported as an AE. All study related indices will be recorded and maintained up to the time of discontinuation. Clinical staff will also follow-up with participant to capture AE, serious adverse events (SAE), and unanticipated problems (UPs)

7.2 PARTICIPANT DISCONTINUATION/WITHDRAWAL FROM THE STUDY

Participants are free to withdraw from participation in the study at any time upon request, or upon the development of an adverse reaction due to exposure to intervention.

PI may discontinue or withdraw participant from the study for the following reasons:

- Significant study intervention non-compliance
- If the participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation
- Death
- Screen failure

The reason for participant discontinuation or withdrawal from the study will be recorded on the ‘Current Status” CRF. 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. Prior to removal from study, efforts will be made to have participant complete a safety visit approximately one week following the last dose of study product.

7.3 LOST TO FOLLOW-UP

A participant will be considered lost to follow-up if he or she fails to return for the three subsequent scheduled visits following baseline visit and is unable to be contacted by the study GENE-FORECAST-SIT staff .
The following actions will be taken if a participant fails to return to the clinic for a required study visit:

- The GENE-FORECAST-SIT staff will attempt to contact the participant and reschedule the missed visit one week 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 GENE-FORECAST-SIT staff 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 medical record or 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 SCREENING PROCEDURES

A listing of former normotensive GENE-FORECAST participants will be generated, including those in the upper tertile with vascular stiffness, by the data base manager that consented for re-contact via the 'Call Back' protocol. The Research Nurse will contact individual to determine interest in participating in GENE-FORECAST-SIT and screen via the telephone for eligibility based on self-reported health information. Participant who agree to participate in study will be scheduled for baseline clinical visit based on availability and subsequently screened again at the Clinical Center. All participants will undergo screening activities upon coming to the NIH. Female participants will undergo a pregnancy test after consent signature. Participants who consent to participate in the trial and subsequently have a positive pregnancy test prior to randomization will be considered a screen failure and not enrolled in the study.

8.2 EFFICACY ASSESSMENTS

1. Salt/Placebo Intervention: Capsules will be dispensed by Clinical Center Pharmacy.
2. Flow Activating Cell Sorting (FACS) Analysis: Characterization of Leukocytes Populations : As part of assessing the whole blood epigenome and transcriptome, the protocol will perform an in-depth characterization of the heterogeneous mixture of leukocytes circulating in the blood and how this profile might be altered in response to the dietary sodium intervention. We will utilize the NHLBI's Flow Cytometry Core in collaboration with Dr. Phil McCoy. We will collect peripheral blood samples at the 4 time points (visit 1, 2 3 and 4). The FACS analysis will define changes in immune cell-type profiles as well as facilitate the isolation of DNA/RNA from specific immune cell sub-types (e.g. CD4 T-cells; CD8 T-cells, Monocytes etc.) for epigenome and transcriptome analysis.
3. Vascular function testing: These tests will be done using specialized non-invasive machines, such as SphygmoCor and EndoPAT, to assess vascular health.
4. 24-hour ambulatory blood pressure monitoring: Participants will be given an ambulatory blood pressure monitoring to device that will be worn for 24 hours within two days of NIH baseline visit and again for 24 hours within two days prior to the return visits.
5 A 24-hour dietary recall will be administered at baseline and at each subsequent visit to assess differential diet and salt intake among each participant. Nutrition staff from the Clinical Center will meet with each participant and ask them to recall any foods or beverages they have eaten over the past 24 hours using an automated dietary analysis software application developed by the US Department of Agriculture for use in the National Health and Nutrition Examination Survey. The intakes for 108 nutrients (including salt) will be calculated for each participant based on individual diet.
6. Psychophysical Taste Task: Sucrose and Salt Detection Thresholds**:** Sucrose and salt detection thresholds will be assessed using a two-alternative forced-choice staircase procedure

was developed at the Monell Center for Adults.[14-16] The two-alternative forced choice is a psychophysical method developed to elicit responses about an individual's experience regarding a stimulus. It focuses on the evaluation of a single attribute (e.g., sweetness or saltiness), and the stimulus is adjusted based on the individual's responses.[17-18] For this study, all testing will take place in a private, comfortable room. Subjects will be fasted for at least one hour before the task and acclimate to the testing room and to the researcher for approximately 15 minutes before testing. For the first trial and each subsequent trial, subjects will be presented with pairs of solutions in random order; within each pair, one solution will be distilled water, and the other will be the taste stimulus. Subjects will be instructed to taste the first solution presented within the pair, swish the solution in their mouth for 5 seconds, and expectorate. This will be repeated for the second solution within the pair. Between solutions, subjects will rinse their mouth with water; they will rinse once within a pair, and twice between successive pairs. After tasting both solutions within a pair, subjects will be asked to point to the solution that has a non-neutral taste. A tracking grid will be used to record subjects' response.[19-21] This method eliminates the need for a verbal response and has been shown to be an effective method for assessing both taste and olfaction in children.[19,22] The task takes approximately 30 minutes to complete.

7. Psychophysical Taste Task: Sucrose and Salt Preference: Sucrose and salt preference will be assessed using a two-series paired comparison-tracking method developed at the Monell Center for Adults.[14-16] Subjects will be presented with pairs of solutions differing in sucrose concentration (3, 6, 12, 24, and 36 g per 100 mL) and salt (0.92–6.14% wt/vol NaCl). They will be asked to taste the solutions without swallowing and point to which of the pair they liked better. Subsequently, each pair presented will be determined by the subject's preceding preference choice. The entire task is then repeated with the stimulus pairs presented in reverse order. After completion of the taste task, the geometric mean of the sucrose concentrations chosen will be determined. This serves as an estimate of the participant's most preferred level of sucrose.[22-23] The task takes approximately 15 minutes to complete.
8. Baecke's Physical Activity Questionnaire: Physical activity will be assessed using 16 questions involving three habitual physical activities (occupational physical activity, physical exercise, and leisure activities) during the previous 12 months.[24]
9. Pittsburgh Sleep Quality Index (PSQI): This is a 19-item self-rated questionnaire for evaluating subjective sleep quality over the previous month.[25]
10. Microbiome: Skin, saliva and stool samples will be collected by the GENE-FORECAST-SIT staff based on the utilization of Norgen kits. Participant will be swabbed in three locations of the skin and will spit in a prepared value. Subjects will be required to not shower within 12 hours of sampling based on the guidelines from the NIH common fund Human Microbiome Project, an initiative to generate resources and facilitate the characterization of the human microbiota (Human Microbiome Project, Nature, 2012). Stool kits will be previously mailed to participant for collection and participant will give stool sample to GENE-FORECAST-SIT staff at the clinic appointment.

It is estimated that participants will spend approximately 5 hours at the Clinical Center during baseline and approximately 3 hours during each of the subsequent visit. Obtaining and explaining the informed consent will take about 20-30 minutes. Female prospective participant who signs consent and subsequently test positive for pregnancy prior to randomization will not be enrolled in the study. Administering the baseline questionnaires will take about 1.45 hours and 75 minutes for the 24-hour diet recall and taste assessments for each subsequent visit. The clinic visit

assessment, plus vascular function test and microbiome collection, will take about 1.30 hours. A member of the clinical study staff will follow-up with participant one week after visit to assess any adverse reaction to consuming intervention product. All procedures and details for each can be found in the study's Manual of Operation (MOP).

