Study Title: **Optimizing Ultrasound-induced Anti-inflammation in Human Subjects**

NCT number: **NCT05685108**

Version Date: **18Feb2025**

OPTIMIZING ULTRASOUND-INDUCED ANTI-INFLAMMATION IN HUMAN SUBJECTS

Short title: Ultrasound anti-inflammation in human subjects

Protocol Number: 1.4

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Principal Investigator: Mark D. Okusa, MD, FASN

Funded by: National Institutes of Health (NIH) and Division of Nephrology, Department of Medicine, University of Virginia

Version Date: 18 February 2025

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STATEMENT OF COMPLIANCE

The trial will be conducted in accordance with applicable United States (US) Code of Federal Regulations (CFR). International Conference on Harmonisation Good Clinical Practice (ICH GCP) guidelines will be incorporated consistent with institutional practice. The Principal Investigator will assure that no deviation from, or changes to the protocol will take place without documented approval from the Institutional Review Board (IRB), except where necessary to eliminate an immediate hazard(s) to the trial participants. All personnel involved in the conduct of this study have completed Human Subjects Protection and GCP Training.

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the IRB for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. 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.

Sponsor-Investigator

_____	_____	_____
Name (print)	**Signature**	**Date**

For single site UVA investigator-initiated trials without an IND/IDE

Principal Investigator

_____	_____	_____
Name (print)	**Signature**	**Date**

ABBREVIATION

AE	Adverse Event
AKI	Acute kidney injury
CAP	Cholinergic anti-inflammatory pathway
CFR	Code of Federal Regulations
CMP	Clinical Monitoring Plan
CONSORT	Consolidated Standards of Reporting Trials
CRF	Case Report Form
DCC	Data Coordinating Center
DSMB	Data Safety Monitoring Board
EC	Ethics Committee
eCRF	Electronic Case Report Forms
ELISA	Enzyme-linked immunosorbent assay
FCCF	Flow Cytometry Core Facility
FDA	Food and Drug Administration
GCP	Good Clinical Practice
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
HIPAA	Health Insurance Portability and Accountability Act
IB	Investigator's Brochure
ICH	International Conference on Harmonisation
IDE	Investigational Device Exemption
IRB	Institutional Review Board
ISM	Independent Safety Monitor
ISPTA	Spatial-peak temporal-average intensity
LPS	Lipopolysaccharide
MedDRA	Medical Dictionary for Regulatory Activities
MI	Mechanical index
MOP	Manual of Procedures
MSDS	Material Safety Data Sheet
NCT	National Clinical Trial
NIH	National Institutes of Health
NSAIDs	Non-steroidal anti-inflammatory drugs
OHRP	Office for Human Research Protections
PI	Principal Investigator
QA	Quality Assurance
QC	Quality Control
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SMC	Safety Monitoring Committee
SOA	Schedule of Activities
SOP	Standard Operating Procedure
UP	Unanticipated Problem
UVA	University of Virginia

1 PROTOCOL SUMMARY

1.1 Synopsis

<table>
<tr><td>Title:</td><td>Optimizing ultrasound-induced anti-inflammation in human subjects</td></tr>
<tr><td>Study Description:

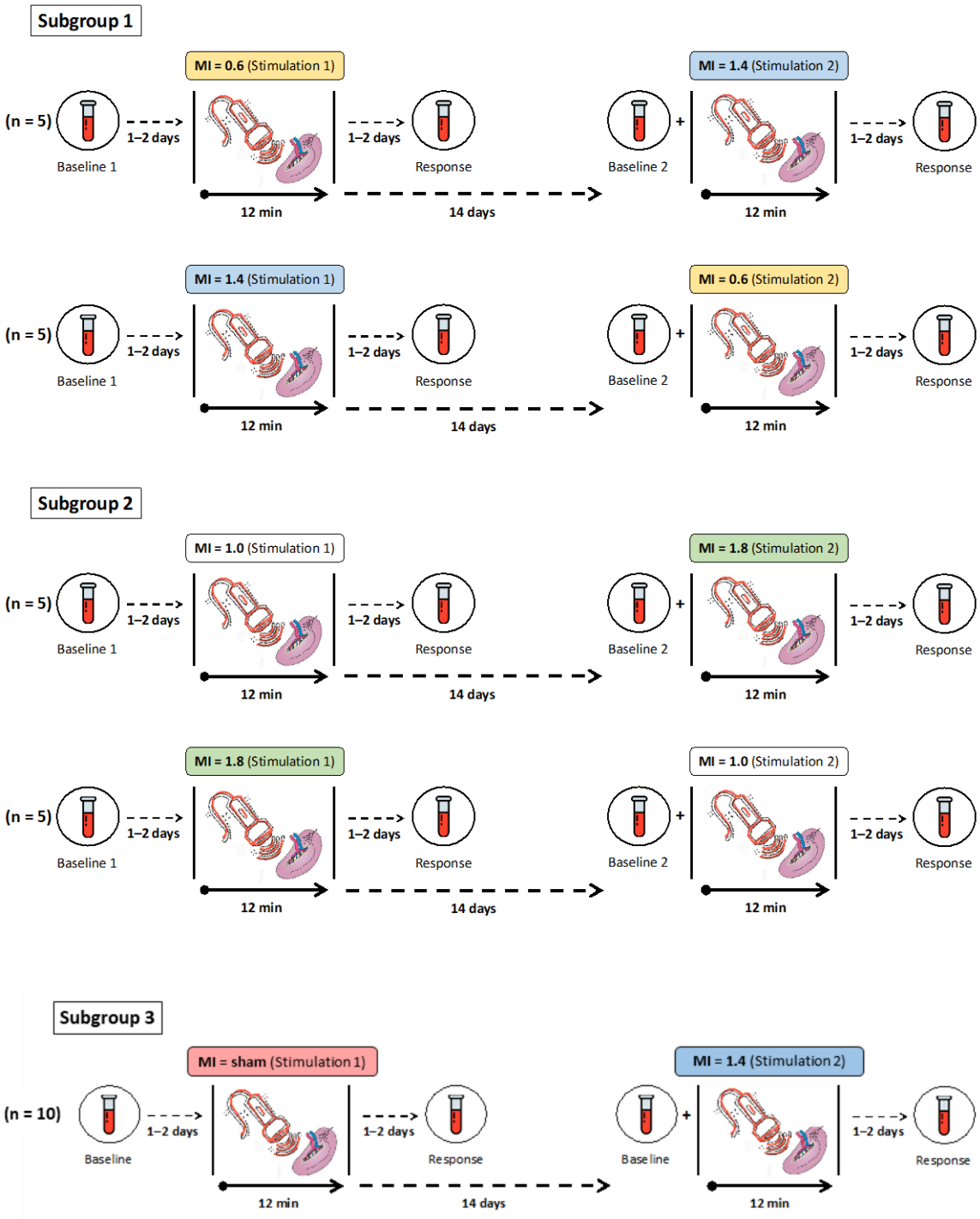
Objectives & Endpoints:</td><td>This study is designed to begin testing the hypothesis that pulsed ultrasound stimulation can be used effectively in human subjects to control pathogenic inflammatory responses through the stimulation of the cholinergic anti-inflammatory reflex pathway, a neuroimmune feedback response. The overall goal of this project is to determine which, if any, ultrasound stimulation protocols are able to restrict the inflammatory response of immune cells collected from healthy subjects post-ultrasound stimulation. Subjects will receive ultrasound and then immune cells will be isolated from their blood and treated ex vivo with inflammatory stimuli to test their inflammatory capacity.
<table>
<tr><th>Objectives</th><th>Endpoints</th></tr>
<tr><td>Primary</td><td></td></tr>
<tr><td>• Objective 1: Determine ultrasound intensities within FDA approved guidelines that limit the inflammatory response
• Objective 2: Determine whether targeting the spleen and/or cervical vagus nerve with ultrasound are effective methods for anti-inflammatory ultrasound delivery</td><td>(Same for both Obj. 1 & Obj. 2) Luminex assays and/or individual enzyme-linked immunosorbent assays (ELISAs) to measure cytokine concentration in the supernatants from ex vivo cultures. Peripheral blood immune cells from pre- and post-ultrasound samples will be cultured with inflammatory stimuli, such as lipopolysaccharide (LPS), for 24 hours prior to collection of the supernatants.</td></tr>
<tr><td>Secondary</td><td></td></tr>
<tr><td>Determine the impact of ultrasound stimulation on the distribution of immune cells in the blood of human subjects.</td><td>Flow cytometry analysis of white blood cells.</td></tr>
<tr><td>Safety assessment</td><td></td></tr>
<tr><td>Verify ultrasound stimulation does not produce any appreciable discomfort in healthy human subjects.</td><td>Survey of participants’ state of mind and physical sensations (i.e., any new experience since receiving ultrasound stimulation such as pain, loss of sensation, and shifts in thought patterns or attitude).</td></tr>
</table>
</td></tr>
<tr><td>Study Population:</td><td>A total of 55 participants will be recruited for this study. Subjects should be in overall good health without acute or chronic medical conditions, of any gender and demographic group, between the ages of 25 and 50 years, and preferably local to the Virginia/Washington D.C. area.</td></tr>
</table>

