Tissue Sodium in Pre-hypertensive Patients

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T. Alp Ikizler, MD, Professor
Division of Nephrology
Vanderbilt University Medical Center
[redacted]

Co-Investigators:

Aseel Alsouqi, MD
Meena Madhur, MD, PhD
David G. Harrison, MD

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Table of Contents:

1.0 Background

Hypertension and cardiovascular disease are age-related. The incidence of heart disease, arthritis, and diabetes increases steeply between the 6th-7 h decades (**Fig. 3**). Blood pressure and pulse-wave velocity, a surrogate parameter for vascular stiffness and an independent predictor for cardiovascular disease,[26] increase continuously with age.[27] *Modifiable risk factors* for cardiovascular disease prevention are hypertension, tobacco use, elevated blood glucose, physical inactivity, unhealthy diet (including excessive salt intake), cholesterol, and overweight/obesity. Age and gender are *non-modifiable risk factors*. Cardio-vascular disease prevention traditionally focuses on modifiable risk factors to prolong ***lifespan***. A more timely approach towards age-related disease management is to improve ***healthspan*** by identifying risk factors that accelerate the aging process.

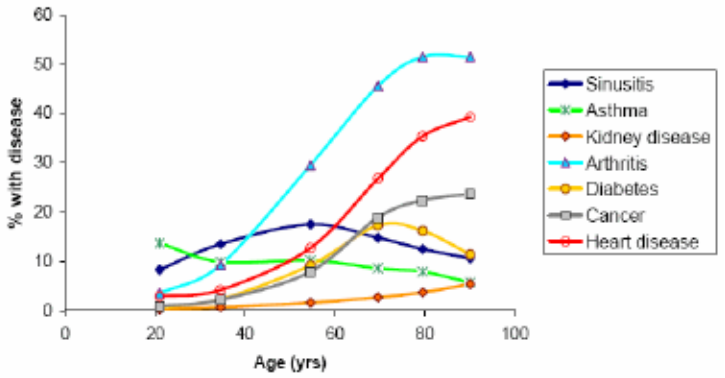


Figure 3. Prevalence of age-related diseases in the United States. *Source: CDC/NCHS, National Vital Statistics System.*

A pro-inflammatory state is associated with the major degenerative diseases of the elderly. Among the causal pathways linking the major age-related diseases associated with a pro-inflammatory state are changes in body composition (obesity) and energy imbalance. Obesity can be summarized as "homeostatic dysregulation" in the aging organism.[25] The current focus of research in age-related diseases is to better understand the origins and consequences of progressive low-level chronic inflammation and to design appropriate interventional approaches to improve healthspan. We believe that the accumulation of salt in the interstitium and inside cells represents a neglected risk factor, which initiates a pro-inflammatory state, chronically increases blood pressure, and leads to systemic energy imbalance. We will explore the concept that Na^+ storage in the skin and in muscle is associated with increased blood pressure, a pro-inflammatory state, and reduced insulin sensitivity. We hypothesize that by reducing age-dependent tissue Na^+ accumulation, healthspan in the general population will be improved and cardiovascular disease can be prevented. We aim to identify Na^+ storage as a novel unexpected risk factor for aging-associated cardiovascular disease. We hope to demonstrate that Na^+ storage can be reduced by dietary intervention or by drug treatment. Our results could eventually progress to end-point trials on long-term tissue Na^+-accumulation prevention to reduce cardiovascular disease risk.

Can Na^+ storage be prevented, and does lower tissue Na^+ reduce CVD risk? The idea that Na^+ is stored in tissue is surprising and seems counterintuitive, because current teaching suggests that body Na^+ content is constant. All bodily cells are bathed in extracellular fluid. The current generally accepted dogma is that extracellular bodily fluids readily equilibrate, resulting in constant electrolyte concentrations in the interstitial space. Na^+ is the major extracellular cation and exerts osmotic pressure. Changing Na^+ concentrations would change osmotic pressures and thereby lead to fluid shifts and swelling or shrinking of cells. Regulatory systems should, if at all possible, prevent any change in interstitial Na^+ content and concentration.[28] The current framework suggests that Na^+ accumulation in the body is almost entirely extracellular and leads to commensurate fluid retention. Accumulation or loss of Na^+ will be strictly prevented by a variety of regulatory steady-state mechanisms, resulting in constant body Na^+ content that is maintained within narrow limits.[29] Steady state thus would be achieved by the kidneys' excreting salt by a variety of renal clearance mechanisms.[30] As a result, the kidneys have been made primarily

responsible for blood pressure regulation. The clarification of Mendelian hypertensive disorders of Na^+ transport has provided additional support for this view.[31] However, genome-wide associations in populations have brought us no closer to physiological understanding of blood pressure regulation in essential hypertension.[32] Cardiovascular researchers remain 'under pressure' to solve this scientific puzzle.[33]

Our findings have added an additional dimension to total-body Na^+ regulation. We have proposed that Na^+ balance is not only a renal affair, but also involves additional regulatory clearance mechanisms locally in muscle and in the skin.[13, 14] We found that large amounts of Na^+ are stored in skin and muscle without commensurate water accumulation.[1-12, 15-18, 34] The resulting increase in tissue Na^+ concentration, which occurs in the absence of significant changes in serum Na^+ concentrations and therefore usually escapes our notice in clinical practice, leads to activation of the immune system. Immune cells entering this hypertonic microenvironment displayed various responses to the osmotic stress, ranging from acute pro-inflammatory action and auto-immune-response[11] to homeo-static immune function for local extrarenal regulation of tissue Na^+ content and blood pressure levels.[8, 9, 12] In an effort to "see" Na^+ distribution and compart-mentalization in man, we developed a ^{23}NaMRI method for non-invasive quantitative visualization of tissue Na^+ and transferred our basic research program into patient-oriented studies (**Fig. 4**).[15-17]

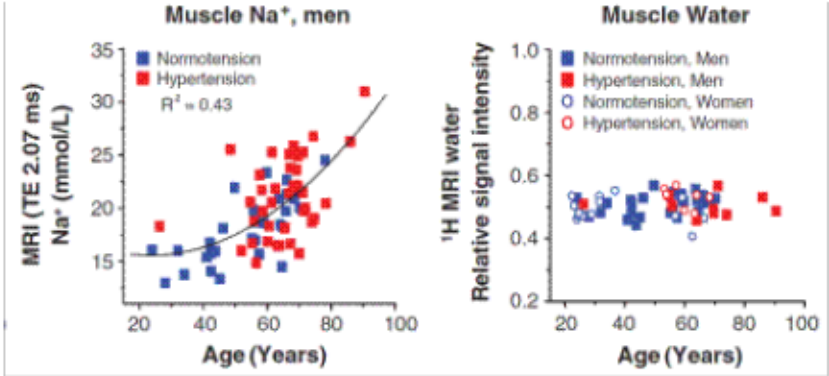


Fig. 4. Demonstration of uncoupling between Na^+ and water content in muscle in humans. While water content remains constant with age, we found that remarkable amounts of Na^+ are stored in muscle. Adapted from Kopp et al., *2013*. MRI: magnetic resonance imaging.

In a cross-sectional analysis of European Caucasian men and women, we found that Na^+ storage in muscle and skin increases with age, is more pronounced in men than in women, and is associated with higher blood pressure, while tissue water remains constant at all ages. This state-of-affairs leads us to the hypothesis that tissue Na^+ storage characterizes a disruption of internal environment composition, which may be causally linked to primary hypertension and insulin resistance. The increase in tissue Na^+ content we observed is similar to the age-dependent increase in blood pressure and pulse wave velocity reported by Vaitkevicius et al. 20 years ago (**Fig. 5 A&B**).[27] Because our preliminary data suggest a strong association between age-dependent tissue accumulation and blood pressure levels, we hypothesize that successful prevention of age-dependent increases in tissue Na^+ content will lower blood pressure, reduce inflammation, improve insulin sensitivity, and will reduce cardiovascular risk long-term. To address this hypothesis, we have initiated a prospective, independent study in European Caucasian men and women across age ranges with careful measurements of skin Na^+ content with ^{23}NaMRI (**Fig. 5C**). This second (unpublished) study confirms the age-related and sex-related effects. The relationship between ^{23}NaMRI measured tissue Na^+ stores and blood pressure suggests that increases in skin Na^+ content account for 50% of the blood pressure increase in men and somewhat less (where we have fewer data) in women. Our overarching hypothesis is that by flattening the time course of tissue Na^+ storage with age, we will improve healthspan and thereby prevent cardiovascular disease (**Fig. 5D**).