Table 1: Estimated length of time for study procedures

NIH Clinic Visit	Estimated time 4 hours	Total Participants	Duration of Study
a) Informed consent (baseline only)	20-30 minutes	40	7 wks
b) Phlebotomy	30 minutes	40	7 wks
c)Physical examination	20 minutes	40	7 wks
d) Baseline Questionnaires: Baecke's Physical Activity Pittsburgh Sleep Quality Index	1.45 hours	40	Baseline
e) 24 diet recall	30 minutes	40	7wks
f) Psychophysical Taste Task: Sucrose and Salt Detection Thresholds	30 minutes	40	7 wks
g) Psychophysical Taste Task: Sucrose and Salt Preference	15 minutes	40	7 wks
h) Microbiome	15 minutes	40	7 weeks
i) Vascular Function	1.0 hrs	40	7 wks

8.2.1 Clinical Evaluations

Physical examination: A physical examination will be performed by the study Physician Assistant to include health/medication history and measurements of height and weight. Vital signs including temperature, pulse, respirations, and blood pressure, will be also be collected.

Radiographic or other imaging assessments:
Radiology or other imaging will not be conducted in this study.

8.2.2 Biospecimen Evaluations

Participants will undergo standardized blood collection for chemistry panel, blood pressure measurement and vascular function test. Biological specimens will include peripheral venous blood collected by phlebotomy, urine, and stool. All biospecimens will be collected at the NIH Clinical Center which is in compliance with Clinical Laboratory Improvement Amendments

(CLIA). Blood, urine and stool samples will be collected from the Clinical Center by the GENE-FORECAST-SIT staff. The samples are brought to PI's lab by research study lab assistants where Laboratory Manager will prepare and store samples in freezers. Shipment of blood and microbiome samples for each visit per participant will be sent overnight for assay in dry ice in compliance with standard procedures. Each sample will be labelled with participant study ID.

Urine sample: (small quantity, 1 cup 90 ml)

1. Urine chemistries (e.g. sodium , chloride, potassium)
2. Urine creatinine and microalbumin (ACR)
3. Urine protein to creatinine ratio (PCR)
1. Osmolality
2. Urea Nitrogen
3. Urine exosomes (RNA/microRNA)

Microbiome samples:
Stool/At home collection kit (quantity = 2 grams):
Skin: swab of inner elbow and behind ears
Oral: saliva

Blood samples:

Fasting samples for clinical chemistries:

Blood samples**:**

Clinical Center Research Lab Test					
Lab test	**Volume Tube (ml)**	**Number of Tubes**	**Total Volume (ml)**	**Total Volume (tablespoon)**	**Tube type**
CBC: CBC with differential count HbA1C: A1C Homocysteine	4	2	8	0.54	Lavender
Fasting lipid panel (serum): Mineral panel Acute Care Panel	3.5	2	7	0.47	green/yellow

Total Protein ApoA1 and ApoB: APOAB Pro-BNP: BNP1 C-reactive protein: CRPHS (high sensitivity CRP)					
Hepatic Panel Serum hCG	3.5	1	3.5	0.24	Gold
Serum Cortisol (CORT) Serum Aldosterone (ALD03,ADS01, ADS02)	8.5	1	8.5	0.57	Red SST
Clinical Center Research Labs Total	**19.5**	**6**	**27**	**1.83**	

Non-Clinical Center Research Lab Test					
Lab test	**Volume Tube (ml)**	**Number of Tubes**	**Total Volume (ml)**	**Total Volume (tablespoon**	**Tube type**
DNA isolation (research, whole blood)	4	1	4	0.27	Lavender/EDTA
RNA/miRNA isolation (whole blood)	2.5	2	5	0.34	PaxGene
Plasma for research archiving	4	1	4	0.27	Green/ Lithium Heparin
Research tube for specialized endothelial function markers (e.g. VCAM-1, VEGF)	3.5	1	3.5	0.24	Red SST

Research tube for specialized inflammatory markers (e.g. IL6, TNF alpha)					
Plasma Renin Activity (PRA, PRAK1)	4	1	1	0.20	Lavendar/EDTA
Serum for research archiving	3.5	1	3.5	0.24	Red SST
FACS Analysis	10	4	40	2.71	Green/ Sodium Heparin
Non-Clinical Center Research Labs Total	**27.5**	**10**	**60**	**4.06**	
Total per visit (4 visits)	**47**	**16**	**87**	**5.88**	

Microbiome:

BODY SITE	SPECIMEN	COLLECTION TUBE
Oral Cavity	Saliva	Saliva DNA Collection, Preservation, and Isolation Kit
Skin	Retroauricular Crease (Left)	Swab Collection and DNA System
	Retroauricular Crease (Right)	
	Antecubital Fossa (Left)	
	Antecubital Fossa (Right)	
GI Tract	Stool	Norgen Stool Nucleic Acid Collection and Preservation System

Total volume of blood collected per visit = 87 ml, which is equivalent to about 5.8-6 tablespoons of blood.

Biological specimens collected from individuals will include peripheral venous blood and urine samples collected by phlebotomy. Oral microbiome samples will be acquired using a spit collection kit. Skin swabs will be used to obtain skin microbiome samples from the left and

right retroarticular crease, as well as, the left and right antecubital fossa**.** Stool microbiome samples will be collected in a stool kit mailed to participants prior to clinic visit. Detailed instructions for the preparation, handling, storage, and shipment of specimens will be explained in the study's MOP.

8.2.3 Correlative Studies for Research/Pharmacokinetic Studies

The protocol will not include correlative studies for research/pharmacokinetic studies.

8.2.4 Samples for Genetic/Genomic Analysis

8.2.4.1 Description of the scope of genetic/genomic analysis

- Analyses will include whole blood epigenetics based on DNA methylation and transcriptomics analysis will use whole blood and urine based on RNAseq.
- Germline and/or somatic analysis will not be conducted.
- Whole blood and urine will be the biospecimens used for analyses. Privacy and confidentiality of medical information/biological specimens will be maximized.
- No personal identifier will be maintained – including protected health information (PHI). Rather, participant will be assigned a unique numerical personal study identifier resulting in the unlikely occurrence of potential identification.
- Clinical and demographic information will be collected on each participant. Participant nor family members can be identified due to the use of a numerical personal study identifier.
- All coded participant, demographic, survey, medical and biological information will be stored in a medical file and a secured NIH database with appropriate IT security measures (e.g. de- identified codes; encryption; password access protection) to maximize the protection of privacy. Only the database manager will have access to the password key for code.
- Pedigree information will not be published.
- The personal identifier will not be released to any third party.
- No personal identifier containing data/sample information will be shared with other investigators. Information will only be deposited into a relevant controlled access database as mandated by NIH policies.
- For additional protection of confidentiality, all hard-copy information will be maintained in a locked file-cabinet with access only by the Research Nurse and Data-Base manager. Hard-copy information will be maintained and destroyed based on the duration mandated by NIH policies. In addition, we will use a custom designed NIH database - Clinical Trial Data Base (CTDB) designed for the collection and storage of study indices from study participants. Access to study participants' information is limited to only a few of the investigators and support staff. The database has password protection, stratified access, and firewall protection.

- Furthermore, to help us protect participant's privacy, this study has obtained a Certificate of Confidentiality from the National Institutes ofHealth. Researchers cannot release identifiable information to any federal, state, or local civil, criminal, administrative, legislative, or other proceedings. Researchers will utilize the Certificate to minimize risks by adding a level of protection to study participants.

- The Certificate cannot be used to resist a demand for information from personnel of the United States Government that is used for auditing or evaluation of federally funded projects or for information that must be disclosed in order to meet the requirements of the federal Food and Drug Administration (FDA).

- The Certificate of Confidentiality does not prevent any participant or a member of their family from voluntarily releasing information about a participant involvement in this research. In the event that an employer, insurer or other person obtains a participant's written consent to obtain research information, then the Certificate of Confidentiality cannot be used to withhold information.