Description of Sites/Facilities Enrolling Participants:	All enrollment and work associated with this study will be performed at the University of Virginia (UVA) in Charlottesville, Virginia, by UVA personnel associated with this project.
Description of Study Intervention:	The intervention used in this study is a pulsed ultrasound stimulation targeted to the spleen or cervical vagus nerve. Ultrasound technology has been used clinically for many years with a variety of applications, but our goal of targeted stimulation of a neuroimmune anti-inflammatory response is novel. The ultrasound device contains several key components. The central component is the transducer that sends and detects ultrasonic sound waves. These waves are generated via a piezoelectric phenomenon in which an electric current is applied to piezoelectric crystals within the transducer. These crystals vibrate in response to the electric current and convert the energy into sound waves with a frequency beyond human hearing (i.e., ultrasonic). When these sound waves interact with solid objects, such as tissue, they are absorbed, scattered, refracted, and reflected. The reflection and scattering allows sound waves to be returned to the transducer and detected in order to generate an image based on the timing and intensity of the returned signal. For our purposes of stimulation, the detection of these waves is a minor element that will only be used in targeting ultrasound bursts to the proper physical location within the subject. According to our hypotheses, the interaction of emitted ultrasound waves of the proper intensity with tissue (spleen or cervical vagus nerve) is capable of stimulating a physiological response that produces an anti-inflammatory state. The other critical element of the device for our study is the programming control unit. This allows for control of the intensity of the ultrasound wave and will enable us to ensure that the device is used in a safe, controlled, and consistent manner. The Sequoia system allows for general programming of the ultrasound parameters and enables us to scale the total energy deposited within the area of ultrasound stimulation to prevent detrimental effects, such as heat-induced tissue damage. The range of intensities chosen for this study have all been used safely in humans before and the Sequoia system will help us ensure that these parameters are not exceeded. The ultrasound machine used in this study is commercially available.
Study Duration:	The study is estimated to take 18 months to complete (from enrollment to completion of data analyses).
Participant Duration:	Each participant will complete a total of 4–6 visits. Each visit should require minimal time to complete and take no more than 1–2 hours of the participant's time. The time between each visit

	is flexible based on the participant's availability and schedule, with the only restriction being the time between ultrasound stimulations (at least 14 days between ultrasound stimulations). This 14-day window is intended to allow any impacts of the previous ultrasound stimulation to dissipate. Realistically, a participant could complete all visits within 1 month, but the study will accommodate the scheduling requirements of the participants.

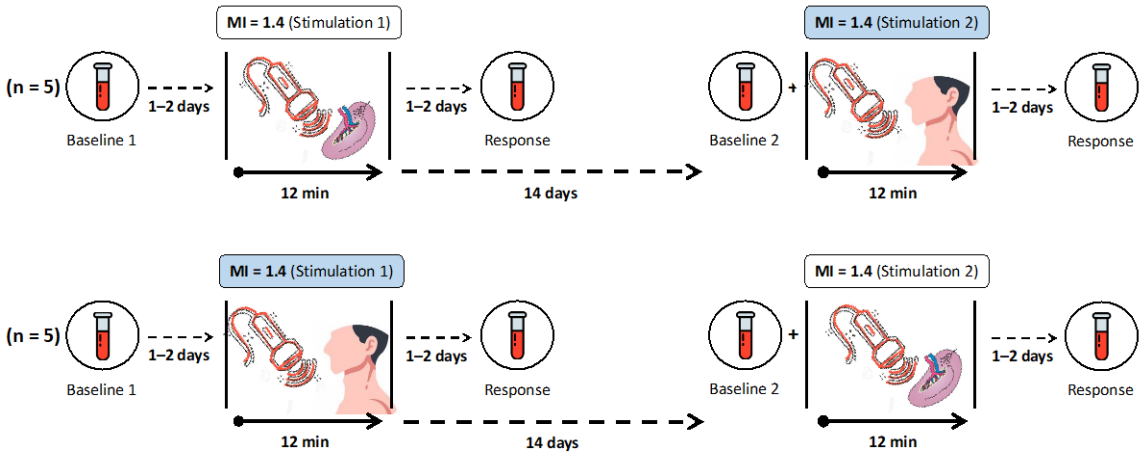
1.2 Schema

OBJECTIVE 1 (GROUP 1; n = 30)



MI, mechanical index.