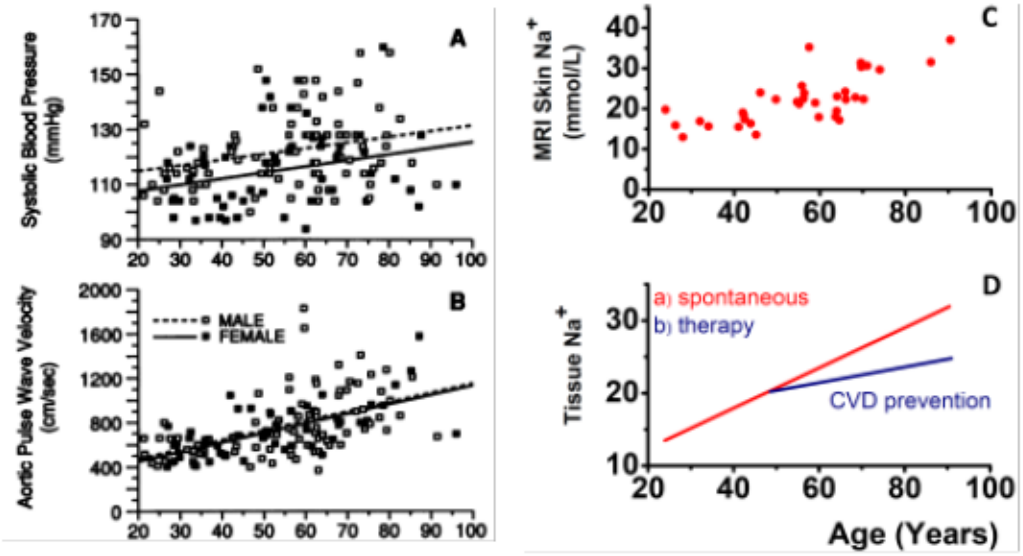


Fig. 5. Panels A – B: Relationship between age and blood pressure and pulse-wave velocity. (Adapted from Vaitkevicius et al.[27]). Our preliminary data show similar age-dependent increases in skin Na^+ storage in Caucasians (**Panel C**). These preliminary findings result in the hypothesis that attenuation of Na^+ storage with age may prevent CVD (**Panel D**).

Can lives be saved with dieta-ry salt reduc-tion? More than 40 years of population stu-dies and short-term (weeks) trials in patients are inconclusive and no convin-cing "hard end-point" studies have been done.[35] Further-more, govern-mental efforts to reduce salt con-tent in processed foods in the United States are not likely to occur.[36] Therefore, we assume that controlling salt intake in the general population is nearly impossible because consumers cannot easily assess salt content in their meals.[37] We will address this problem in our studies on tissue Na^+ content. *First*, one of our study arms will provide subjects with a prepared nutritious diet containing 6 g salt/day (2.4 g sodium/day) for 8 consecutive weeks to test the hypothesis that recommended levels of salt intake will reduce tissue Na^+ content. *Second*, we will test the hypothesis that blockade of the mineralocorticoid receptor (MR) will lower skin and muscle Na^+ content. Our preliminary cross-sectional analysis of tissue Na^+ content in Caucasians suggests that *spironolactone* may effectively reduce tissue Na^+ content (**Fig. 6**).[17] *Third*, we will test whether or not treatment with the diuretic *hydrochlorothiazide* lowers tissue Na^+ content. The drug intervention hypotheses will be tested in a prospective, double-blinded, randomized controlled trial.

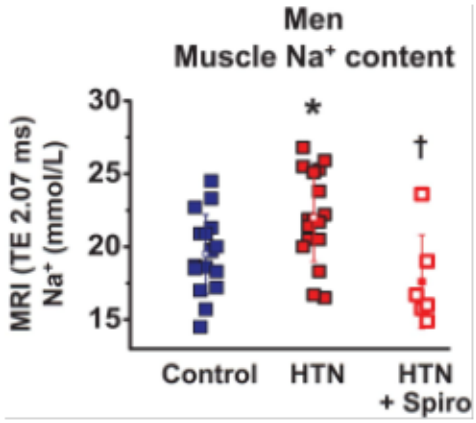


Fig. 6. Cross-sectional analysis of men with normal blood pressure (control), arterial hypertension (HTN), and men with HTN treated with the MR antagonist spironolactone (Spiro). Patients with the MR antagonist showed reduced Na^+ storage in muscle. * P(HTN) < 0.05; † P(Spiro)< 0.05. Adapted from Kopp et al., 2013.

Na^+ storage and immune cell response

We have shown in animal experiments that Na^+ storage in the skin and the resulting disequilibrium in interstitial Na^+ concentration attracts macrophages, which then regulate clearance of electrolyte via cutaneous lymph capillaries, thereby controlling blood pressure by secretion of vascular endothelial growth factor C (VEGF-C).[8, 9, 12] We also demonstrated that Na^+ storage-driven

immune responses can be pro-inflammatory and worsen auto-immune disease.[11] We now aim for rapid transfer of these novel mechanistic insights on immune function into the clinical arena. Our general hypothesis is that age is associated with reduced beneficial homeostatic immune function, resulting in impaired tissue electrolyte clearance and accumulation of Na^+ in the skin. Tissue Na^+ accumulation in turn may promote separate pro-inflammatory immune-cell responses. The resulting vicious circle may lead to chronic inflammation and age-dependent development of cardiovascular disease. Our preliminary findings support this hypothesis. We found an inverse correlation between serum VEGF-C levels and skin Na^+ content in our European subjects (**Fig. 7**), suggesting that age-dependent Na^+ accumulation is due in part to reduced lymph capillary-driven tissue Na^+ clearance. In our clinical prevention study proposed, we will test the hypothesis that increased Na^+ storage is associated with a pro-inflammatory response and an anti-lymphangiogenic serum profile, which can be improved by therapeutic reduction of tissue Na^+ content.

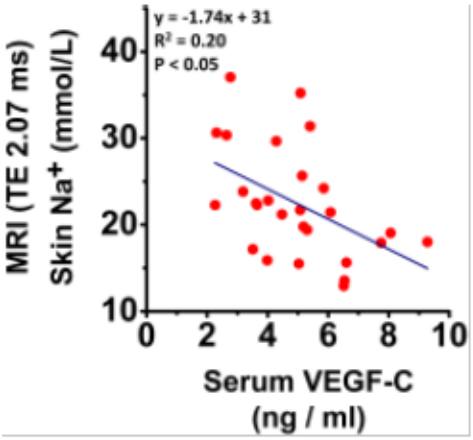


Fig. 7. Serum VEGF-C levels and skin Na^+ content in humans. Skin Na^+ accumulation is associated with low VEGF-C levels, suggesting impaired clearance of tissue Na^+ via cutaneous lymph capillaries. Unpublished.