8.2.4.2 Management of Results

Participants will be mailed a letter following baseline visit at the NIH Clinical Center (which is a CLIA certified lab) with results from their clinical visit, including Body Mass Index with health definitions, glucose, blood pressure.

8.2.4.3 Genetic counseling

Genetic counseling will not be provided. We are not doing gene sequencing as a part of the study, therefore reporting the possibility of incidental findings is not applicable.

8.2.4.4 Safety and Other Assessments

Table 3: List and description for study procedures

NIH Clinic Visit	Description
a) Physical examination	Height, weight, temperature, pulse, respirations, blood pressure conducted by NIH Clinical Center Nursing Staff, Physician Assistant will perform physical examination.
b) Phlebotomy	Standard clinical laboratory chemistry- including whole blood for DNA methylation epigenome and RNAseq transcriptome collected by Clinical Center phlebotomy
c) Urine	Urine sample for RNAseq transcriptome collected by Clinical Center phlebotomy
d) Microbiome Skin, saliva, stool	Swab, saliva and stool kits will be used to assess microbiome collected by study GENE-FORECAST-SIT staff
e) Baseline Questionnaires	Pittsburgh Sleep Quality Index (PSQI**):** This is a 19-item self-rated questionnaire for evaluating subjective sleep quality over the previous month. Assessment will be completed in about 20 minutes. This will only be administered at baseline. Baecke's Physical Activity Questionnaire: Physical activity will be assessed using 16 questions involving three habitual physical activities (occupational physical activity, physical

	exercise, and leisure activities) during the previous 12 months. Assessment will be completed in about 15 minutes. This will only be administered at baseline. Questionnaires conducted by study GENE-FORECAST-SIT staff
f) 24 diet recall	Nutrition staff from the Clinical Center will meet with each participant and ask them to recall any foods or beverages they have eaten over the past 24 hours using an automated dietary analysis software application developed by the US Department of Agriculture for use in the National Health and Nutrition Examination Survey. The intakes for 108 nutrients (including salt) will be calculated for each participant based on individual diet.
g) Psychophysical Taste Task: Sucrose and Salt Detection Thresholds	Sucrose and salt detection thresholds will be assessed using a two-alternative forced-choice staircase procedure was developed at the Monell Center for Adults. The two-alternative forced choice is a psychophysical method developed to elicit responses about an individual's experience regarding a stimulus. It focuses on the evaluation of a single attribute (e.g., sweetness or saltiness), and the stimulus is adjusted based on the individual's responses. After tasting both solutions within a pair, subjects will be asked to point to the solution that has a non-neutral taste. A tracking grid will be used to record subjects' response.[19-21] Assessment conducted by Associate Investigator Dr. Paule Joseph.
h) Psychophysical Taste Task: Sucrose and Salt Preference	Sucrose and salt preference will be assessed using a two-series paired comparison-tracking method developed at the Monell Center for Adults.[14-16] Subjects will be presented with pairs of solutions differing in sucrose concentration (3, 6, 12, 24, and 36 g per 100 mL) and salt (0.92–6.14% wt/vol NaCl). They will be asked to taste the solutions without swallowing and point to which of the pair they liked better. Subsequently, each pair presented will be determined by the subject's preceding preference choice. The entire task is then repeated with the stimulus pairs presented in reverse order. After completion of the taste task, the geometric mean of the sucrose concentrations chosen will be determined. This serves as an estimate of the participant's most preferred level of sucrose.[22-23] The task takes approximately 15 minutes to complete. Assessment conducted by Associate Investigator Dr. Paule Joseph.
i) Vascular Function	These tests will be done using specialized non-invasive machines, such as SphygmoCor and EndoPAT, to assess vascular health conducted by study Physician Assistant.

Biological specimen collection and laboratory evaluations:
Biological specimen

Clinical Center Research Lab Test					
Lab test	**Volume Tube (ml)**	**Number of Tubes**	**Total Volume (ml)**	**Total Volume (tablespoon)**	**Tube type**
CBC: CBC with differential count HbA1C: A1C Homocysteine	4	2	8	0.54	Lavender
Fasting lipid panel (serum): Mineral panel Acute Care Panel Total Protein ApoA1 and ApoB: APOAB Pro-BNP: BNP1 C-reactive protein: CRPHS (high sensitivity CRP)	3.5	2	7	0.47	green/yellow
Hepatic Panel Serum hCG	3.5	1	3.5	0.24	Gold
Serum Cortisol (CORT) Serum Aldosterone (ALD03,ADS01, ADS02)	8.5	1	8.5	0.57	Red SST

Clinical Center Research Labs Total	**19.5**	**6**	**27**	**1.83**

Non-Clinical Center Research Lab Test					
Lab test	**Volume Tube (ml)**	**Number of Tubes**	**Total Volume (ml)**	**Total Volume (tablespoon)**	**Tube type**
DNA isolation (research, whole blood)	4	1	4	0.27	Lavender/EDTA
RNA/miRNA isolation (whole blood)	2.5	2	5	0.34	PaxGene
Plasma for research archiving	4	1	4	0.27	Green/ Lithium Heparin
Research tube for specialized endothelial function markers (e.g. VCAM-1, VEGF) Research tube for specialized inflammatory markers (e.g. IL6, TNF alpha)	3.5	1	3.5	0.24	Red SST
Plasma Renin Activity (PRA, PRAK1)	4	1	1	0.20	Lavendar/EDTA
Serum for research archiving	3.5	1	3.5	0.24	Red SST
FACS Analysis	10	4	40	2.71	Green/ Sodium Heparin
Non-Clinical Center Research Labs Total	**27.5**	**10**	**60**	**4.06**	

Total per visit (4 visits)	**47**	**16**	**87**	**5.88**

Microbiome:

BODY SITE	SPECIMEN	COLLECTION TUBE
Oral Cavity	Saliva	Saliva DNA Collection, Preservation, and Isolation Kit
Skin	Retroauricular Crease (Left)	Swab Collection and DNA System
	Retroauricular Crease (Right)	
	Antecubital Fossa (Left)	
	Antecubital Fossa (Right)	
GI Tract	Stool	Norgen Stool Nucleic Acid Collection and Preservation System

Total volume of blood collected per visit = 87 ml, which is equivalent to about 5.8-6 tablespoons of blood. More detailed description of all procedures can be found in the study MOP.

Laboratory evaluations:
DNA and RNA extractions will be done by ReproCELL USA, Inc. DNA and RNA sequencing will be done by the NIH Intramural Sequencing Center (NISC). Microbiome assay will be done by University of Pennsylvania Microbiome Center. Flow Activating Flow Cell Sorting (FACS) Analysis will be conducted by the NHLBI's Flow Cytometry Core in collaboration with Dr. Phil McCoy.

Adverse Events and Serious Adverse Events
8.2.5 Definition of Adverse Event
We will define an adverse event (AE) as any untoward medical occurrence associated with the use of an intervention in humans, whether considered intervention relation based on 21 CFR 312.32(a).

8.2.6 Definition of Serious Adverse Events (SAE)
8.2.7 Classification of an Adverse Event
We will define a SAE or suspected SAE as "serious" if, in the view of either the investigator or sponsor, 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 of 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, 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 the 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.

8.2.7.1 Severity of Event

All Adverse Events (AEs) will be assessed by the Staff Clinician. For 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 interferences with functioning.

Severe: Events interrupt a participant's usual daily activities 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".