OBJECTIVE 2 (GROUP 2; n = 10)



MI, mechanical index.

A detailed description of the schemata is provided in section 6.2.

2 INTRODUCTION

2.1 Study Rationale

Inflammatory conditions are an imminent threat to human health, happiness, and survival. Major examples of these include sepsis, bodily trauma (burns or organ damage), and autoimmune disorders such as lupus erythematosus or rheumatoid arthritis. Such conditions are common within the human population and it is likely that the majority of people will experience one or more of them during their lifetime. While the etiology and manifestation of these conditions may be varied, there is now the potential for therapies that intentionally trigger inherent anti-inflammatory mechanisms that are hard-wired into our physiology to ameliorate or even nullify the impacts of pathologic inflammation. These pathways are applicable to multiple inflammatory conditions and provide a means to exploit an aspect of our physiologic “source code” to combat excessive or inappropriate inflammation. While the most common procedure for triggering these effects is to stimulate the vagus nerve electrically, this strategy requires invasive surgery and does not lend itself to clinical treatment or repeated therapy.

We discovered that pulsed ultrasound stimulation of mice can recapitulate the outcomes of electrical stimulation and protect mice from pathologic inflammation to a large degree (1, 2). The use of ultrasound technology has multiple advantages given that it noninvasively provides accurate imaging test of the body, thus allows targeted stimulation, and is widely accessible in routine clinical practice. Ultrasound stimulation is already used clinically for the treatment of pain, physical injuries, and tumor ablation (3-5). Thus, it is a far more viable option for clinical translation to ameliorate inflammatory conditions and has the potential to provide much needed relief to a broad spectrum of patients. To this end, we are seeking to better understand the mechanisms of and requirements for ultrasound-induced anti-inflammation, as well as how this treatment impacts the human system. We hypothesize that both spleen-targeted and cervical vagus-targeted ultrasound will produce an anti-inflammatory effect that will be reflected in reduced cytokine production, and that this will have a threshold of efficacy dependent on the intensity of ultrasound delivered. We also hypothesize that there will be measurable fluctuations in the distribution of immune cells in the blood and will seek to characterize this as a secondary endpoint. The enhanced understanding gained by this study will help ensure that this powerful technique is used safely and appropriately to maximize the benefit to patients as well as provide input for future study and treatment design.

2.2 Background

The cholinergic anti-inflammatory pathway (CAP) represents one of the best studied neuroimmune-modulatory pathways (6-9). The CAP has been elucidated as a way for the nervous system to restrict inflammation and rescue many of the detrimental effects of inflammatory disease. By stimulating the vagus nerve, the neurotransmitter acetylcholine is produced in distal tissues and can be detected by immune cells expressing nicotinic acetylcholine receptors. For reasons unknown, this interaction leads to an anti-inflammatory state, in which immune cells produce lesser amounts of inflammatory cytokines (10, 11). Interestingly, since the receptors for acetylcholine are nicotinic, treatment of rodents or cells with nicotine can also induce anti-inflammation and improve the survival of mice with experimental sepsis (11, 12). This neuro-immune crosstalk has also been shown to protect mice from acute kidney injury (AKI) following renal ischemia-reperfusion (13, 14). Additional work with mice showed that pulsed ultrasound stimulation recapitulates the renal protection observed following electrical vagus nerve stimulation (1, 2, 15, 16). This showed pulsed ultrasound stimulation has the potential to modulate

immune cell distribution and cytokine production in vivo, providing indications of anti-inflammation that likely contribute to the observed renal protection (7). Given the invasiveness and/or questionable efficacy of currently available vagus nerve stimulation protocols and devices, the potential for non-invasive ultrasound to induce the same effects in humans is an attractive and viable clinical approach to ameliorate inflammation in a controlled fashion.

2.2.1 Pre-Clinical Experience

While vagus nerve stimulation has received a great deal of attention for its anti-inflammatory capability, work with pulsed ultrasound-mediated anti-inflammation is less prevalent. Studies previously performed by the Okusa lab are some of the only ones that have used a stimulation protocol in mice that is similar to the approach used in this trial (mechanical index [MI] between 0.5 and 1.9, single treatment, short duration of ultrasound exposure). As stated, these studies have shown that ultrasound targeted to the flanks of mice is capable of providing protection from AKI and potentially sepsis (1, 2). However, protection is lost when the spleen is removed prior to ultrasound stimulation (2, 15). This reliance on an intact spleen strengthens the similarities between vagus nerve stimulation and ultrasound stimulation and provides a rationale for targeting the spleen in this study. In addition to this work, another group has tested the impacts of a range of ultrasound intensities targeted to the spleen and liver of mice (7). This study exhibited a “dose response” with increasing ultrasound intensities and demonstrated that the inflammatory and hyperglycemic effects of lipopolysaccharide (LPS) injection were ameliorated comparably to vagus nerve stimulation (7). Another pre-clinical study used a similar ultrasound protocol to investigate the impacts of the treatment on hyperglycemic myocardial ischemia-reperfusion injury and found that spleen-targeted ultrasound was able to reduce infarct size (17). In addition, that study also investigated the efficacy of cervical vagus (neck)-targeted ultrasound, given that it may have an effect similar to spleen-targeted ultrasound through upstream vagus nerve modulation. The investigators found that cervical vagus ultrasound stimulation was similarly effective compared to spleen-targeted stimulation (17). This provides rationale for our second study group in which we will compare the efficacy of ultrasound delivered to the spleen and neck of subjects.

2.2.2 Relevant Clinical Experience

While the parameters selected for this study are within the Food and Drug Administration (FDA) limits for ultrasound imaging, there are no published studies that have explored the impacts of pulsed ultrasound stimulation for protecting human subjects from inflammatory conditions. While ultrasound technology has been used clinically for many years for purposes ranging from diagnostic imaging to treating sports injuries, it’s use as a therapeutic anti-inflammatory agent is essentially unexplored, aside from a single pre-publication study (18) (clinicaltrials.gov: NCT03548116 & NCT03690466). This study employed a MI of 1.4 and targeted the spleen of study participants for a 3-minute stimulation period. The authors showed that this stimulation protocol was sufficient to downregulate cytokines and associated pathways in healthy subjects and patients with rheumatoid arthritis without compromising the adaptive immune response or any negative outcomes or side effects of note (18).