Na^+ storage and insulin sensitivity

Figure 8A shows evidence that removal of an aldosterone-secreting tumor markedly reduces muscle Na^+ content in a patient with Conn's syndrome. In our animal experiments, we routinely monitored plasma glucose levels. We found that increased plasma glucose levels paralleled intracellular Na^+ storage in skeletal muscle in deoxycorticosterone acetate (DOCA)-treated rats (**Fig. 8B**). This finding is particularly interesting, because Conn initially reported that 52% of the patients with primary hyperaldosteronism *"exhibited diminished carbohydrate tolerance"* in glucose tolerance tests.[38] The increases in intracellular Na^+ content we have measured by element analysis in muscle in DOCA treated animals, as well as the increases in muscle Na^+ content in humans as consistently shown in all our pilot Na-MRI studies,[15, 16, 39] raise questions about cellular and systemic energy metabolism in the aging organism. Na^+-dependent and independent glucose transport uses downhill Na^+ or osmolyte gradients to transport glucose inside cells. We hypothesize that elevated intracellular Na^+ levels impair mitochondrial calcium accumulation, which results in net oxidation of the redox state of NAD(P)H/$NADP^+$, and leads to energy deficit with reduced glucose oxidation and increased reactive oxygen species (ROS) production, and reduced insulin sensitivity. To explore this

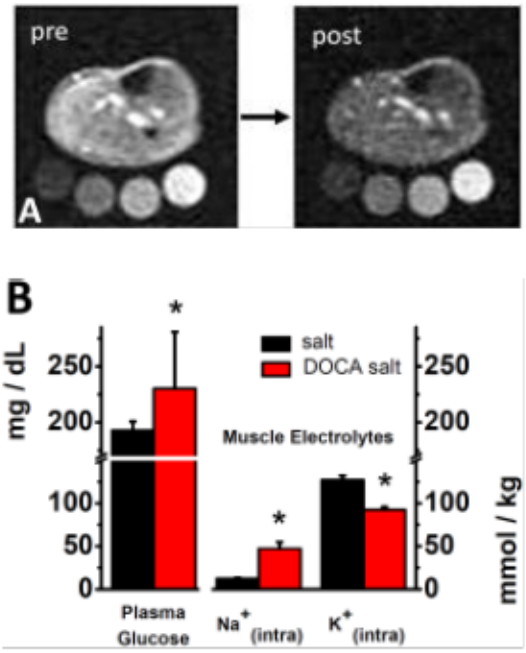


Fig. 8 *Panel A*: Reduction of muscle Na^+ content in a patient with primary hyperaldosteronism before (pre) and after (post) removal of an aldosterone-producing tumor. *Panel B*: Plasma glucose levels (unpublished) and intracellular muscle Na^+ and K^+ in rats with and without DOCA treatment. *Adapted from Ziomber et al.*[20] * $P_{(DOCA)} < 0.05$.

hypothesis clinically, we will investigate insulin sensitivity in patients with high and low ^{23}NaMRI Na^+ content in muscle.

2.0 Rational and Specific Aims

We studied the biology of Na^+ storage and elucidated findings that moved us beyond the equilibrium theory of Na^+ balance.[1-12] Our findings add an additional dimension to total-body Na^+ regulation.[13, 14] We propose that Na^+ balance is not solely a renal affair, but instead involves additional regulatory clearance mechanisms locally in muscle and skin. Our overarching hypothesis is that Na^+ is stored in the skin and in muscle without commensurate water retention. The resulting increase in tissue Na^+ concentration leads to activation of the immune system (**Fig. 1**). Immune cells entering this hypertonic microenvironment respond to the osmotic stress, and exhibit both acute pro-inflammatory reactions and regulatory responses,[11] which modulate tissue Na^+ content and blood pressure.[8, 9, 12] These findings have opened an entirely new perspective on immune functions that extend beyond ancient protection from invaders to physiological adaptation to environmental conditions. Besides skin Na^+ storage, we found that large amounts of Na^+ are stored inside skeletal muscle cells. Intracellular Na^+ storage occurs in animal models with activation of the mineralocorticoid receptor (MR).[4, 5, 7] Our preliminary data suggests that cellular Na^+ balance is most intimately coupled with metabolism. Muscle Na^+ storage seems to lead to energy deficit, insulin resistance, and propensity to diabetes mellitus.

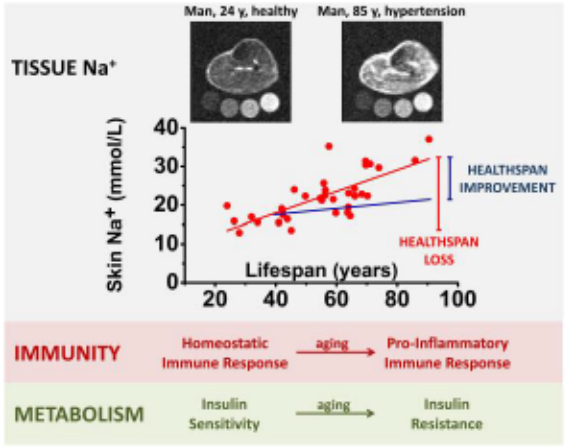


Fig. 1. ^{23}NaMRI relationship between tissue Na^+ accumulation and aging is shown. Inflammatory immune response and insulin sensitivity in the aging organism are hypothetical. Aging is paralleled by dramatic Na^+ accumulation in skin and in muscle. Interstitial Na^+ accumulation promotes a pro-inflammatory immune cell phenotype. Similarly, muscle Na^+ storage may predispose to insulin resistance. Bending the relationship between age and tissue Na^+ accumulation will reduce pro-inflammation and blood pressure, and improve insulin sensitivity. The resulting improved healthspan will prevent cardiovascular disease.

We have invested considerable effort to extend this new biological concept to clinical medicine.[15-18] We understood that Na^+ storage occurs in the absence of significant changes in serum Na^+ concentrations or body fluid content, and therefore usually escapes our notice in clinical practice. To "quantify and visualize" Na^+ distribution and compartmentalization disorders in man, we have developed a ^{23}NaMRI method for non-invasive detection of tissue Na^+ storage and transferred our basic research program into patient-oriented studies.[15-17] We confirmed many mechanistic insights from our animal research program in humans. Na^+ is stored in muscle and in skin in humans and MR activation leads to excess Na^+ storage in patients with primary hyperaldosteronism.[16] By visualizing Na^+ at the tissue level, we have established that age-dependent increases in skin Na^+ content are associated with increases in blood pressure.[17] This recent work has shown that skin Na^+ content actually explained more of the variability in blood pressure than did age, especially in men. Based on these exciting preliminary data, we hypothesize that tissue Na^+ accumulation may be a relevant and potentially reversible pathophysiological component for the development of cardiovascular disease in aging populations. Cumulative evidence suggests that hypertension is an inflammatory disease.[19-24] Pro-inflammatory immune cell activation in the interstitium of Na^+ overloaded tissues may be the

missing link towards a new and better understanding of the chronic pro-inflammatory state that leads to cardiovascular disease with aging (**Fig. 1**).[25] Similarly, Na^+ stored in muscle may predispose to energy deficit and insulin resistance. Tissue Na^+ storage thus may not only be pathophysiologically linked to arterial hypertension, but also with diabetes mellitus, another highly relevant cardiovascular risk factor. Successful therapeutic bending of the slope relationship between age and tissue Na^+ accumulation (**Fig. 1**) therefore promises to become a novel, important approach for cardiovascular disease prevention.

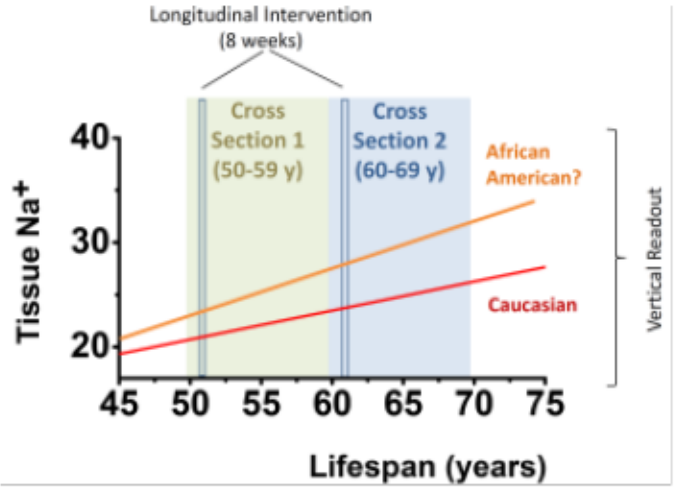


Fig. 2. Proposed cohort for the study of the relationship between tissue Na^+ storage and cardiovascular disease. Cross-sectional analysis will primarily address the effect of race on tissue Na^+ content. We hypothesize that African Americans have increased skin Na^+ storage, which is associated with increased arterial stiffness and higher blood pressure *(Aim 1)*. In a proof-of-concept approach, we then will prospectively test the hypothesis that diet or drug intervention for 8 weeks can lower tissue Na^+ content (*Aim 2*). Finally, we will test the hypothesis that subjects with increased tissue Na^+ content show pro-inflammatory immune-cell polarization (cooperation with basic science project), and reduced insulin sensitivity (*Aim 3*).