8.2.7.2 Relationship to Study Intervention

All adverse events (AEs) must have their relationship to study intervention assessed by the PI, in consultation with clinical study staff, who will examine and evaluate the participant based on temporal relationship and his clinical judgement. The degree of certainty about causality will be graded using the categories below. In a clinical trial, the study product must always be suspect.

Related: The AE is known to occur with the study intervention, when 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.

OR

Definitely Related: There is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out. The clinical event, including an abnormal laboratory test result, occurs in a plausible time relationship to study intervention administration and cannot be explained by concurrent disease or other drugs or chemicals. The response to withdrawal of the study intervention should be clinically plausible. The event must be pharmacologically or phenomenologically definitive, with use of a satisfactory re-challenge procedure if necessary.

Probably Related: There is evidence to suggest a causal relationship, and the influence of other factors is unlikely. The clinical event, including an abnormal laboratory test result, occurs within a reasonable time after administration of the study intervention, is unlikely to be attributed to concurrent disease or other drugs or chemicals, and follows a clinically reasonable response on withdrawal (de-challenge). Re-challenge information is not required to fulfill this definition.

Potentially Related: There is some evidence to suggest a causal relationship (e.g., the event occurred within a reasonable time after administration of the trial medication). However, other factors may have contributed to the event (e.g., the participant's clinical condition, other concomitant events). Although an AE may rate only as "possibly related" soon after discovery, it can be flagged as requiring more information and later be upgraded to "probably related" or "definitely related", as appropriate.

Unlikely to be Related: A clinical event, including an abnormal laboratory test result, whose temporal relationship to study intervention administration makes a causal relationship improbable (e.g., the event did not occur within a reasonable time after administration of the study intervention and in which other drugs or chemicals or underlying disease provides plausible explanations (e.g., the participant's clinical condition, other concomitant treatments).

Not Related: The AE is completely independent of study intervention administration, and/or evidence exists that the event is definitely related to another etiology. There must be an alternative, definitive etiology documented by the clinician.

8.2.7.3 Expectedness

The Staff Clinician will be responsible for determining whether and 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.

8.2.8 Time Period and Frequency for Event Assessment and Follow-Up

The occurrence of an AE or SAE may come to the attention of clinical study staff during study visits and follow-up after study visit to assess reaction(s) to intervention product.

All AEs including local and systemic reactions not meeting the criteria for SAEs will be capture on the appropriate Case Report Form (CRF). Information to be collected includes events description, time of onset, Staff Clinician's assessment of severity, relationship to study product and time of resolution/stabilization of the event. All AEs occurring while on study will be documented appropriately regardless of relationship. All AEs will be followed to adequate resolution.

Any medical condition that is 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, 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 require documentation of onset and duration of each episode.

The study's Physician Assistant will record all reportable events with start dates occurring any time after informed consent is obtained until 7 days (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation. At each study visit, the PI will inquire about the occurrence of AE/SAEs since the last visit. Events will be followed for outcome information until resolution or stabilization.

8.2.8.1Adverse Event Reporting

The study PI will report all nonserious AEs to the Independent Safety Monitor and to the NIH IRB at the time of the continuing review. The PI will be responsible for signing off of all AE reports. Full details of procedures, including flow chart, will be included in the study's Manual of Operating Procedures (MOP). Potential side effects associated with the intervention product (i.e. sodium chloride) may include discomfort while swallowing, increased thirst, indigestion, nausea, vomiting, headaches, mild elevations in blood pressure, mild changes in blood chemicals (electrolytes), lightheadedness, tiredness, mild swelling of arms/legs, and/or diarrhea, and rash. There are no disease related conditions related to the intervention product.

8.2.8 Serious Adverse Event Reporting

The study PI will immediately report to the NIH IRB any serious adverse event, whether or not considered study intervention related, including those listed in the protocol and will include an assessment of whether there is a reasonable possibility that the study intervention caused the event. Study endpoints that are SAEs (e.g., all-cause mortality) must 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).

All SAEs will be followed until satisfactory resolution or until the PI deems the event to be chronic or the participant is stable. Other supporting documentation of the event will be made available to the Independent Safety Monitor or Intramural Program as soon as possible upon request.

8.2.9 Events of Special Interest

Not applicable

8.2.10 Reporting of Pregnancy

If a participant test positive for pregnancy during baseline visit, she will not be enrolled. If the participant test positive at any subsequent visit or becomes pregnant in between visits, she will be discontinued, and this will be reported as an AE to the NIH IRB during the time of continuing review.

8.3 UNANTICIPATED PROBLEMS

8.3.1 Definition of Unanticipated Problems (UP)

Any incident, experience, or outcome that meets all of the following criteria shall be considered an UP:

- Unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol related documents, such as the IRB-approved research protocol and informed consent documents; and (b) the characteristics of the participant population being studied; and

- 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 (which many include research staff, family members or other individuals not directly participating in the research) at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or expected.

8.3.2 Unanticipated Problem Reporting

The investigator will report unanticipated problems (UPs) to the NIH Institutional Review Board (IRB) as per Policy 801.

8.3.3 NIH Intramural IRB Reporting of IND Safety Reports

Only IND Safety Reports that meet the definition of an unanticipated problem will be reported to the NIH Intramural IRB.

9 STATISTICAL CONSIDERATIONS

The protocol will not have a formal Statistical Analytical Plan (SAP).

9.1 STATISTICAL HYPOTHESIS

1) Increased dietary sodium intake will be associated with vascular dysfunction and increased blood pressure in the treatment group compared to the placebo group.
2) Increased dietary sodium intake will be associated with changes in the whole blood epigenome as assessed by DNA methylation in the treatment group compared to the placebo group.
3) Increased dietary sodium intake will be associated with changes in the whole blood and urine transcriptome as assessed by RNA-seq in the treatment group compared to the placebo group.
4) Increased dietary sodium intake will be associated with changes in the skin, oral and gut microbiome in the treatment group compared to the placebo group.
5) Increased dietary sodium take will be associated with blood epigenome/transcriptome molecular signature in the treatment group compared to the placebo group.

Endpoints:

Primary:
Vascular function
Microbiome

Secondary:
Blood pressure
Whole blood epigenome
Whole blood and urine transcriptome

9.2 SAMPLE SIZE DETERMINATION

Data will be tested for normality using the Shapiro-Wilk test. Continuous variables will be described as mean ± standard deviation (SD) if normally distribute. Variables with skewed distribution (i.e. aldosterone, BNP, etc.) were logarithmically transformed prior to analyses and are presented as median and interquartile range (IQR). Categorical variables will be expressed as numbers (percentages).
When data are normally distributed, comparisons between the treatment (sodium intake) and control (placebo) groups will be made using paired Student's t-tests, and when data are non-