2.3 Risk/Benefit Assessment

2.3.1 Known Potential Risks

While ultrasound carries risks related to thermal and mechanical effects, these are dependent on the amount of energy deposited to the tissue site over time. Tissue heating

can cause damage to cells, including nerve cells. Mechanical forces can lead to shear stress from fluid streaming effects within tissue and cavitation of gas bubbles due to rapid changes in pressure generated by ultrasound pulses. Both of these can damage cells or tissue structures if not managed properly. These elements have led to the FDA establishing the upper limit for MI at 1.9 (19). MI is calculated by dividing the peak negative pressure of the ultrasound wave by the square root of the wave frequency (20). Significant tissue damage could lead to both immediate and long-range risks if it results in pain, reduced organ function, and/or chronic injury. However, by limiting the exposure to and power of our ultrasound parameters and remaining within the FDA prescribed limits for imaging ultrasound, we do not view these as substantial risks in this study. We anticipate to gain additional insights on tolerability and safety of the ultrasound procedure by measuring urinary AKI biomarkers and spleen measures post-stimulation. Furthermore, the cumulative maximal volume of whole blood drawn during the entire study period will not exceed 48 ml, which is far below the 45 CFR 46.110-defined blood drawing limits.

2.3.2 Known Potential Benefits

The animal and human work outlined above capture the potential benefits of this study. By enhancing our knowledge of how ultrasound stimulation impacts the human system, we come closer to its effective use in the clinic, which will potentially provide relief from inflammatory conditions for millions of patients. The studies above exhibit benefits in the form of reduced pro-inflammatory cytokine production, reduced inflammation-associated tissue injury, and potentially reduced sepsis-related morbidity and mortality, if ultrasound stimulation is functioning similarly to vagus nerve stimulation (7, 8).

2.3.3 Assessment of Potential Risks and Benefits

The potential risks in this study have been considered and managed in the design. Due to the wide use of ultrasound in the clinic for other procedures, there is a long track record of safe use that has informed our design. We have designed our intervention to minimize the total energy deposited at tissue sites through a pulsed wave procedure, with defined MI intensities and short total duration, and have ensured that we stay beneath the FDA prescribed threshold for imaging ultrasound with our chosen MI range (19). Thus, we view the potential for detrimental outcomes as low in this study. The potential benefits, on the other hand, are far-reaching and would be incredibly meaningful to patients suffering from acute or chronic inflammation, a substantial portion of which have life-threatening conditions.

3 OBJECTIVES AND ENDPOINTS

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Primary		
• Objective 1: Determine ultrasound intensities within FDA approved guidelines that are able to limit the inflammatory response • Objective 2: Determine whether targeting the spleen and/or cervical vagus nerve are effective	Luminex and/or individual ELISA assays to measure cytokine production from peripheral blood immune cells collected before and after ultrasound stimulation and cultured with inflammatory stimuli. Blood for analysis will be collected at the baseline visits 1–2 and	Evaluating cytokine production post-ex vivo stimulation with known inflammatory agents is a relatively simple means of assessing the behavior of immune cells and does not require the induction of inflammation in the human

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
methods for anti-inflammatory ultrasound delivery	24–48 hours after each ultrasound stimulation. Luminex technology allows the simultaneous measurement of multiple cytokines via a flow cytometry-based assay. This essentially leverages the principles of flow cytometry to perform multiple ELISA tests in a single assay. This analysis will enable us to assess the concentration of a broad range of cytokines in the supernatants from our ex vivo cell stimulations. This will provide robust data for evaluating the efficacy of ultrasound in limiting the inflammatory capacity (i.e. cytokine production) of immune cells. Additional ELISA assays may be necessary if we decide there are additional cytokines we would like to measure that do not have Luminex reagents available, but Luminex analysis will be the primary method for determining cytokine production.	subjects enrolled in the study. Thus, this provides us with an effective strategy to assess the efficacy of ultrasound with minimal risk of discomfort or adverse events in the subjects. The use of immediate baseline analysis and subsequent post-ultrasound sample collection will allow for the use of each subject as their own control in a standardized assay. This will provide us with the opportunity to identify the impacts of ultrasound on the inflammatory response for each individual subject and will yield the most consistent and reliable data for analysis.
Secondary		
Determine the impacts of ultrasound stimulation on the distribution of immune cells in the blood of human subjects.	Flow cytometry analysis of blood cells. This will be performed using a portion of the same blood samples collected for the primary objective (samples from immediate baseline and after each ultrasound). By using cell surface markers to identify individual immune cell populations in subjects' blood, we will be able to quantify the abundance of populations of interest and track any ultrasound-induced changes in their relative distribution. This is a relevant measure both separate from and related to the cytokine production measurements in the primary objective. Changes in the distribution of immune cells could alter the ability of subjects to respond to certain types of infection or other disruptions to homeostasis. While anti-	This endpoint was chosen since it supplements the primary endpoint and is achievable without additional sample collection. It provides an additional level of understanding to the results and context for interpretation. Further, results from this endpoint will provide information for forming hypotheses for follow-up studies.

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
	inflammation is the goal, tracking this measurement will also inform potential side effects and situations that clinicians should be aware of when considering ultrasound therapy. Altered prevalence of immune cells will also provide context for analysis of the cytokine data. If there are reductions in the production of specific cytokines post-ultrasound, this will help us assess if the reduction is due to effects on the cells directly or if a reduction in cell numbers in the culture condition could explain the difference.	
Safety evaluation		
Verify ultrasound stimulation does not produce any appreciable discomfort in healthy human subjects or kidney stress or damage.	Survey of participants' state of mind and physical sensations (i.e., any new experience since receiving ultrasound stimulation such as pain, loss of sensation, and shifts in thought patterns or attitude). Information will be collected at each visit after the baseline visit and may include a final follow-up survey if responses during the study warrant additional attention. This endpoint will be achieved with a simple survey of patient feedback in which we will enquire about their overall experience post-ultrasound. We will enquire if they have experienced any noticeable changes in thought, mood, or sensation. This will be designed as an open-response inquiry to encourage honest, undirected feedback and track any concerns the subjects may have during the course of the study. Assessment of urinary AKI biomarkers to assess kidney stress potentially related to ultrasound stimulation. Optional spot urine samples for biomarkers will be collected at Visits 1–4 (i.e., immediately before ultrasound stimulation, and directly after and	This endpoint has been included because, although we do not anticipate any adverse responses to the ultrasound stimulation, the use of ultrasound to stimulate what we believe to be a neuroimmune regulatory pathway is a novel approach. The potential neurological element is of interest and we want to confirm that we are targeting inflammatory mechanisms without any detrimental patient experience or kidney stress/damage.

