Statistical Analysis Plan

1 SAP Signatures

I give my approval for the attached SAP entitled Visualizing Vascular Mechanisms of Salt Sensitivity dated 11/01/2023.

Date: 11/03/2023

Principal Investigator

Name: Rachelle Crescenzi

Signature: Rachelle L Crescenzi

Date: 11/03/2023

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3 Abbreviations and Definitions

SSBP	Salt Sensitive Blood Pressure
AHA	American Heart Association
MR	Magnetic Resonance
HDLs	High-Density Lipoproteins
BMI	Body-Mass-Index
SAT	Subcutaneous Adipose Tissue
IRB	Institutional Review Board
VUMC	Vanderbilt University Medical Center
VUIIS	Vanderbilt University Institute of Imaging Science
LSD	Low Salt Diet
HSD	High Salt Diet
SS	Salt Sensitive
SR	Salt Resistance
DANTE	Delay Alternating Nutation for Tailored Excitation
ASL	Arterial Spin Labeling
pCASL	pseudo-continuous ASL sequence
KSP	Key study personnel

4 Introduction

4.1 Preface

Salt sensitive blood pressure preferentially affects black Americans

Salt sensitive blood pressure (SSBP) is a physiologic phenotype defined by a parallel change in blood pressure in response to dietary salt load. SSBP is fundamentally related to the body's inability to clear salt, leading to elevated blood pressure and a life-long increased risk of cardiovascular disease. SSBP was determined to be an independent risk factor for developing cardiovascular disease in hypertensive patients almost two decades ago[1], and more recently SSBP was found to portend all-cause mortality[2]. It is a prevalent condition that affects up to 50% of normotensive, 75% of hypertensive[3], 74% of black American adults[4], and is estimated to be 6% more prevalent among females than males[5, 6]. Black Americans are preferentially affected by this phenotype and represent one of the most at-risk population for cardiovascular diseases related to SSBP. Salt sensitivity is increased with the occurrence of metabolic syndrome associated with abdominal obesity[7] and insulin resistance[8]. Current tests for SSBP require dietary salt loading, are time-intensive and, more importantly, provide limited mechanistic information on the incompletely characterized mechanisms of tissue salt storage.

4.2 Scope of the analyses

Our overarching hypothesis is that impaired lymphatic clearance is associated with elevated tissue sodium and adipose storage in persons with SSBP. The overall goal of this work is to apply noninvasive, clinical imaging tools to assess the biological systems involved in the pathophysiology of tissue salt storage, which will enable future investigations of mechanisms and development of treatment strategies for SSBP in a clinical population.

5 Study Objectives

5.1 Impacts

Impact of Aim 1: Over half of the American population is estimated to be salt sensitive and yet research of this pathophysiology is limited by time-consuming and specialized protocols to identify salt sensitivity. 1) A radiologic phenotype of SSBP, based on unique distributions of tissue salt and adipose content, has the potential to serve as a surrogate test for salt sensitivity. Methods applied here can be performed in routine clinical imaging centers, making its potential translation to clinical research possible. 2) Establishing a radiologic phenotype of salt sensitivity is a necessary prerequisite to studying mechanistic hypotheses *in*

vivo about how tissue salt is stored and its relationship to a known risk factor for cardiovascular disease, adiposity.

Impact of Aim 2: Identifying for the first time the extent of lymphatic dysfunction in SSBP could motivate the exploration of understudied lymphatic mechanisms of salt sensitivity, as current models of salt sensitivity are incomplete. Successful completion of this aim will provide the necessary lymphatic imaging technologies to evaluate emerging therapies that hold promise for improving lymphatic pumping and lymphangiogenesis[28], and potentially tissue sodium clearance to improve the management of patients with salt sensitivity and increased risk of cardiovascular disease.

Impact of Aim 3: Localized, noninvasive imaging metrics of renal circulation within the cortex, where sodium-filtering nephrons are located, relative to peripheral tissue sodium storage have potential to portend salt sensitive hypertension if their relationship to SSBP is established.

6 Study Methods

6.1 Data Acquisition and Analysis

Blood Pressure Measurements. During screening and active treatment, outpatient BP will be measured with an aneroid sphygmomanometer (Welch Allyn, Skaneateles Falls, NY), using the appearance and complete disappearance of the Korotkoff sounds (K1 and K5) as systolic blood pressure and diastolic blood pressure. The mean of three seated measurements will be used. During study days, BP will be measured using an automated oscillometric recording device (Dinamap, Critikon, Carlsbad, CA) while the subjects are supine. The mean of three measurements one min apart will be used. Change in blood pressure as a primary outcome measure of salt sensitivity will be calculated as the mean arterial systolic pressure during LSD minus the mean arterial systolic pressure during HSD (ΔBP, mmHg, in absolute units).