In this clinical project, we will take the first step towards therapeutic "bending" of the age/Na^+ accumulation relationship to a flatter slope (**Fig. 2**). We will establish a cohort of 200 subjects with pre-hypertension, age: 30 to 80 years. The cohort will be controlled for gender, race, and age so that cross-sectional analysis of the cohort characteristics will provide us with new information on the effect of race on tissue Na^+ content (Aim 1, **Fig. 2**). In a prospective, double-blinded randomized-controlled trial, we then will test the hypothesis that 8 weeks of a reduced salt diet or drug treatment with either the MR antagonist spironolactone, or with the diuretic hydrochlorothiazide, will reduce skin and muscle Na^+ content (Aim 2, short-term longitudinal readout, **Fig. 2**). Tissue Na^+ content will be our primary endpoint in Aims 1 and 2. As secondary endpoints, we will read out differences in blood pressure, skin forearm blood flow by BOLD MRI, and arterial stiffness by measurement of pulse wave velocity. Finally, we speculate that lower tissue Na^+ content reduces a pro-inflammatory immune cell phenotype and improves insulin sensitivity (Aims 3 and 4). We will address the following specific aims:

Specific Aim 1: To test the hypothesis that African Americans are characterized by increased tissue Na^+ storage, which is paralleled by higher blood pressure, reduced forearm blood flow, and enhanced pulse wave velocity

Specific Aim 2: To test the hypothesis that treatment with spironolactone reduces tissue Na^+ content (Primary Outcome)

Specific Aim 3: To test the hypothesis that Na^+ storage leads to immune cell activation (Secondary Outcome)

Specific Aim 4: To test the hypothesis that the accumulation of salt in skin and muscle is associated with decreased insulin sensitivity and propensity to diabetes mellitus (Secondary Outcome)

3.0 Animal Studies and Previous Human Studies

Our views on body electrolyte and water homeostasis were transformed by studying defined electrolyte input and output under the rigorously controlled conditions of the simulated "missions to the MIR station or to Mars".[34, 40] These were the Russian-sponsored simulated space flights of 135, 110, and 510 days where we monitored Na^+ intake and quantified output daily – for months (**Fig. 9**). Quantification of the corresponding changes in body Na^+ content showed that large amounts of Na^+ had been stored and released in the body – without parallel changes in body water content. The obvious next question, namely, *"Where is the salt?"* could not be addressed in clinical studies at that time. We therefore established a basic animal research program to reinvestigate Na^+ metabolism.[1-9, 11, 12] We abandoned accepted assumptions on the composition of interstitial and intracellular fluid spaces and relied on chemical quantitative analysis of tissue electrolyte and water content with more precise methods than employed previously. We found that body Na^+ content indeed was not at all maintained within narrow limits, but was in contrast highly variable. Addressing the question where all this variable Na^+ could be hidden, we found that with increasing body Na^+ content, Na^+ was stored in muscle and in skin, without commensurate water retention.[2-7] We concluded that Na^+ must be stored in extrarenal tissues, independent of direct renal regulation, suggesting additional extrarenal regulatory mechanisms for tissue Na^+ homeostasis.[14] We showed that Na^+ storage in the skin and the resulting disequilibrium in interstitial Na^+ concentration attracts macrophages, which then regulate clearance of electrolyte via cutaneous lymph capillaries and thereby control blood pressure.[8, 9, 12] Hence, we concluded that extracellular body fluids are significantly in disequilibrium and that these unexpected extracellular electrolyte gradients are controlled by novel extrarenal regulatory processes at the tissue level. This initial hypothesis on the existence of hidden Na^+ reservoirs resulted in the first report on immune-cell driven clearance of stored extracellular electrolytes via cutaneous lymph capillaries.[8] The finding has opened an entirely new perspective on immune function that extends innate immunity's ancient protection from invaders to physiological adaptation to environmental conditions and helpful blood pressure regulators. This idea also has provided new research avenues for autoimmune disease. For

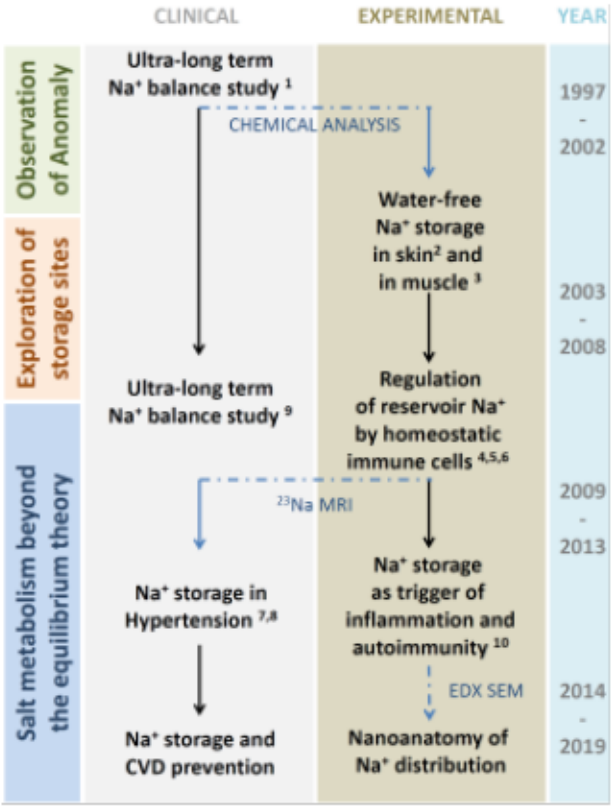


Figure 9. Previous and anticipated work of the applicant since his graduation from medical school, 1996. The 10 most relevant references to this work are 1. *Am J Kidney Dis.* 2002; 40:508-516; 2. *Am J Physiol Renal Physiol.* 2003;285:F1108-1117; 3. *Am J Physiol Renal Physiol.* 2008;295:F1752-1763; 4. *Nat Med.* 2009;15:545-552; 5. *Hypertension.* 2010;55:755-761; 6. *J Clin Invest.* 2013; 123: 2803-2815; 7. *Hypertension.* 2012;59:167-172; 8. *Hypertension.* 2013;61:635-640; 9. *Cell Metabolism.* 2013; 17:125-131; 10. *Nature.* 2013;496:518-522. Research-program shifting methodological improvement is depicted. with blue arrows.

instance, Na^+ storage-driven immune responses greatly worsened experimental encephalomyelitis.[11]