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
	24–48 hours after ultrasound stimulation).	

4 STUDY DESIGN

4.1 Overall Design

This is a single-site pilot study that contains 2 major Groups to address questions about both dose response (Objective 1) and delivery route (Objective 2) for pulsed-ultrasound induction of anti-inflammation in humans. Thus, healthy subjects have been chosen as the study population for this analysis in order to determine therapeutic potential and possible best practices. In this small-scale study, subjects will be used as their own control for data analysis.

Group 1 of the study (Objective 1) will be used to assess dosing thresholds for induction of anti-inflammation. We will test four different levels of ultrasound intensity ("doses") as well as sham ultrasound treatment to determine which of them are capable of producing an anti-inflammatory effect. The doses will be defined in terms of MI and each subject will be randomly assigned to receive two different MI doses of ultrasound. The MIs to be tested are sham, 0.6, 1.0, 1.4, and 1.8. In animal studies (1, 2), a MI of 1.2 was sufficient to generate a protective anti-inflammatory response and we have used this as a guide while keeping in mind the MI limit previously set by the FDA of 1.9 (19). Each subject will receive two separate doses of ultrasound stimulation (one per treatment visit, two treatment visits) with a MI difference of 0.8. Subgroup 1 will be tested at MI = 0.6 & 1.4, Subgroup 2 will receive MI = 1.0 & 1.8, and Subgroup 3 will receive MI = sham US & 1.4. The two doses will be administered in separate visits with at least 14 days between each stimulation to allow any effects of the first stimulation to dissipate. We assume that 14 days represent an acceptable duration of time to dissipate any effects of ultrasound stimulation. This assumption is based on experimental data from the Okusa lab indicating that there is no significant efficacy of ultrasound-induced anti-inflammation in mice when ultrasound stimulation is applied ≥7 days before induction of ischemia-reperfusion injury (1). We have doubled this period to 14 days to ensure ample time is given for the effects of the first treatment to wane off. However, since there are no human data available for the duration of ultrasound-induced anti-inflammation, we have opted to perform two reciprocal stimulation set-ups within each group and subgroup. For example, in Group 1 Subgroup 1, five participants will receive the lower MI dose in the first visit and the lower MI dose in the second visit while the other five participants will receive the doses in the reverse order. This will allow us to compare the relative effects of each MI as a first dose vs. as a second dose to verify that there is no residual impact of receiving a prior ultrasound treatment.

Group 2 of the study (Objective 2) will be used to test the efficacy of ultrasound delivered to the spleen versus targeted to the cervical vagus nerve at the neck. In animal studies, splenectomy prior to delivery of ultrasound to the flank removes the bulk of protection offered by the treatment (2, 15), thus we believe this is a central tissue for generating the anti-inflammatory effect. However, human subjects vary widely in size and shape in the abdominal region and localization of the spleen may vary somewhat between individuals, so this may not be the ideal location for consistent ultrasound stimulation delivery in humans. Since we believe this treatment may be functioning through a vagus nerve-mediated response and electrical stimulation of the vagus nerve produces anti-

inflammation (10, 11), we will target both the spleen and cervical vagus nerve in separate treatment sessions for this subject group. The same reciprocal setup is used in this spleen-vs- neck-targeting group as in Group 1. Five participants will receive spleen ultrasound in the first treatment while the other five will receive neck ultrasound as the first treatment, with 14 days being allowed between treatment sessions. The ultrasound stimulations will be performed at a uniform MI of 1.4 for spleen vs. neck testing.

Sample and data collection will be the same for both groups and will consist of research blood draws of ~3 ml prior to each treatment visit and 24–48 hours after each treatment visit. The interval of 24–48 hours was chosen based on experimental data from the Okusa lab showing that the efficacy of the ultrasound-induced anti-inflammation wanes in a time-depended manner when ultrasound stimulation is applied ≥3 days before induction of ischemia-reperfusion injury (1). The blood will be divided into two portions with one being used for flow cytometry analysis of immune cell populations and the other used for setting up ex vivo stimulation assays to measure cytokine production. Analysis of each stimulation condition (stimulation 1 and 2, respectively) will be compared to the immediate baseline values (baseline 1 or 2, respectively) from the same subject. We will quantify the difference between the means of the baseline and treatment, for example as fold change or percent of baseline, for each subject's samples. The magnitudes of difference will then be treated as an additional test statistic and will be grouped by treatment protocol (e.g., all MI = 0.6 differences from baseline will be compiled into one group, all MI = 1.0 will be compiled into a group, etc.). The compiled magnitudes will be compared to those from the other treatment conditions to assess the relative impact of each ultrasound stimulation protocol. We believe this will yield the most relevant and reliable data for this study.

4.2 Justification for Dose

As stated above, we have chosen our stimulation MI based on animal studies and FDA guidance for diagnostic ultrasound. The target sites for ultrasound delivery have also been chosen based on animal studies and current knowledge regarding the potential mechanism of ultrasound-induced anti-inflammation.

4.3 End of Study Definition

Primary completion date is the date that the final participant was examined or received an intervention for the purposes of final collection of data for the primary outcome, whether the clinical study concluded according to the pre-specified protocol or was terminated. In the case of clinical studies with more than one primary outcome measure with different completion dates, this term refers to the date on which data collection is completed for all of the primary outcomes defined as the final date for the collection of data for the primary endpoint.

Study completion date is the date the final participant was examined or received an intervention for purposes of final collection of data for the primary and secondary outcome measures and adverse events (AE; for example, last participant's last visit), whether the clinical study concluded according to the pre-specified protocol or was terminated.