Standardized ^{23}Na (sodium) MRI. Mentor Dr. John Gore, who has experience with multi-nuclear imaging and development, will oversee application and analyses. Sodium MRI will be acquired from the dominant upper and lower extremities using the standardized clinical sodium MRI protocol[40] previously used to determine sodium stores in hypertensive patients[26]. Briefly, the subject's calf will rest on a platform embedded with four standard sodium solutions (aqueous NaCl in the physiologic range of tissue sodium content 10-40 mmol/L) inside a quadrature coil tuned for sodium signal reception, and the sodium MRI protocol performed. Next, the subject will be repositioned to place their forearm inside the coil, and sodium MRI performed. Each sodium image acquisition time is approximately 15 minutes. The MR signal intensity in tissues will be calibrated to signal intensity within the standard sodium solutions to produce a map of standardized tissue sodium content (**Figure 2B-C**). **Standardized tissue sodium content (mmol/L)** will be measured in the muscle (primary observable of interest) but will also be quantified in skin and subcutaneous adipose tissues for exploratory analyses.

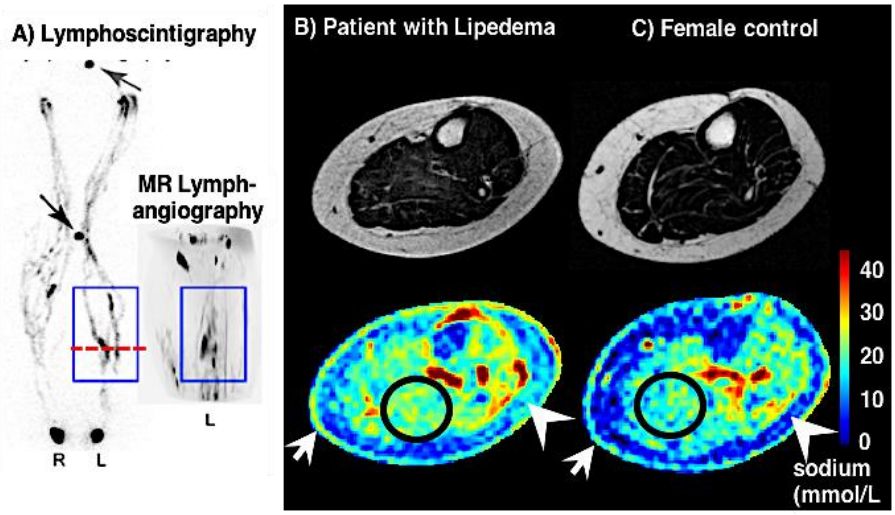

Figure 2. (A) Validation of MR lymphangiography with clinical gold-standard lymphoscintigraphy in a patient with edema of the left leg. Gray arrow=pubic marker, black arrow=knee marker. Lymph stasis is visible 40 min post-contrast injection on lymphoscintigraphy, which can also be visualized in 10 min at higher spatial resolution using MR lymphangiography (blue box). Note that the position was repositioned between scans with these different modalities, however the proximity of stasis (hypointensity) is identical. The red dashed line indicates the location of sodium and adipose imaging. **(B-C)** MRI of tissue sodium and adiposity in the calf of a patient with lymphatic dysfunction due to lipedema and a female control matched for age, race, and BMI. Structural measures of calf circumference and subcutaneous adipose tissue (SAT) area are similar. However, total tissue fat/water fraction is elevated (lipedema vs. control values, 0.95 vs. 0.87 ratio). Tissue sodium content is higher in the patient with lipedema in all regions, including skin (15.7 vs. 12.6 mmol/L, arrow), SAT (13.3 vs. 8.2 mmol/L, arrowhead), and muscle (19.8 vs. 14.3 mmol/L, circle). **These findings demonstrate the potential for tissue sodium and adiposity to indicate lymphatic impairment which may have relevance to SSBP.**

Body composition MRI. Mentor Dr. John Gore will oversee application of the AMRA® Body Composition Protocol available at our center. The AMRA® Body Composition Protocol employs the Dixon MRI method for separating fat and water signals from a single acquisition to provide fat-weighted and water-weighted images. We will apply this method optimized for whole-body imaging from the head to ankle. Images in the legs will be analyzed for this study's purpose, and whole-body image acquisitions preserved for potential future analyses of visceral fat, intramuscular fat, total abdominal fat index, lean muscle tissue, and liver fat. Here, we will use automated image-processing routines developed in our preliminary study[10] to measure **tissue fat/water fraction (ratio)** in the identical slice where sodium imaging was acquired. Tissue fat/water fraction will be calculated as the ratio of the volume of subcutaneous and intramuscular adipose compared to the volume of muscle and skin tissues.