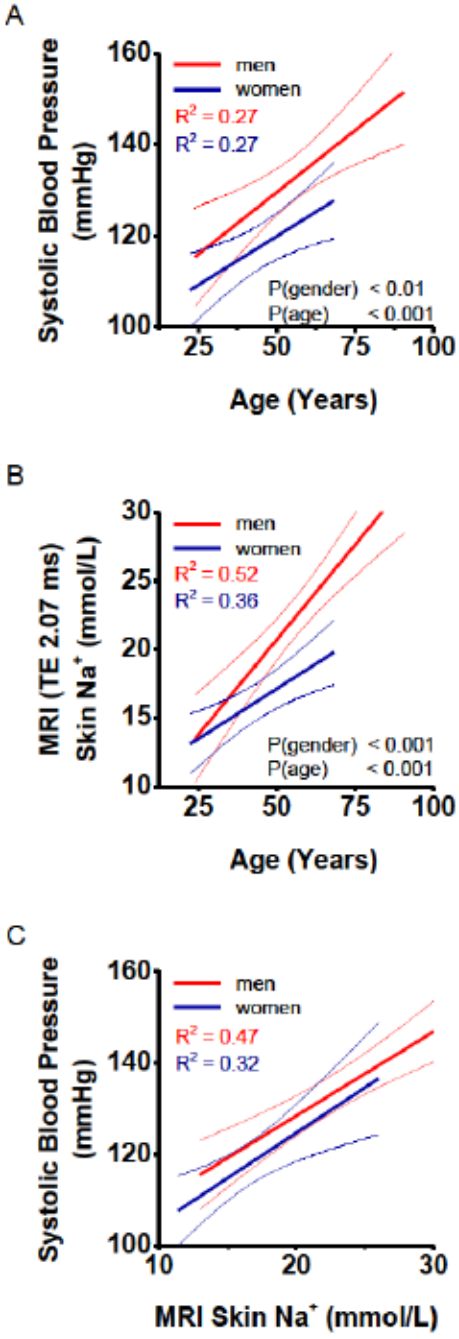


Figure 10. Panels A-B: Gender differences in the relationship between age, blood pressure, and skin Na^+ content. Panel C: Relationship between skin Na^+ content and blood pressure in both sexes. These preliminary data are from 34 men and 24 women (Causcasian) at age 22 to 90 years. *Linear regression line with 95% confidence levels. Unpublished.*

We next decided to again focus on a research program combining both basic scientific studies with clinical research (**Fig. 9**). We therefore have developed ^{23}Na MRI technology (http://patents.justia.com/inventor/jens-titze) to quantitatively and non-invasively visualize Na^+ reservoirs in humans.[15-17] We now can proceed with a complementary basic-science and patient-oriented clinical research approach. This arrangement predisposes us for bidirectional collaboration within the Research Center team. The information on tissue electrolyte composition in experimental animals that we can provide by chemical analysis and by energy-dispersive x-ray spectroscopy / scanning electron microscopy (EDX-SEM), as well as the genetically targeted animals from our NIH-sponsored basic research program (R01 HL118579-01), will be useful for Dr. Harrison's/Dr. Madhur's basic science project. Furthermore, our clinical research project within the Center interacts with Dr. Wang's research project. We will provide with ^{23}Na MRI readout on the reversibility of Na^+ storage in muscle and in skin in patients with hydrochlorothiazide treatment, which is the diuretic component of the *polypill* used in the population project.

Preliminary data from ^{23}Na MRI pilot studies. In European Caucasians, we have shown earlier that tissue Na^+ content increases with age. Controlled for age, tissue Na^+ is further elevated in patients with refractory hypertension.[16, 17] We now have prospectively screened skin Na^+, muscle Na^+, and systolic blood pressure in 34 men (age: 55.8±16.2 years) and in 24 women (age: 44.8±14.1 years) to investigate the effect of age and gender on tissue Na^+ content and blood pressure in humans. We have confirmed that blood pressure continuously increases with age, albeit at lower levels in women (**Fig. 10A**). Interestingly, we found a very similar increase in skin Na^+ content with age, which again was more pronounced in men than in women (**Fig. 10B**), while increases in skin Na^+ content were associated with parallel, as if dose-dependent, increases in blood pressure in both sexes (**Fig. 10C**). Skin Na^+ content explained more of the variability in blood pressure than age, especially in men. Based on these preliminary data, we hypothesize that tissue Na^+ accumulation may be a relevant and potentially reversible pathophysiological component for the development of hypertension in the aging population. This hypothesis leads to 3 subsequent questions. *First*, are populations with a higher incidence of hypertension (African Americans) characterized by higher tissue Na^+ content? *Second,* could tissue Na^+ content be therapeutically modified to attenuate the age-dependent increase in tissue Na^+ content? *Third*, will successful reduction of tissue Na^+ content lower blood

pressure and reduce pulse wave velocity (PWV)? We will address these questions in a prospective, double-blinded, randomized controlled trial on the efficacy of diet and different drugs to lower tissue Na^+ content (Aim 2).

Transfer of basic research findings into clinical study. In animals, we have demonstrated that macrophages are regulators of salt and water balance. These cells infiltrate Na^+-storage sites in skin and control tissue electrolyte removal. This local, extrarenal clearance of electrolyte clearance from skin is mediated by vascular endothelial growth factor C (VEGF-C), which promotes Na^+ and Cl^- removal from the skin via cutaneous lymph capillaries. Failure of this immune cell driven clearance process leads to augmented electrolyte accumulation in the skin and increased blood pressure.[8, 9, 12] We assume that VEGF-C, and its soluble receptor, sFLT4, can be used as a biomarker for enhanced or inhibited clearance of electrolyte from tissue in patients. More recently, we have shown that T cells polarize into an autoimmune and pro-inflammatory phenotype when exposed to osmotic stress in Na^+ storage sites.[11] While Dr. Harrison's/Dr. Madhur's basic research project will focus on mechanisms how Na^+ regulates adaptive and innate immune cells to cause hypertension, we will focus on transferring these findings into the clinical arena and test whether tissue Na^+ storage leads to similar macrophage / T-cell responses in humans (Aim 3). Our most recent animal experiments suggest that Na^+ storage in muscle elevates glucose levels. Although the underlying mechanisms are unclear, we aim for rapid transfer of this entirely new perspective for Na^+ homeostasis research into our patient-oriented research program by testing the hypothesis that humans with high muscle Na^+ content have reduced insulin sensitivity (Aim 4).

4.0 Inclusion/Exclusion Criteria

There will be no restriction on gender (except pregnant women), race, age (except less than 30 or greater than 80 years old) or disease etiology for subject selection or exclusion except as stated below.

Inclusion Criteria:

1. Age 30 to 80 years;
2. Systolic blood pressures between 110 to 150 mmHg and/or diastolic blood pressure between 80-99 mmHg;
3. Ability to give informed consent.

Exclusion Criteria:

1. Pregnancy;
2. Intolerance to study protocols;
3. Acute cardiovascular events within the previous 6 months;
4. Impaired renal function (estimated GFR < 45 ml/min/1.73m^2);
5. Current or recent treatment with systemic glucocorticoid therapy (within 1 month of enrollment);
6. Current use of anti-hypertensive medication (except calcium channel blockers and beta blockers);
7. Diabetes mellitus requiring medical therapy;
8. Morbid obesity (BMI > 45);
9. Prior adverse reaction to a thiazide or spironolactone;
10. Claustrophobia preventing the patient from having an MRI or other contraindications to MRI;
11. Impaired hepatic function (aspartate amino transaminase and/or alanine amino transaminase > 1.5x upper limit of normal range);
12. Current illicit drug use.

13. Sexually active women of childbearing potential** who are unwilling to practice adequate contraception during the study (adequate contraceptive measures include stable use of oral contraceptives or other prescription pharmaceutical contraceptives for 2 or more menstrual cycles prior to screening; intrauterine device [IUD]; bilateral tubal ligation; vasectomy; condom plus contraceptive sponge, foam, or jelly, or diaphragm plus contraceptive sponge, foam, or jelly).

    Postmenopausal women must be amenorrheic for at least 12 months in order **not to be considered of child bearing potential. Pregnancy testing and contraception are not required for women with documented hysterectomy or tubal ligation.

5.0 Enrollment/Randomization

The study will be conducted at the Vanderbilt University Medical Center. Institutional Review Board approval and written informed consent will be obtained from all study subjects.

We propose to study a total of 200 subjects controlled for race (50% African Americans, 50% Caucasians) and gender (50% female, 50% male). Subjects will be recruited from the Outpatient Clinics at VUMC (including the General Medicine Clinic and the Hypertension Clinic), as well as by emails, flyers, and other recruitment tools. All participants will have a baseline assessment, as well as on-going monitoring, of their health status as documented in their medical record.