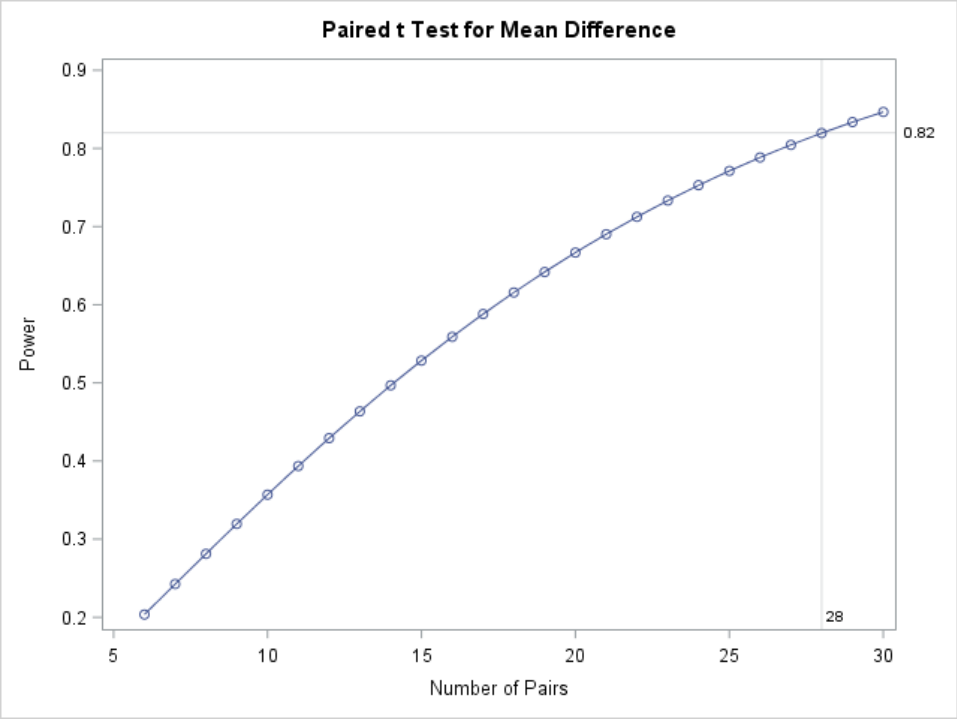
normally distributed, the Mann-Whitney U test will be employed. Chi-square test will be used for categorical variables. Tests for sodium intake effect on the primary outcomes without correction for period effect will be done using Mann-Whitney test on the differences. Tests for period effect will be assessed using two-sample Wilcoxon tests on the differences between treatment groups, and tests for carry-over effect will be assessed by a two-sample Mann-Whitney test on the averages in the two treatment periods. Correlations were determined with Pearson's correlation coefficient.

Additionally, we will evaluate the effects of sodium intake on changes in blood pressure, 24-hr ambulatory blood pressure monitoring (ABPM), biochemical markers, vascular functions, and specific transcriptome and epigenome by using a linear mixed model, with subject as a random effect, thereby accounting for the period, sequence, and carryover effects and to model the various sources of intra-individual and inter-individual variability. All statistical tests will be two-sided and P values <0·05 will be regarded as significant. Statistical analyses will be carried out using SAS software (SAS Institute, Inc., Cary, NC, USA).

Sample size and statistical power: We expect to enroll and randomize a total of 40 eligible subjects for this protocol. The sample size is assessed to detect meaningful effects of sodium intake on the study's primary outcomes and secondary outcomes, including changes in vascular function (PWV m/s), sitting SBP and DBP (mm Hg), ABPM (mm Hg), biochemical markers (plasma aldosterone, pg/mL, brain natriuretic peptide (pg/mL), as well as epigenome, transcriptome, and microbiome under an assumption about the effect size, standard deviation outcomes, correlation of measurements within individual. We estimated that a sample size of 28 participants will be required in a cross-over study to detect changes in PWV between high salt intake and control groups of 0.6 m/s (estimates based on Pimenta et al. [47]), with 80% power and $P < 0.05$, assuming common SD of 0.9 m/s for high sodium intake and control groups, and correlation of 0.3 within subjects (Figure 2). Accounting for a potential of 20% dropout during the study, the sample size would be 34.

In addition, the estimated sample size will provide us with more than 80 percent power to detect a difference of 6 mm Hg (SD=9 mm Hg) in sitting SBP between sodium intake and the placebo treatment at 0.05 significance level (estimates based on Tzemos et al. [48]). Similarly, the proposed sample size would give more than 90% power to detect significant changes in plasma aldosterone (pg/ml) and brain natriuretic peptide (pg/mL) between high sodium intake and control at 0.05 significance level [47,48]. The sample size calculation was performed using a paired *t* test assuming no carryover effect and no interactions between subjects, treatments, and periods.

Figure 2.



9.3 POPULATIONS FOR ANALYSES

We will use the per-protocol analysis dataset for our study. We are confident that participants will comply with the protocol sufficiently to ensure that the data will be likely to represent the effects of exposure to treatment and placebo arms based on the underlying scientific model.

9.3.1 Evaluable for toxicity

9.3.2 Evaluable for objective response

Not applicable

9.3.3 Evaluable Non-Target Disease Response

Not applicable

9.4 STATISTICAL ANALYSES

9.4.1 General Approach

For descriptive statistics, categorical variables will be assessed via chi-square and presented as percentages. Continuous variables will be assessed via t-test and will be presented as means with standard deviations. We will check assumptions underlying statistical procedure and correct procedure if necessary.

9.4.2 Analysis of the Primary Endpoints

The two primary endpoints are vascular function and microbiome.
First, vascular function difference between placebo and treatment (salt intake) will be estimated. Then, microbiome differential between treatment and placebo will be identified and estimate for each of the microbiome collection sides (skin, oral and gut). Details of the vascular function and microbiome analyses with respect to treatment and placebo are provided below.

Vascular function (e.g. pulse wave velocity, PWV) will be first analyzed as a continuous variable. Its relationship with salt intake will be investigated through multiple linear regression with vascular function as outcome and salt intake (a binary variable with two categories:

treatment and placebo) as exposure variable. For this analysis only samples with complete data for vascular function and salt intake will be considered. The outcome variable will be log transformed if it departs markedly from the Gaussian distribution. The aim of this first analysis is to estimate the effect of salt intake on vascular function. The effect size, p-value and 95% confidence interval will be provided as results.

The second endpoint is microbiome; the microbiome data of each of the all the collection sites (oral, gut and skins) will be analyzed separately. Prior to investigating the relationship between microbiome and salt intake, the raw microbiome data requires extensive processing and quality controls (QC) because of challenges posed by this type of data [30,31] and the potential of spurious results if appropriate QC are not applied. The raw data will be processed in mainly 5 steps: (a) reads preparation (assembly of paired reads, filtering of low quality reads and identification of unique sequences), (b) Operational Taxonomic Units - OTU clustering (selection of unique OTU sequences), (c) quality controls (identification and removal of artifacts from amplicon reads, in OUT sequences). These tasks will be carried out using pipelines included in the standards operating protocols (SOPs) developed and used by the NIH Human Microbiome Project [31,32]. For the metagenomic microbiome data, the *HUMAnN2* [33] pipeline will be used and for the 16s microbiome data the *DADA2* [34] pipeline will be used.

The output of the pipelines are count data which are discrete, not continuous, and include only positive values [30,33,34]. The counts, C_{ij}, are the observed number of microbes for the i^{th} sample and j^{th} feature where the features are the OTUs or microbial taxa. The data are normalized because library sizes (total counts for each sample, across all features) often have a large magnitude difference, which can cause a bias [35]. The data are right skewed with, usually, a large number of data points having the value 0. Such data follows a Poisson distribution and therefore a Poisson regression model type (e.g. negative binomial model) will be used to investigate the relationship between microbiome and salt intake. For each feature, a model will be fitted with the feature as outcome and salt intake (a binary variable with two categories: treatment and placebo) as covariate. The differential count (fold change difference) between treatment and placebo will be estimated for each feature (OTU). To adjust for the multiple testing of several OTUs, a false discovery rate penalty will be applied and only fold changes with an adjusted p-value (Benjamini-Hochberg multiple test correction) smaller or equal to 0.05 will be considered statistically significant.