5 STUDY POPULATION

5.1 Inclusion Criteria

In order to be eligible to participate in this study, an individual must meet all of the following criteria:

1. Male or female, aged 25–50 years

2. Provision of signed and dated informed consent form
3. Able to comprehend the study goals and procedures, stated willingness to comply with all study procedures, and availability for the duration of the study
4. Considered English proficient so that the subject can follow verbal commands during the ultrasound procedure
5. In good general health, as evidenced by medical history
6. Laboratory results indicating normal blood count and adequate organ function per the following criteria:

System	Laboratory Value
Hematological	
White blood cell count	≥ 4.00 k/μL
Platelets	≥ 150 k/μL
Hemoglobin	≥ 11.0 g/dL
Renal	
Estimated glomerular filtration rate	≥ 60 mL/min/1.73 m^2
Blood urea nitrogen	≤ 1.5 x ULN
Hepatic	
AST and ALT	≤ 2.5 x ULN
Other	
Fasting blood sugar	<126 mg/dL
Hemoglobin A1c	< 6.5%

7. Agreement to adhere to Lifestyle Considerations (see *Section 5.4*) throughout study duration.

5.2 Exclusion Criteria

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

1. Chronic medical conditions, including cancer (in remission or active cancer), cerebrovascular disease, chronic kidney disease, heart conditions (such as heart failure, coronary artery disease, cardiomyopathies), lung disease, liver disease, hypertension, diabetes mellitus type 1 and 2, human immunodeficiency virus infection, primary immunodeficiencies, solid organ or hematopoietic cell transplantation, tuberculosis, and cystic fibrosis, autoimmune disorders (e.g., rheumatoid arthritis, inflammatory bowel disease), sickle cell anemia or other anemia syndromes
2. Mean systolic and diastolic blood pressure at screening ≥160 and ≥100 mm Hg, respectively, or on non-selective beta-blockers and/or alpha-methyl dopa, or hypertension requiring more than two anti-hypertension medications
3. Obesity (body mass index ≥30 kg/m^2)
4. Use of anti-inflammatory or immunomodulatory medication, such as non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, or other immunosuppressants, within one week of receiving ultrasound delivery
5. Use of anticoagulant drugs (e.g., coumadin, direct oral anticoagulants) or antiplatelet drugs (e.g., aspirin, clopidogrel) within one week of receiving ultrasound delivery
6. Pregnancy, breastfeeding, or planning to become pregnant during the study
7. Active bacterial or viral infection; febrile illness within 2 weeks
8. Known allergic reactions to ultrasound gel

9. Treatment with another investigational drug or other intervention within 1 month
10. Any vaccination received within 1 month
11. Current smoker or nicotine use within 2 weeks
12. Use of recreational drugs within 2 weeks
13. History of arrythmia (e.g., clinically significant bradycardia, atrial flutter, atrial fibrillation, ventricular arrythmias)
14. History of deep vein thrombosis or pulmonary embolism
15. History of bleeding disorder
16. History of seizure
17. History of unilateral or bilateral vagotomy
18. Participants with an implantable medical device, such as pacemaker, hearing aid implant, or any implanted electronic device
19. Surgery or traumatic injury (e.g., visceral injury, cerebral injury) in the past 3 months
20. Prior surgery on thyroid or parathyreoid glands, esophagus, stomach, or spleen
21. Participant considered by the Investigator, after reviewing medical and psychiatric history, physical examination, and laboratory evaluations, to be unsuitable for any other reason that may either place the patient at increased risk during participation or interfere with the interpretation of the study outcomes.

5.3 Justification for Study Population

For the purposes of this pilot study, we want a population that is immunologically mature and without extensive age-associated immunological decline. Pregnancy is considered as exclusion criteria given that current guidelines recommend to limit MI less than 1 during obstetric diagnostic ultrasound imaging (21). The inclusion criteria are broad to allow for timely completion of enrollment with good representation of the general population without known medical conditions. Blood pressure will be measured three times in a seated position and averaged. Relevant hypertension in exclusion criteria is defined as grade 2 or higher hypertension per 2020 International Society of Hypertension Global Hypertension Practice Guidelines (22) or when treated with non-selective beta-blockers and/or alpha-methyl dopa, hypertension requiring more than two antihypertension medications. Diabetes mellitus is defined as fasting blood sugar ≥126 mg/dL or hemoglobin A1c ≥6.5%, or use of diabetic medication (23) or if self-reported. The estimated glomerular filtration rate is calculated using the 2021 Chronic Kidney Disease Epidemiology Collaboration creatinine equation (24). Participants who get vaccinated or develop an active bacterial or viral infection after study enrollment would have to wait a minimum of 4 weeks before an ultrasound stimulation can be delivered. This 1-month time period should allow for any restoration of the immune system after vaccination or infection and, therefore, dissipate their impact on outcome measures after ultrasound stimulation.

5.4 Lifestyle Considerations

During this study, participants are asked to:

- Abstain from alcohol for 24 hours before the start of each visit
- Participants who have used nicotine products in the past will be instructed that use of nicotine-containing products (including nicotine patches) will not be permitted while they are in the trial
- Abstain from strenuous exercise for 24 hours before each blood collection
- Abstain from NSAIDs, if any taken, before the start of each visit up to each blood collection based on current NSAIDs discontinuation recommendations (22).

If subjects become ill or require new medications or treatments during the course of the trial, the nature of the condition will be examined on a case-by-case basis to determine continued eligibility. If the illness or condition is not deemed to be linked to an effect of the ultrasound treatment and does not produce long term changes in the subject's health status, future visits will be postponed until the condition resolves. Otherwise, the subject will be withdrawn from the trial.

5.5 Screen Failures

Screen failures are defined as participants who consent to participate in the clinical trial but are not subsequently entered in 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 (for NIH studies) and to respond to queries from regulatory authorities. Minimal information includes demography, screen failure details, eligibility criteria, and any serious adverse event (SAE). We do not anticipate many screen failures for this study since our inclusion criteria is broad and the major restrictions are age range, general good health without known medical conditions, no nicotine use, and not pregnant. Information regarding these criteria will be communicated to potential subjects during consent acquisition and further screening should generally not be necessary.

5.6 Strategies for Recruitment and Retention

The general strategy for timely recruitment will focus on spreading word within the UVA community (graduate students, personnel, patient areas) as a first strategy. We will generate e-mail and social media announcements and flyers to post around UVA grounds. If we fail to reach target enrollment or believe the process is moving too slowly, we will extend the reach of our recruitment materials via social media and communication with other nearby medical centers/hospitals. As a retention strategy, participants will be compensated for each blood draw session after screening they complete at a rate of $100/session. We will also encourage retention via maintaining contact with the study participants. Thus, the total compensation would come to $400 for participants that complete all study procedures. We believe this will encourage healthy volunteers to consider enrolling in and completing the study without providing undue financial incentive.