Subjects will be randomly assigned to one of the study arms (diet, spironolactone, chlorthalidone, or placebo). Randomization will be determined using a stratified permuted-block randomization method. Stratification will be based on gender and race.

One treating physician will be unblinded as to whether subjects are receiving spironolactone or chlorthalidone and will guide decisions regarding subjects who develop abnormal serum potassium levels.

To specifically address Aims 1, 3 and 4, we will also enroll subjects who will only complete visits through Week 0. In addition, to aid data analysis, we will also re-enroll subjects who will complete a follow-up visit consisting of a blood draw.

6.0 Study Procedures

The design is a randomized, double-blind (except for those in the diet group), placebo-controlled study. Once the subject is determined to be eligible, we will randomly assign him/her to one of the study arms (diet, spironolactone, chlorthalidone, or placebo) as depicted in **Figure 1**. Each subject will be treated for 8 weeks. During the study, subjects will receive (1) a diet containing 6 g salt per day, (2) oral administration of spironolactone (50 mg per day), (3) oral administration of chlorthalidone (25 mg/day) or (4) oral administration of matching placebo. All medications and matching placebo will be prepared and dispensed by Vanderbilt's Investigational Drug Services (IDS). Except for the diet arm, the investigators and the study subjects will be blinded to the treatment. ^{23}NaMRI, Pulse wave velocity (PWV) and a hyperinsulinemic euglycemic clamp (HEGC) study will be performed at the beginning and at the end of the study. An optional DNA blood sample may also be collected.

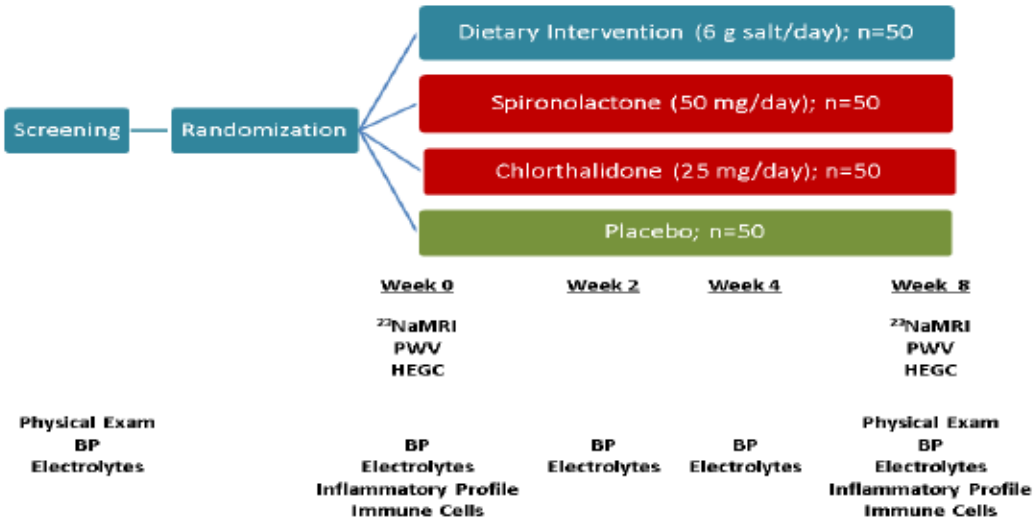


Figure 1: Study Protocol

Safety Precautions: In this protocol we have the following safety precautions: (1) Anti-hypertensive/diuretic medications (like the ones used in this study) may lower blood pressure excessively and/or alter serum electrolyte levels. Accordingly, we will monitor blood pressure and electrolyte levels throughout the study. Subjects who develop electrolyte imbalance will receive an electrolyte supplement or be asked to slightly change their diet. (2) Since the use of diuretics during pregnancy introduces unnecessary risk, female subjects will have a urine pregnancy test at each study visit before any research procedures are performed. (3) Since the presence of any metal introduces unnecessary risk, a screening X-ray exam will be performed to confirm the absence of any metal in subjects where that is a possibility.

Permanent stopping criteria: A patient will be terminated from the study for any of the following reasons:

- Persistent hyperkalemia (K > 5.5 mmol/L) occurs;
- Any allergies to the drugs develop;
- Women who become pregnant;
- There are any adverse events or clinical events that the PI judges increases the risk to the patient;
- There are any signs or symptoms of decline or deterioration of their health status.

Screening Visit: Within about two weeks up to the start of the Treatment Phase (which begins after the Baseline Visits) and after the subject has provided informed consent, the following information will be collected:

- Demographics
- Medical history
- Concomitant medications

Also, the following procedures and assessments will be performed:

- Vital signs (including blood pressure)
- Physical exam

- CMP (comprehensive metabolic panel) unless there is a documented CMP within 1 month of the stipulated study entry date as part of routine medical care
- Pregnancy test
- X-ray exam

Baseline Visits (Week 0): Subjects that qualify for the study will be randomized to one of the study arms, and will be asked to come in a fasted state on two separate days (preferably consecutive but no more than about 4 weeks apart) to the General Clinical Research Center (GCRC). On one of the visits, a hyperinsulinemic euglycemic clamp (HEGC) study will be performed. At the other visit, ^{23}NaMRI and pulse wave velocity (PWV) will be performed, as well as a hand grip test. These procedures are described below.

At the Baseline Visits, the following information will be collected:

- Concomitant medications (medication reconciliation)
- Medical history and concurrent illnesses

Also, the following procedures and assessments will be performed:

- Pregnancy test
- Vital signs (including blood pressure)
- CMP (including electrolytes)
- Blood samples for research labs, which include hsCRP, IL-6, TNFα, F2-isoprostanes, ADMA, s-ICAM, s-VCAM, insulin, leptin, adiponectin, resistin, C-peptide, lipid panel, FFA, and immune cell analysis
- Urine samples for research labs

Subjects in the diet arm will be counseled on the details of the low salt diet. Subjects in the other arms will be dispensed study drug. After the second Baseline visit, the intervention phase of the study begins.

Week 2 Visit: At the Week 2 Visit, subjects will be asked to come to the GCRC. The following information will be collected:

- Concomitant medications
- Adverse Events (AEs)

Also, the following procedures and assessments will be performed:

- Pregnancy test
- Vital signs (including blood pressure)
- CMP (including electrolytes)
- Urine samples for research labs
- Dietary counseling for those in the diet arm

Week 4 Visit: At the Week 4 Visit, subjects will be asked to come to the GCRC. The following information will be collected:

- Concomitant medications
- Adverse Events (AEs)

Also, the following procedures and assessments will be performed:

- Pregnancy test
- Vital signs (including blood pressure)
- CMP (including electrolytes)
- Urine samples for research labs
- Dietary counseling for those in the diet arm
- Study drug dispensing for those in the other arms

Week 8 Visits: At the Week 8 Visit, subjects will be asked to come in a fasted state on two separate days (preferably consecutive but no more than about 4 weeks apart) to the GCRC. On one of the visits, a hyperinsulinemic euglycemic clamp (HEGC) study will be performed. At the other visit, ^{23}NaMRI and pulse wave velocity (PWV) will be performed, as well as a hand grip test. These procedures are described below.

- Concomitant medications
- Adverse Events (AEs)

Also, the following procedures and assessments will be performed:

- Pregnancy test
- Vital signs (including blood pressure)
- Physical exam
- CMP (including electrolytes)
- Blood samples for research labs, which include hsCRP, IL-6, TNFα, F2-isoprostanes, ADMA, s-ICAM, s-VCAM, insulin, leptin, adiponectin, resistin, C-peptide, lipid panel, FFA, and immune cell analysis
- Urine samples for research labs

Diet Recalls: For subjects in the diet arm, study dietitians will conduct dietary recalls via telephone interviews after the Screening Visit, as well as at Week 4 and at Week 8 to confirm compliance with the low-salt diet. We will utilize a two-day diet recall that includes one week day and one weekend day to capture variability in eating. Dietary recall data will be collected and analyzed using Nutrition Data System for Research. For subjects in the other arms, dietitians will conduct the dietary recalls 2 times—after the Screening Visit and at Week 8.