Statistical analysis procedures for Aim 1:
Statistical analyses will be overseen by Dr. Donahue and members of the Center for Quantitative Sciences

(see mentor support letter). The primary statistical objective of Aim 1 is to test the hypothesis that adults who are salt sensitive have elevated baseline tissue sodium relative to adults who are not salt sensitive. To test this, we will apply a previously-published, informed cutoff to dichotomize participants into those that are salt sensitive (SS, ≥5 mmHg reduction in ΔBP depletion) or salt resistant (SR, <5 mmHg reduction in ΔBP), according to a review of methods and recommendations from Kurtz *et al.*[11]. The metric ΔBP represents a continuous variable of SSBP; tests for normality and homoscedasticity will be made. Depending on normality, a Mann Whitney U test or a Student's t-test will be applied to determine whether muscle sodium content is significantly different in SS and SR groups, matched for race and accounting for biological sex as a covariate. Based on a prior study of tissue sodium storage in adults with hypertension (n=10) and normotensive (n=12)[26] we anticipate a similar difference in tissue sodium of 6±4 (mmol/L) between SS and SR adults. With a sample size of 20 in each group, our study design will provide at least 80% power to detect differences in tissue sodium with a two-sided significance of $p<0.05$.

Our secondary statistical objective of Aim 1 is to test the hypothesis that muscle sodium content and potential risk factors for SSBP, including biological sex and tissue fat/water fraction as a sensitive surrogate of BMI, are related to ΔBP as a continuous variable. We will apply multivariate regression analysis using muscle sodium, fat/water fraction, and biological sex as independent variables, and ΔBP as the dependent variable among all participants. With a sample size of 40, we will have sufficient power to evaluate three predictors of ΔBP at the

Statistical analysis procedures for Aim 2:
The primary statistical objective of Aim 2 is to test the hypothesis that tissue sodium content is elevated in tissues affected by impaired lymphatic flow. To test this hypothesis, the same statistical considerations outlined in Aim 1 will be applied. Next, we will calculate either the Pearson's-*r* or Spearman's-rho correlation coefficient, depending on normality, between lymphatic flow velocity and tissue sodium content in adults who are SS or SR and matched for age, biological sex, and BMI. The correlation coefficient and adjusted p-values and 95% confidence intervals will be reported for each test, with the hypothesis that the relationship will be significant in SS but not SR individuals. Based on our preliminary data in normotensive black women who display a Spearman's-rho=0.49 between tissue sodium and fat/water fraction (**Table 1**), we anticipate a similar or greater correlation with lymphatic function; with 16 subjects in each group, our design will provide 80% power to detect a correlation with two-sided significance of $p<0.05$ between tissue sodium content and lymph flow velocity. To test whether the relationship between lymphatic flow velocity and muscle sodium is unique for the different groups, a linear regression with robust errors and an interaction term will be applied. The covariates will be biological sex and fat/water fraction. The coefficient for the interaction will be quantified as the difference between the slopes.

Statistical analysis procedures for Aim 3: The primary statistical objective of Aim 3 is to test the hypothesis that elevated tissue sodium content is associated with reduced renal perfusion in adults with SSBP. To test this hypothesis, we will calculate either the Pearson's-*r* or Spearman's-rho correlation coefficient, depending on normality, between renal cortical perfusion and peripheral tissue sodium content in adults who are SS or SR and matched for age, race, and BMI, and accounting for biological sex as a covariate. The correlation coefficient and adjusted p-values and 95% confidence intervals will be reported for each test. Based on our preliminary data in normotensive black women who display a Spearman's-rho=0.49 between tissue sodium and fat/water fraction (**Table 1**), we anticipate a similar or greater correlation with renal perfusion; with 20 subjects in each group, our design will provide 80% power to detect a correlation with two-sided significance of $p<0.05$ between tissue sodium content and renal cortical perfusion. Next, to compare the relationships in the SS group between i) tissue sodium and lymph flow velocity and ii) tissue sodium and renal cortical perfusion, a linear regression for each relationship with robust errors and an interaction term will be applied. The covariates will be biological sex and fat/water fraction. The coefficient for the interaction will be quantified as the difference between the slopes.