The analysis describes above using a Poisson regression model assumes the features (OTUs) are independent but it has been shown that microbial communities do interact in such way that changes in one feature affect another feature [36-38]. However, the number of interactions can be large due to the number of features, and not easily fitted in a conventional regression model. Therefore, a machine learning algorithm (Random Forest or Neural Network) will be used to investigate the combined response of the features to salt intake. These algorithms can incorporate multiple and complex interactions between features without making an assumption about their distribution. The features will be used as predictor variables to predict salt intake (a binary variable with two categories: treatment and placebo). The results will be presented as the area under the curve (AUC) of a receiver operating curve (ROC) that reflect how well the features can predict treatment and placebo. Because these ensemble techniques do not assume the predictors

variable (OTUs) are independent, no multiple testing penalty is required but rather cross-validation will be undertaken.

9.4.3 Analysis of the Secondary Endpoint(s)

The secondary endpoints will be assessed independent of the results associated with the primary endpoints; secondary endpoints include blood pressure, whole blood epigenome, and whole blood and urine transcriptome. The effect of treatment and placebo will be investigated will be investigated for each secondary endpoint, as detailed below.

The association between blood pressure (systolic and diastolic) and salt intake will be assessed using multiple linear regression with salt intake (two categories: treatment and placebo) as the exposure variable and blood pressure outcome variable. The effect size, p-value and 95% confidence interval of the regression model will be provided as results.

The effect of salt intake on gene expression will be investigated in a differential expression analysis to identify genes whose expression differs significantly between treatment and placebo. The gene expression data will be normalized, using the weighted trimmed mean of M-values (TMM) method, an optimal method for the normalization of mRNA count data [39]. Genes with an expression less than 1 Count Per Million (CPM = count/sum [counts] x 1million) in at least 2 samples will be excluded because results from genes with extremely low expression are not reliable. Gene expression is generated as count data which follows a Poisson distribution; therefore, a negative binomial model will be fitted using the R library *edgeR* [40] developed to handle the specificities of mRNA sequencing data. The model computes likelihood ratio tests for the coefficients whilst taking into account dispersion. A deviance of goodness of fit test will be carried out to identify genes where the model fit was poor indicating that the dispersion estimate is away from the average dispersion. Dispersion outliers will not be considered for the results because the deviance indicates low quality or marked expression difference for the gene. Finally, genes with a log fold change not equal to 0 and a false discovery rate adjusted p-value $\leq$ 0.05 will be reported as significantly differentially expressed between treatment and placebo. Multiple testing correction, for the total number of genes considered, will be carried out using the Benjamini-Hochberg method.

The epigenetic effect of salt intake will be investigated trough a two-sample t–test to identify methylation sites with significant methylation level difference between treatment and placebo. At a minimum of 5X coverage and an average coverage of 30X, the SureSelect-MethSeq (SS-Methseq) platform captures between 2.6 and 2.3 million methylation sites [41]. After thorough processing and QC of the methylation data, using the Bismark pipeline [42], samples and methylation sites that passed all QC filters will be considered for the analysis. Each methylation site will be treated as a continuous variable and salt intake will be treated as a nominal variable with two categories (treatment and placebo), in the t-test. In such analysis, the methylation sites are assumed to be independent and there is, hence, a high burden of multiple testing due to the millions of sites being tested. Therefore, a significance threshold of p-value $< 9 \times 10^{-8}$ will be applied to control for false positive findings; this threshold was derived from empirical work [43] based on the Illimunia EPIC array which has a lower density than the SS-Methseq platform but includes carefully selected sites that span the whole genome. The estimated mean difference, p-value and 95% confidence interval of the t-test will be presented as the results.

After the transcriptome, epigenome and microbiome data have been considered separately in the analyses described above and to understand the links between gene expression and methylation, an ensemble technique such as Random Forests will be used to leverage the high dimensionality of the data (number of features >> number of observations) [44,45] and identify the best combination of predictors of salt intake in the transcriptome and epigenome data. Subsequently, the OTUs identified in the microbiome data will be integrated to find out if microbiome information improves the prediction accuracy of the omics data [46]. This will provide further insights into the interplay between the three domains (microbiome, transcriptome and epigenome).

9.4.4 Safety Analyses
Not applicable.

9.4.5 Baseline Descriptive Statistics
Not applicable.
Baseline analysis stratified by treatment and placebo arms will be assessed based on clinical and demographic characteristics. Chi-square will be used to assess categorical variables and t-test will be used to assess continuous variables.

9.4.6 Planned Interim Analyses
Not applicable

9.4.7 Sub-Group Analyses
Primary and secondary endpoints will be compared based on age and sex differences stratified by treatment and placebo groups.*)*.

9.4.8 Tabulation of individual Participant Data
Individual participant data will not be presented by time point.

9.4.9 Exploratory Analyses
Exploratory analyses will not be conducted.

10 REGULATORY AND OPERATIONAL CONSIDERATIONS

10.1 INFORMED CONSENT PROCESS

10.1.1 Consent/Assent Procedures and Documentation
Consent will be obtained at the NIH Clinical Center during baseline visit. The GENE-FORECAST-SIT staff is experienced in administering consent and will escort potential participant to a private area of the Clinical Center to discuss the consent form.

The GENE-FORECAST-SIT staff will provide the individual a copy of the consent forms, read all sections of the consent form and solicit for any questions. The consent process will take between 20-30 minutes. The GENE-FORECAST-SIT staff is experienced in obtaining informed consent for NIH trials and will not coerce or influence potential participant to enroll in the trial. Potential participant will be given the opportunity to consult with others prior to providing consent. Consent for minors when they reach the age of majority
Not applicable. Our study population will be 21-65 years of age.

10.1.2 Considerations for Consent of NIH staff, or family members of study team members

Consent from staff members will be obtained by an individual independent of the staff member's team whenever possible. Otherwise, the consent procedure will be independently monitored by the CC Department of Bioethics Consultation Service in order to minimize the risk of undue pressure on the staff member.

10.1.3 Participation of Subjects who are/become Decisionally Impaired

Adults unable to provide consent are excluded from enrolling in the protocol. However, it is possible that subjects enrolled in the protocol may permanently lose the capacity to consent for themselves during the course of this study. In the event this occurs, the subjects will be withdrawn from the study. .

10.2 Study Discontinuation and Closure

Study participant can discontinuation participation in protocol if any potential side effects occur due to consumption of study product (e.g., nausea, dizziness, rash). The possibility of a temporary suspension of the study is highly unlikely.

10.3 Confidentiality and Privacy

Participant confidentiality and privacy is strictly held in trust by the PI, participating investigators, and study staff. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of study participant.

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

Representatives of the NIH IRB and/or regulatory agencies may inspect all documents and records required to be maintained by the PI, including but not limited to, medical records (office, clinic) and pharmacy records for the participants in this study. The NIH Clinical Center will permit access to such records.

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.

Study participant research data, which is for purposes of statistical analysis and scientific reporting, will be transmitted to and stored on dedicated and secured NIH servers and the NIH electronic Clinical Trial Database (CTDB). This will not include the participant's contact or, identified by a unique study identification number.

The study data entry and study management systems used by the research staff will be secured and password protected. At the end of the study, all study databases will be identified and archived on PI's dedicated NIH server.

To further protect the privacy of study participants, a Certificate of Confidentiality has been issued by NIH. This certificate protects identifiable research information from forced disclosure. It allows the investigator and others who have access to research records to refuse to disclose

identifying information on research participation in any civil, criminal, administrative, legislative, or other proceedings, whether at the federal, state, or local level. By protecting researchers and institutions from being compelled to disclose information that would identify research participants. Certificates of Confidentiality help achieve the research objectives and promote participation in studies by helping assure confidentiality and privacy to participants.

10.4 Future use of Stored Specimens and Data

No unintended specimens or residual specimens will be retained after the study is completed.