- The anticipated accrual rate is 5 patients per month
- The single-center study will be conducted at the UVA Kidney Center Clinic in Charlottesville, VA
- Sources of participants and recruitment venues will include outpatient clinics, UVA campus, and general public
- Potential participants will be invited via written invitations put out at public places, local flyers, and via social media channels
- Measures to increase participant retention will include maintaining contact with the participants via phone and/or email with the aim to ensure participants are kept well-informed of the study's progress, and to identify barriers encountered throughout the study to organize strategies to enhance retention
- We do not have specific strategies for recruitment of women or underrepresented minority populations since this is a proof-of-concept pilot study with broad enrollment criteria. We will accept all willing participants who meet inclusion criteria and determine the gender/societal distribution upon study conclusion.

6 STUDY INTERVENTION

6.1 Description of Study Interventions

The interventions performed in this study are designed to test the impacts of different ultrasound stimulation procedures on the inflammatory capacity of circulating immune cells. This consists of two main approaches: 1) varying ultrasound intensity (MI = sham, 0.6, 1.0, 1.4, and 1.8,) and 2) varying the physical location targeted by the ultrasound stimulation (spleen-targeted vs. cervical vagus-targeted). The ultrasound machine used in this study is commercially available and the ultrasound transducer used in this study is paired with a clinically approved diagnostic scanner within current FDA regulatory limits of MI $\leq$1.9 and spatial-peak temporal-average intensity (ISPTA) <720 mW/m^2. Depending on the imaging depth used, the focal depth and power setting will be adjusted to achieve the target MI at the desired tissue location. The manufacturer of the transducer also assures that the instantaneous skin contact surface does not heat up and that skin contact materials are skin compatible and that these surfaces can be adequately cleaned and sterilized by one of any already existing protocols in use at UVA Health for transducer-based scanning.

Description of device and parameters:

- Device model: Acuson Sequoia 512
- Description of each component: Clinical Acuson Sequoia 512 ultrasound system (Siemens Healthcare Diagnostics, Inc, Tarrytown, NY) with a paired curvilinear Acuson 4C1 transducer intended for spleen ultrasound and a linear Acuson 15L8 transducer intendent for neck ultrasound
- Device settings and programming:
  - for spleen ultrasound: frequency 3 MHz; burst MIs 0.6–1.8 (further description provided in 4.1); burst duration: 4 sec; burst application repetition frequency 10 sec; duration of exposure: 12 min
  - sham ultrasound will consist of turning the US system on, locating the stimulation area, and holding the US transducer in place for 12 minutes without delivery of higher-intensity pulses.
  - for neck ultrasound: frequency 10 MHz; burst MI 1.4; burst duration: 4 sec; burst repetition frequency 10 sec; duration of exposure: 12 min
- Technically, the Sequoia is no longer supported commercially – but the settings being used were part of an FDA compliant and approved software release during the commercial life of the device. No modifications are being made to the device and the machine's user interface will not allow, under any circumstance, for the settings to be modified outside of FDA compliant conditions. The use is technically "off label" since the settings of interest were intended for use in combination with ultrasound contrast agent, and no contrast agent is being used in our protocol. However, it can be scientifically argued that use of these ultrasound settings without contrast agent represents a lower risk than when used in conjunction with a contrast agent, since those agents can contribute to tissue damage and injury. This ultrasound device has been used in the clinic for many years and its safety has been well established. We are not testing the safety of the device in this trial, but rather the efficacy of the ultrasound protocol for inducing an anti-inflammatory state. While we do not anticipate this treatment protocol will cause any harm to the participants, we will verify its safety by monitoring the physical and mental state of participants throughout the trial.

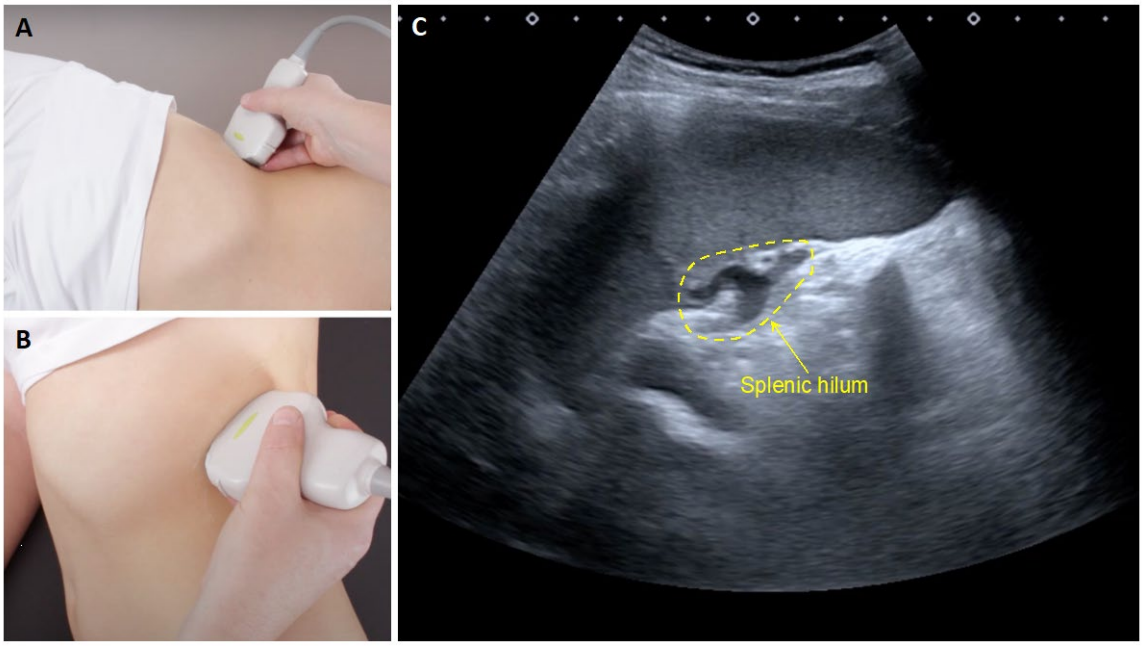
6.2 Dosing and Administration

As stated above, different doses (i.e., ultrasound intensities) will be delivered in one of the main groups. All doses chosen for this study were selected to cover a range of MI but is below the FDA established maximum MI threshold allowed for ultrasound diagnostic imaging.