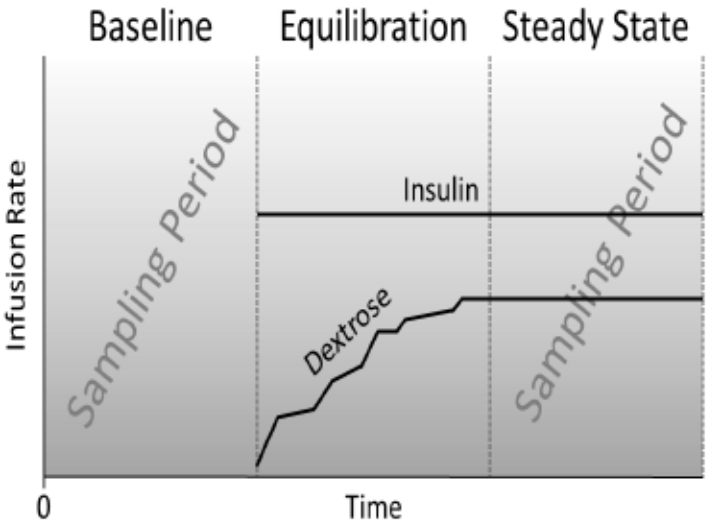
Standard Methods (all performed for research purposes only):

MRI Measurements of tissue Na^+ content (Primary Endpoint): We will quantify Na+ and water content in skin and muscle by ^{23}Na MRI and ^{1}H MRI as reported previously using a 3.0 T Phillips Achieva scanner, equipped with a double resonant ^{1}H/^{23}Na coil and a mono resonant ^{23}Na coil from Rapid, Germany. This will take about an hour.

Aortic Pulse Wave Velocity (PWV): This test will show us the stiffness of the arteries. This test will be measured with an ultrasound machine by a member of the research team. This test does not involve any shots, radiation or invasive procedures. This will take about 1 hour. Any arrhythmia or a problem with the rate or rhythm of the heartbeat may not allow participation in this test.

Hyperinsulinemic Euglycemic Clamp Study: Insulin sensitivity will be assessed with the hyperinsulinemic euglycemic clamp as previously described in the literature. Patients will report to the GCRC the morning of the study in a fasted state. Two indwelling catheters will be inserted, one for infusion of insulin and dextrose, and another for blood sampling (**Figure 2**). At time zero, a fasting blood glucose measurement will be obtained, and a blood sample will be drawn to assess baseline values of inflammatory, hormonal, and oxidative stress markers. Also starting at time zero, blood samples will be drawn at 5-minute intervals for 30 minutes to assess basal levels of glucose. At 30 minutes, a prime continuous infusion of human regular insulin will be started at a rate of 2.0 mU/kg/min and maintained throughout the clamp study (about 2 to 3 hours duration). At the same time, a variable infusion of 20% dextrose will be started and will be adjusted to reach the stead-state goal of the basal blood glucose level. Throughout the clamp study, blood samples for determination of plasma glucose concentration will be drawn every 5 minutes. After steady-state conditions are achieved (dextrose infusion rate no longer being adjusted and glucose measurements consistently on target), blood samples will be drawn at 5-minute intervals for 30 minutes to assess the total body glucose disposal rate (**M-value**), the basic clamp-derived IS index, which is the average value of the glucose infusion rate during the final 30 minutes of the steady state period. The M-value will be also normalized to body weight (**Mbw**) for reasons of comparison. The mean of the plasma insulin measurements obtained at the beginning and end of the final 30-minute steady state period will be used to standardize the M-values, as the respective **M/I** indices.

Figure 2. Clamp Study Design



We will also monitor serum potassium levels every 30 minutes and administer potassium chloride to maintain eukalemia.

Once the study is completed, the insulin infusion will be discontinued and subjects will be fed. The dextrose infusion will be gradually decreased over time with blood glucose being checked every 15 minutes. Once the blood glucose level has stabilized, the dextrose infusion will be discontinued. Standard discharge instructions will be provided to the subject by the GCRC including contact information.

Markers of inflammation and T cell activation: The inflammatory profile will include plasma levels of IL-17A, IFNγ, IL-6, IL-10, and hsCRP. In addition, T cells will be isolated from 40 ml of blood and divided for two studies. One half will be subjected to flow cytometry for detection of surface markers. These will include markers for chemokine receptors, T regulatory cells, and other T cell subtypes. The remaining cells will be cultured on anti-CD3 plates at $1x10^6$ cells/ml and release of cytokines including IL-17A, IFNγ, TNFα, IL-10 and IL-5 will be measured. These measures will be obtained before and after the 8-week intervention.

Activation of monocytes and dendritic cells: In additional experiments, we will determine if tissue sodium stores in humans, determined from ^{23}NaMRI data, affects levels of isoketal-protein

adducts in human monocytes and the propensity of human monocytes to differentiate into monocyte-derived dendritic cells (MoDCs) in a chimeric NOG mouse model. We will obtain 40 ml of heparinized blood from the subjects enrolled above. Twenty subjects will be selected with high baseline sodium stores and 20 with low baseline sodium stores. The below experiments will be performed at baseline and following 8 weeks of treatment. From 10 ml of this sample, buffy coats will be isolated by centrifugation and residual RBCs lysed. Cells will be stained for CD45, CD11b, CD14, CD3, CD19, CD83 and 7-AAD (to exclude dead cells). In addition, the cells will be fixed, permeabilized, and stained for IsoK-protein adducts. We will identify monocytes as $CD45^+/CD11b^+/CD14^+$ cells and determine both the percent and mean fluorescence intensity for isoKs staining. We will also examine $CD19^-/CD83^+$ cells in plasma, which are circulating dendritic cells. These are a very small percent, but their level of isoketals will be of interest.

From the other 30 ml, we will sort monocytes using negative selection. Human monocytes will be adoptively transferred to NOG mice. Five days later, the NOG mice will receive either normal or 4% salt chow. One week later, aortas, spleen and lymph nodes will be harvested, single cell suspensions prepared, and samples stained as described above. We will determine the number of cells that infiltrate the aorta, spleen and lymph nodes and have converted to $CD14^+/CD83^+$ and $CD14^-/CD83^+$. Because we start with a pure population of $CD14^+/CD83^-$ monocytes, this transformation represents conversion to DCs. Using PCR, we will examine mRNA in the aortas for human cytokines produced by DCs that influence the inflammatory milieu, including IL-6, IL-1β, IL-23, TGFβ and IL-12.

Schedule of Procedures

Procedure	Screening	Week 0	Week 2	Week 4	Week 8
Physical exam	✓				✓
Vital signs	✓	✓	✓	✓	✓
Blood collection	✓	✓	✓	✓	✓
Pregnancy test	✓	✓	✓	✓	✓
CMP	✓	✓	✓	✓	✓
Urine collection	✓	✓	✓	✓	✓
X-ray exam	✓				
^{23}NaMRI		✓			✓
PWV		✓			✓
HEGC		✓			✓
Hand grip test		✓			✓
Research labs		✓	✓	✓	✓
Immune cell analysis		✓			✓
Study drug/placebo dispensing		✓		✓	
Diet recalls		✓		✓	✓