10.5 Safety Oversight

We will have an Independent Safety Monitor (ISM) responsible for safety oversight. The ISM will be composed of individuals with appropriate expertise (e.g., physician, nurse, epidemiologist), will be independent from the study and free of conflict of interest. The ISM will meet at least semiannually to assess safety and efficacy data on each arm of the study. The ISM will provide input to the study PI and study staff.

10.6 Clinical Monitoring

Clinical site monitoring will be conducted to ensure 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 International Conference on Harmonisation Good Clinical Practice (ICH GCP), and with applicable regulatory requirements.

10.7 Quality Assurance and Quality Control

The database manager will perform internal quality management of study conduct, data and biological specimen collection, documentation and completion. An individualized quality management plan will be developed to describe quality management.

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 to the PI and other relevant study staff for clarification/resolution.

Following written (Manual of Operating Procedures (MOP), the database manager will verify that the study is conducted and data are generated and biological specimens are collected, documented (recorded), and reported in compliance with the protocol, ICH GCP, and applicable regulatory requirements (e.g., Good Laboratory Practices (GLP), Good Manufacturing Practices (GMP).

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

10.8 Data Handling and Record Keeping

10.8.1 Data Collection and Management Responsibilities

Data collection will be the responsibility of the clinical staff (i.e., Research Nurse and Physician Assistant) under the supervision of the PI. The PI is ultimately responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported.

All source documents will be completed in a neat, legible manner and recorded in electronic CTDB to ensure accurate interpretation of data.

Hardcopies of the study visit worksheets will also be used 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.

All study related data (including AEs, concomitant medications, and expected adverse reaction data) and clinical laboratory data will be entered into the CTDB on the PIs server. The CTDB will be password protected and internal quality checks conducted by the database manager, such as automatic range checks, to identify data that appear inconsistent, incomplete, or inaccurate. Data will be entered directly from the source documents.

10.8.2 Study Records Retention

Study documents will be retained for a minimum of 2 years or until at least 2 years have elapsed since the formal discontinuation of the study or as per the NIH Intramural Records Retention Schedule.

10.9 PROTOCOL DEVIATIONS

It is the responsibility of the PI to use continuous vigilance to identify and report deviations to the NIH Institutional Review Board as per Policy 801. The PI responsible for knowing and adhering to the reviewing IRB requirements.

10.9.1 NIH Definition of Protocol Deviation

A protocol deviation will be any change, divergence, or departure from the IRB approved research protocol and defined as:

- Major deviations: Deviations from the IRB approved protocol that have, or may have the potential to, negatively impact the rights, welfare or safety of the subject, or to substantially negatively impact the scientific integrity or validity of the study.
- Minor deviations: Deviations that do not have the potential to negatively impact the rights, safety or welfare of subjects or others, or the scientific integrity or validity of the study.

10.10 PUBLICATION AND DATA SHARING POLICY

10.10.1Human Data Sharing Plan

This study will be conducted in accordance with the following publication and data sharing policies and regulations:
NIH Public Access Policy, which ensures that the public has access to the published results of NIH funded research. This requires scientists to 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 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 the ClinicalTrials.gov, and results from this trial will be submitted to ClinicalTrials.gov. In addition, every attempt will be made to publish results in peer reviewed journals.

10.10.2Genomic Data Sharing Plan

Not applicable.

.

10.11 COLLABORATIVE AGREEMENTS

Not applicable.

10.11.1 Agreement Type

Not applicable.

10.12 CONFLICT OF INTEREST POLICY

The independence of this study from any actual or perceived influence, such as by a pharmaceutical industry, is critical. Therefore, 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. 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 on the design and conduct of the study. The study leadership in conjunction with the NHLBI 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.

11 ABBREVIATIONS

AE	Adverse Event
AI	Associate Investigator
CFR	Code of Federal Regulations
CLIA	Clinical Laboratory Improvement Amendments
CMP	Clinical Monitoring Plan
COC	Certificate of Confidentiality
CRF	Case Report Form
CONSORT	Consolidated Standards of Reporting Trials
CTDB	Clinical Trial DataBase
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
FACS	Flow Activated Cell Sorting
GCP	Good Clinical Practice
GENE-FORECAST:SIT	Genomics, Environmental Factors and Social Determinants of Cardiovascular Disease In African Americans: Sodium Intervention Trial
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
GWAS	Genome-Wide Association Studies
HIPAA	Health Insurance Portability and Accountability Act
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
LSMEANS	Least-squares Means
MOP	Manual of Procedures
NCT	National Clinical Trial
NIH	National Institutes of Health
NIH IC	NIH Institute or Center
NIH IDOU	NIH Investigational Drug & Research Section Outsourcing Unit
NHGRI	National Human Genome Research Institute
NHLBI	National Heart, Lung, and Blood Institute
OHRP	Office for Human Research Protections
PI	Principal Investigator
QA	Quality Assurance
QC	Quality Control
RVS	Research Volunteer System
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SMC	Safety Monitoring Committee
SOA	Schedule of Activities
SOP	Standard Operating Procedure
TMAO	Trimetylamine-oxidase
UP	Unanticipated Problem

12 ADDENDUM FOR TRANSFER OF GENEFORECAST DATA

GENE-FORECAST (GENomics, Environmental FactORs and the Social DEterminants of Cardiovascular Disease in African Americans Study) was established to explore the intricate relationship between the unique genomic variations characteristic of African-Americans (AA) and the broader array of social determinants and environmental factors, collectively known as the 'exposome', that impact the development of cardiovascular disease (CVD) in this population.
The Sodium Intervention Trial (SIT) is a clinical trial protocol (000070) aimed at investigating the impact of dietary salt on the cardiovascular system, leveraging the existing pool of participants of the GENE-FORECAST protocol (18-HG-0048). Initially embedded within GENE-FORECAST, SIT sought to enroll participants who had previously consented to be re-contacted. However, as guidelines prohibited the inclusion of a sub-study within an ongoing natural history protocol, SIT was separated from GENE-FORECAST after recruiting an initial cohort of 20 participants.
Following the division of the protocols, an additional 8 participants were enrolled in SIT, resulting in a total of 28 participants. To conduct comprehensive analyses of the SIT clinical trial data, it is imperative to have access to the baseline characteristics of all 28 participants recruited from the

GENE-FORECAST protocol. Therefore, we kindly request the transfer of data pertaining to these 28 participants from the GENE-FORECAST protocol to the SIT protocol.