6.2 Inclusion-Exclusion Criteria and General Study Population

Inclusion Criteria:
- Men and women ages 18-55 years
- BMI 25 to < 140/90 mmHg at screening
- Identification as black race
- Willing to adhere to study diets

• Able to provide informed consent and communicate with study personnel

Exclusion criteria:
• Prevalent cardiovascular disease or use of medications for cardiovascular disease
• Current or prior history of hypertension or use of blood pressure lowering medications
• Current or prior history of diabetes mellitus or use of anti-diabetic medications
• Prevalent renal disease (eGFR < 60 ml/min/1.73m2), abnormal serum sodium or potassium
• Current or prior smoker
• Current pregnancy, or use of hormone replacement therapy or oral contraceptive
• Current steroid use
• Contraindications to MRI
• Active infection or open wounds on the top of the feet or hands
• Also excluded are prisoners and subjects who are non-English speaking

6.3 Randomization and Blinding

All subjects will undergo the same interventions. However, the order of the study interventions will be randomized using a randomized crossover design.

This is not a blinded study.

6.4 Study Assessments

Visit	Screening	Baseline	Diet 1	Washout	Diet 2	MRI
Target day of visit		1	7	14	21	28+
Phlebotomy	x	x	x	x	x	
24-hour urine collection		x	x	x	x	
MRI						x
History & Physical	x					
Study Diet			x		X	
Pregnancy test	X					
Biophysical exam		x	x	x	x	X
Adverse event monitoring		x	x	x	x	x

6.5 Time Windows

Visit	Time Windows allowed
Screening	N/A
Baseline	N/A
Diet 1	After exactly 7 days adherence to consecutive study diet.
Washout	Begins on the first day after Diet 1 is completed and is no fewer than 7 days.
Diet 2	After exactly 7 days adherence to consecutive study diet and no fewer than 7 consecutive days of not adherence to a study diet.
MRI	After no fewer than 7 consecutive days of not adherence to a study diet.

7 General Analysis Considerations

7.1 Timing of Analyses

The final analysis will be performed after all imaging data and labs has been processed.

8 Summary of Study Data

All continuous variables will be summarized using the following descriptive statistics: n (non-missing sample size), mean, standard deviation, median, maximum and minimum. The frequency and percentages (based on the non-missing sample size) of observed levels will be reported for all categorical measures. In general, all data will be listed, diet and subject, and when appropriate by visit number within subject. All summary tables will be structured with columns.

8.1 Demographic and Baseline Variables

Demographics will be assessed at baseline to confirm all participants adhere to inclusion/exclusion criteria prior to enrollment.

8.2 Treatment Compliance

Treatment compliance verification will include KSP check in with participants during diet intervention as well as quality checks of data and lab results.

9 Efficacy Analyses

All efficacy variables will be listed by subject within study center. Data will be summarized by treatment group. N, Mean, Standard Deviation, Minimum and Maximum will summarize continuous efficacy variables, whereas number and percent will summarize categorical efficacy variables.

All analyses of the continuous efficacy variables (e.g. salt sensitivity) will be performed as analysis of variance with treatment group adjusting for study center and surgical category. Treatment groups will be tested at the 2-sided 5% significance level.

All assumptions for regression models will be assessed by viewing plots of the residual values.

9.1 Primary Efficacy Analysis

Define the primary analysis that will provide the main result of the trial in this section. Note the use of “analysis” singular. This section of the document should be structured in parallel with 5.2 in terms of the ordering of endpoints considered. If appropriate use standard text: “The summary statistics will be produced in accordance with section 9.”

10 Safety Analyses

Per-protocol all randomized subjects who have been gone through diet intervention in the study without major protocol deviations that may significantly impact the interpretation of efficacy results. Detailed protocol deviation criteria will be determined at the latest before database lock. Data will be used for the primary efficacy outcome and safety analysis. Subjects enrolling in study need to satisfy all the following basic criteria: (1) Meet all the eligibility criteria specified in the study protocol; (2) The subjects were randomized and received the assigned interventions. (3) Adverse Events

When calculating the incidence of adverse events, or any sub-classification thereof by treatment, time period, severity, etc., each subject will only be counted once and any repetitions of adverse events will be ignored; the denominator will be the total population size.

Only incidence of AEs that are judged to be related to the treatment will be reported.

10.1 Clinical Laboratory Evaluations

All labs will be run through same center to avoid different values during processing. If duplicate laboratory test within study period are found, only one sample per study period per subject will be used for analysis.