7.0 Risks of Investigational Agents/Devices (side effects)

- Inconvenience of reporting to the GCRC.
- Eating a low salt diet for 8 weeks may be inconvenient.
- Fasting for 8 hours may be inconvenient.
- X-ray exam: For subjects who may have metal in their body, this research study involves exposure to radiation from 1 X-ray exam. The total amount of radiation that you might receive by participating in this study is equal to your body receiving 9 months of radiation from your natural surroundings, or about 5% of the amount allowed in a year for people exposed to radiation as part of their work.
- Having to lie still during the ^{23}NaMRI scan may be uncomfortable.
- Aortic pulse wave velocity measurement is a non-invasive test and poses no known risks. The only inconvenience is time spent performing the test.
- The insulin infusion during the clamp study can lower blood potassium levels. If this drops too low, the subject's heart rhythm may be affected. We will closely monitor potassium levels (every 30 minutes), and place the subject on a machine that monitors the heart to decrease this risk and to observe any complications. If a decrease in potassium occurs, it will be replaced intravenously.
- Replacing the potassium may cause slight burning or irritation and the subject's heart rhythm may be affected. Again, we will monitor the subject's heart to decrease this risk and adjust the rate of replacement accordingly.
- There is a small risk of low blood sugar during the clamp study. If this occurs, the subject may experience any of these symptoms: feeling lightheaded, headache, fast heartbeat, sweating, blurred vision, hunger, and rarely seizures. We will check blood sugar every 5 minutes. If the blood sugar level drops below normal, it will be restored intravenously.
- Inconvenience of having gelled electrodes placed on the chest and being connected to a heart monitor during the clamp study. The gelled electrodes used for ECG monitoring may cause skin irritation.
- Pain, redness, soreness, bruising, or infection may occur at the needle stick site. Rarely some people faint. The study doctor may put some cream (called EMLA) on the skin to numb the area so the subject will not feel the needle stick as much. The numbing cream may make the skin or the area have a change in skin color, but this is rare.
- The study drugs may lower blood pressure excessively or alter electrolyte levels. Electrolyte imbalance may occur. We will monitor blood pressure and electrolyte levels throughout the study, and give electrolyte supplements or slightly change diet as needed. If persistent electrolyte imbalance occurs, the subject may be withdrawn from the study.
- The study drugs may produce allergic reactions. We will exclude subjects with allergies to these drugs and if allergies to these drugs develop during the course of the study, we will discontinue the subject.
- The study drugs should not be used during pregnancy. As a precaution we will exclude pregnant women. We will check urine pregnancy tests before each study day. If a woman self-reports pregnancy to us, we will immediately discontinue study medication and withdraw that subject. Risk is further minimized by the short duration of the study.

- The most commonly reported side effect of the study drugs is gastrointestinal upset (diarrhea, cramping, nausea/vomiting, etc.).
- Other less common side effects include weakness, dizziness, headache, leg cramps, and drowsiness.
- Spironolactone may produce reversible breast tenderness or enlargement in a minority of male subjects. This risk will be minimized due to the short duration of the study. Subjects have the option of withdrawing from the study if this side-effect necessitates.

As with all research studies, there are unidentifiable and unforeseeable risks that may occur during this study.

8.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

Safety information will be assessed by subject interview. As these results are collected, all adverse events (AE) will be identified and reported to the principle investigator within fourteen (14) days. Adverse events will be reported as described below. The principal investigator (PI) is responsible for evaluating each AE and for determining whether the AE affects the risk/benefit ratio of the study and whether modifications to the protocol and consent form are required.

Treatment will be discontinued for any subject who develops conditions during the course of the study that are felt to significantly alter the risk/benefit profile for the individual subject.

Adverse event grading. All adverse events will be graded as follows:

Severity
0 = No adverse event or within normal limits
1 = Mild—did not require treatment
2 = Moderate—resolved with treatment
3 = Severe—required professional medical attention
4 = Life-threatening or disabling
5 = Death

Related to study drug (there is a reasonable possibility that the experience may have been caused by the drug)
0 = Unrelated
1 = Unknown
2 = Related

Unexpected event (an AE with specificity or severity not consistent with the risk information in the protocol/application or an AE that has not been previously observed)
0 = No
1 = Yes

Serious (any AE occurring at any dose that results in death; a life-threatening adverse drug experience; inpatient hospitalization or prolongation of existing hospitalization; a persistent or significant disability/incapacity; a congenital anomaly/birth defect; or any important medical event that, based on medical judgment, jeopardizes the subject and may require medical or surgical intervention to prevent one of the above outcomes)
0 = No
1 = Yes

Adverse event reporting. Any adverse experience associated with the use of drug that is serious, unexpected and possibly or definitely study related will be reported in writing to the IRB within 10 working days of the PI's first knowledge of the occurrence. The PI will review all expedited adverse event reports. In addition, any deviations from protocol will be reported to the IRB.

The annual summary of all adverse events and any audit reports will be sent to the IRB and to the study DSMB.

Data Safety and Monitoring Board (DSMB). We will establish a DSMB for the purposes of this study. The board will consist of the following member from the Vanderbilt University Medical Center:

Italo Biaggioni, MD (Associate Director, CRC); Chair

The DSMB will regularly monitor study progress. On an annual basis, the DSMB will also review adverse event logs and any preliminary data. DSMB summary reports will be provided to the IRB according to the IRB's policies and procedures. No interim analysis is planned. Annual progress reports will be submitted to VICTR.

9.0 Study Withdrawal/Discontinuation

Subjects who are non-compliant with the study protocol, or no longer wish to participate in the study will be withdrawn from the study.

10.0 Statistical Considerations

Power and sample size calculations: For Aim 1, 100 per race group (200 in total) provides 85% power to detect an effect size of 0.42 in blood pressure, forearm blood flow or PWV with two-sided type I error = 5%. For Aim 2, assuming a reduction in muscle Na+ of 5 mmol/L and SD=6.0 mmol/L by drug treatment, 85% power and Bonferroni adjusted 2-sided type I error (significant level) test at level 1%, a sample size of 41 subjects per group (161 in total) is needed. For Aim 3, the proposed sample size 200 enable us to detect the Pearson Correlation of 0.21 or higher (absolute value) between Na+ storage change and immune cell activity change with two-sided type I error = 5%. For Aim 4, the sample size calculation is the same as Aim 3.

Data collection and analysis: For Aim 1, summary statistics of blood pressure, forearm blood flow and PWV at week 0 will be provided for both African American group and Caucasian group. We will test the difference of these variables among two race group using two sample t-test. For Aim 2, we will conduct univariate analysis for comparing the magnitude of the Na+ tissue content among each pair of treatment groups using two sample t-test, and the Bonferroni correction will be applied to control the type I error due to multiple comparisons. In addition, partial linear model with be used to estimate the linear treatment effect and interaction effect while adjusting the nonlinear effect such as age and gender. For Aim 3, we will calculate the Pearson correlation of Na+ storage change (before and after treatment) and the immune cell activity change. Alternatively, Spearman correlation or Kendall's tau will be calculated to capture the nonlinear correlation. We will also perform linear regression with all covariates and pairwise interactions in the model to adjust the impact of factors such as age, sex, and body weight on tissue sodium. For Aim 4, we focus on the association between tissue sodium change and insulin sensitivity change using the same analysis methods as in Aim 3.

11.0 Privacy/Confidentiality Issues

All efforts, within reason, will be made to keep personal information in the research record confidential but total confidentiality cannot be guaranteed. Each participant will be assigned a unique study identification number and will be referred to by this number to protect their identity over the course of the study. The numbers will be assigned sequentially. Only key study personnel will have access to these numbers. The codes will be kept in a password protected database on the VU server. No information will be given to anyone without permission from the subject. All data will be identified with the assigned study identification number unique to the subject.

All access to protected health information (PHI) as defined by current and future federal standards will be carefully managed. Best effort will be made to de-identify PHI in all case report forms. All physical records (including case report forms that contain PHI) are stored in a locked office and only study personnel will have access. All computers containing patient information are behind locked doors in rooms with limited access.

The database is housed on the Vanderbilt University secured computer network and is password protected. Only members of the study team will have access to this database.

Adverse event reports and annual summaries will not include subject-identifiable material but only the assigned study identification numbers.

12.0 Follow-up and Record Retention

The study will be conducted over the course of five years. Records will be retained indefinitely.

Data will be stored on the Vanderbilt University computer network in a password protected database. Only members of the study team will have access. Only Key Study Personnel will have access to the subject's code. The code will be destroyed after study closure. Paper case report forms and other pertinent paper documentation will be kept in a locked office and only study personnel will have access.

Only personnel directly involved with the study will have access to source data and the electronic database. All research data will be maintained by the PI after study closure. The PI will maintain the research data indefinitely.

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