13 REFERENCES

1. Estruch, R. et al. Primary prevention of cardiovascular disease with a Mediterranean diet. N Engl J Med 368, 1279-90 (2013).
2. Sacks, F.M. et al. Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet. DASH-Sodium Collaborative Research Group. N Engl J Med 344, 3-10 (2001).
3. Farquhar, W.B., Edwards, D.G., Jurkovitz, C.T. & Weintraub, W.S. Dietary sodium and health: more than just blood pressure. J Am Coll Cardiol 65, 1042-50 (2015).
4. Schmidlin, O., Sebastian, A.F. & Morris, R.C., Jr. What initiates the pressor effect of salt in salt-sensitive humans? Observations in normotensive blacks. Hypertension 49, 1032-9 (2007).
5. Rayner, B.L. & Spence, J.D. Hypertension in blacks: insights from Africa. J Hypertens 35, 234-239 (2017).
6. Mell, B. et al. Evidence for a link between gut microbiota and hypertension in the Dahl rat. Physiol Genomics 47, 187-97 (2015).
7. Binger, K.J. et al. High salt reduces the activation of IL-4- and IL-13-stimulated macrophages. J Clin Invest 125, 4223-38 (2015).
8. Brian, MS, et al. Dietary sodium and nocturnal blood pressure dipping in normotensive men and women. J Hum Hypertens 31, 145-150 (2017).
9. Cava, A et al. The role of cyclo-oxygenase-1 in high salt diet-induced microvascular dysfunction in human. J Physiol 593, 5313-24 (2015).
10. Cho, H.Y., Park, H.C. and Ha, S. K. Salt sensitivity and hypertension: a paradigm shift from kidney malfunction to vascular endothelial dysfunction. Electrolyte Blood Press 13, 7-13 (2015).
11. Liu, Y. et al. CD8+ T cells stimulate Na-Cl co-transporter NCC in distal convoluted tubules leading to salt-sensitive hypertension. Nat Commun 8, 14037 (2017).
12. Tang, W.H. & Hazen, S.L. The Gut Microbiome and Its Role in Cardiovascular Diseases. Circulation 135, 1008-1010 (2017).
13. Tang, W.H. et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med 368, 1575-84 (2013).
14. Cowart, B. and G. Beauchamp, Early development of taste perception. Psychological basis of sensory evaluation, 1990: p. 1-17.
15. Mennella, J.A., et al., Evaluation of the Monell forced-choice, paired-comparison tracking procedure for determining sweet taste preferences across the lifespan. Chem Senses, 2011. 36(4): p. 345-55.
16. Pepino, M.Y. and J.A. Mennella, Effects of cigarette smoking and family history of alcoholism on sweet taste perception and food cravings in women. Alcohol Clin Exp Res, 2007. 31(11): p. 1891-9.
17. Bartoshuk, L.M., The psychophysics of taste. Am J Clin Nutr, 1978. 31(6): p. 1068-77.
18. Jogan, M. and A.A. Stocker, A new two-alternative forced choice method for the unbiased characterization of perceptual bias and discriminability. J Vis, 2014. 14(3): p. 20.

19. Bobowski, N.K. and J.A. Mennella, Disruption in the Relationship between Blood Pressure and Salty Taste Thresholds among Overweight and Obese Children. J Acad Nutr Diet, 2015. 115(8): p. 1272-82.
20. Joseph, P.V., D.R. Reed, and J.A. Mennella, Individual Differences Among Children in Sucrose Detection Thresholds: Relationship With Age, Gender, and Bitter Taste Genotype. Nurs Res, 2016. 65(1): p. 3-12.
21. Pepino, M.Y., et al., Obese women have lower monosodium glutamate taste sensitivity and prefer higher concentrations than do normal-weight women. Obesity (Silver Spring), 2010. 18(5): p. 959-65.
22. Mennella, J.A., et al., Preferences for salty and sweet tastes are elevated and related to each other during childhood. PLoS One, 2014. 9(3): p. e92201.
23. Mennella, J.A. and N.K. Bobowski, Psychophysical Tracking Method to Measure Taste Preferences in Children and Adults. J Vis Exp, 2016(113).
24. Baecke JAH, Burema J, Frigters JER, A short questionnaire for the measurement of habitual physical activity in epidemiological studies. Am J Clin Nutr. 1982; 36:936-942.
25. Buysse DJ, Reynolds CF, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh sleep quality index: A new instrument for psychiatric practice and research. Psychiatry Research. 1989; 28(2):193-213.
26. Krishnan K, Chen T, Paster BJ. A practical guide to the oral microbiome and its relation to health and disease. Oral Dis. 2017 Apr;23(3):276-286
27. Sánchez-García S, Heredia-Ponce E, Juárez-Cedillo T, Gallegos-Carrillo K, Espinel-Bermúdez C, de la Fuente-Hernández J, García-Peña C. Psychometric properties of the General Oral Health Assessment Index (GOHAI) and dental status of an elderly Mexican population. J Public Health Dent. 2010 Fall;70(4):300-7.
28. Grice, E., Segre, J. The skin microbiome. Nat Rev Microbiol 9, 244–253 (2011).
29. Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC; NISC Comparative Sequencing Program, Bouffard GG, Blakesley RW, Murray PR, Green ED, Turner ML, Segre JA. Topographical and temporal diversity of the human skin microbiome. Science. 2009 May 29;324(5931):1190-2.
30. Bolyen E, Knight R, and Caporaso JG. et al. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37: 852–857.
31. A framework for human microbiome research. Human Microbiome Project Consortium, Nature, 486 (2012), pp. 215-221
32. Structure, function and diversity of the healthy human microbiome. Human Microbiome Project Consortium, Nature, 486 (2012), pp. 207–214
33. Franzosa EA*, McIver LJ*, Rahnavard G, Thompson LR, Schirmer M, Weingart G, Schwarzberg Lipson K, Knight R, Caporaso JG, Segata N, Huttenhower C. Species-level functional profiling of metagenomes and metatranscriptomes. Nat Methods 15: 962-968 (2018)
34. Callahan, B., McMurdie, P., Rosen, M. et al. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13, 581–583 (2016).
35. Weiss, S., Xu, Z.Z., Peddada, S. *et al.* Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome* **5,** 27 (2017).
36. Pacheco, A.R., Moel, M. & Segrè, D. Costless metabolic secretions as drivers of interspecies interactions in microbial ecosystems. *Nat Commun* **10,** 103 (2019).

37. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR, Muehlbauer MJ, Ilkayeva O, Semenkovich CF, Funai K, Hayashi DK, Lyle BJ, Martini MC, Ursell LK, Clemente JC, Van Treuren W, Walters WA, Knight R, Newgard CB, Heath AC, Gordon JI. Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science. 2013 Sep 6;341(6150):1241214.
38. Weiss S, Van Treuren W, Lozupone C, et al. Correlation detection strategies in microbial data sets vary widely in sensitivity and precision. *ISME J*. 2016;10(7):1669-1681.
39. Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. Genome biology. 2010;11(3):R25.
40. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26(1):139–40.
41. Walker DL, Bhagwate AV, Baheti S, Smalley RL, Hilker CA, Sun Z, Cunningham JM. DNA methylation profiling: comparison of genome-wide sequencing methods and the Infinium Human Methylation 450 Bead Chip. Epigenomics. 2015;7(8):1287-302.
42. Krueger F, Andrews SR. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics. 2011 Jun 1;27(11):1571-2. doi: 10.1093/bioinformatics/btr167. Epub 2011 Apr 14.
43. Mansell, G., Gorrie-Stone, T.J., Bao, Y. *et al.* Guidance for DNA methylation studies: statistical insights from the Illumina EPIC array. *BMC Genomics* **20,** 366 (2019).
44. Breiman, L. *Classification and regression trees*, x, 358 p. (Wadsworth International Group, Belmont, Calif., 1984).
45. Boulesteix, A.L., Janitza, S., Hapfelmeier, A., Van Steen, K. & Strobl, C. Letter to the Editor: On the term 'interaction' and related phrases in the literature on Random Forests. *Brief Bioinform* **16**, 338-45 (2015)
46. Shankar J. Insights into study design and statistical analyses in translational microbiome studies. *Ann Transl Med*. 2017;5(12):249. doi:10.21037/atm.2017.01.13
47. Pimenta E, Gaddam KK, Oparil S, Aban I, Husain S, Dell'Italia LJ, Calhoun DA. Effects of dietary sodium reduction on blood pressure in subjects with resistant hypertension: results from a randomized trial. Hypertension.
48. Tzemos N, Lim PO, Wong S, Struthers AD, MacDonald TM. Adverse cardiovascular effects of acute salt loading in young normotensive individuals. Hypertension. 2008 Jun;51(6):1525-30.