Procedure for ultrasound delivery:
Following screening and enrollment, participants will be scheduled in non-fasting state for the first visit to collect blood for laboratory testing to confirm good health, perform baseline flow cytometry analysis, and set up ex vivo stimulation to assess cytokine production. Participants will be assigned to the study groups while attempting to keep equal representation of age, sex, and ethnicity in each, but we will aim to complete enrollment of Group 1 first (Group 1; n = 20: dose response; Group 2, n = 10: site of stimulation spleen vs. neck). Subgroup 1 of Group 1 (n = 10) will receive MI doses of 0.6 and 1.4. Subgroup 2 of Group 1 (n = 10) will receive MIs of 1.0 and 1.8. Both subgroups will be divided in respect to the sequence in which the MI doses are delivered (higher dose first or lower dose first). Subgroup 3 will receive the sham treatment first and the 1.4 MI dose second. Optional spot urine samples will be collected prior to ultrasound stimulation to measure AKI biomarkers. No study-specific medications will be administered in this study. There is no strict timing schedule or requirements aside from the 14-day minimal interval between ultrasound stimulations, so it is not possible for doses to be delayed or missed. For Group 2, 5 participants will receive the first ultrasound stimulation targeting the spleen and the second targeting the cervical vagus in the neck, both at MI of 1.4, while in the other 5 participants the sequence will be flipped (first targeting the neck, then the spleen).

To expose the splenic region of the abdomen, the participants will be asked to lie in the right lateral recumbent position (Figure 1A) or to sit upright (Figure 1B; depending on the ultrasonographic visibility of the spleen), with both arms above their head aiming. Second, ultrasound gel will be applied to the left high flank region and ultrasound will be performed using a standardized intercostal imaging approach to detect the spleen and assess its dimensions in the B mode setting (23). Third, the ultrasound probe will be positioned targeting the splenic hilum as the focal landmark for ultrasound delivery (Figure 1C) and an image will be electronically stored to document the position and the device settings.

Figure 1: Ultrasound of the splenic hilum



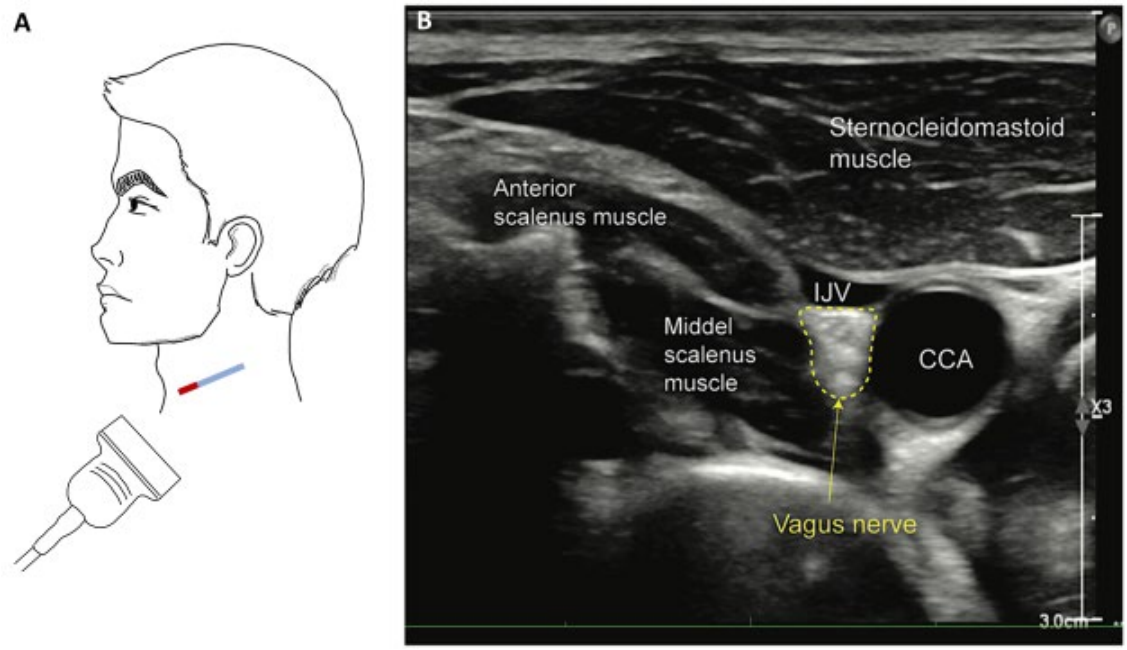
Adapted from Amboss.com/de (24). Panels A and B show two representative participant positions to examine the spleen with ultrasound, respectively. Panel C shows the corresponding B-mode ultrasound image of the spleen and the splenic hilum (yellow dashed circle and arrow) in a healthy individual.

Throughout the ultrasound procedure, the ultrasonographer will check the location of the probe using the imaging mode to ensure that the spleen hilus remains the target throughout the ultrasound stimulation and participants will be asked to breath shallowly to limit movement of the spleen. Approximately 30 minutes after the ultrasound stimulation, the participants will be asked to provide a second spot urine sample for AKI biomarkers. Participants will be scheduled for another visit 1 to 2 days after the first ultrasound stimulation to collect the third spot urine sample for AKI biomarkers and draw blood for flow cytometry analysis and stimulation of blood cells to assess cytokine production. In addition, B mode ultrasonography will be repeated to measure the spleen diameters. No additional ultrasound stimulation will be delivered. A subject experience survey will be performed prior to blood collection to determine if they are experiencing any noticeable effects of the stimulation. Once the survey and blood collection are complete, the next stimulation visit will be scheduled and the participant reminded that they can contact us at any point if they experience any changes that may be related to the procedures. Surveying and scheduling will be performed the same for all groups.

For cervical vagus-targeted ultrasound, first the participants will be asked to lie down in supine position with the neck hyperextended. Second, ultrasound gel and the ultrasound probe will be placed on the left side of the neck in the transverse plane (Figure 2A) using the B mode image setting (25). Third, the ultrasound probe will be positioned over the major neurovascular bundle (region of interest: lateral margins of the anterior cervical region beneath the sternocleidomastoidei muscles) as the focal landmark for ultrasound delivery (Figure 2B) and an image will be electronically stored to document the position and the device settings. Ultrasound stimulation will be applied with the same procedure (pulse repetition and duration) as stated previously. Before each ultrasound delivery, the ultrasonographer will check the location of the probe using the imaging mode to ensure

that the major neurovascular bundle remains the target throughout the ultrasound stimulation. In addition, the ultrasonographer will assess the dimensions of the spleen in the B mode setting. Approximately 30 minutes after the ultrasound stimulation, the participants will be asked to provide a second spot urine sample for AKI biomarkers.

Figure 2: Ultrasound of the cervical vagus nerve



Adapted from Ottaviani et al. (26). Panel A shows a schematic representation of the left cervical region highlighting the conventional placement of the ultrasound probe to visualize the major neurovascular bundle. Panel B shows the corresponding B-mode ultrasound image including the central carotid artery, inferior jugular vein, vagus nerve (yellow dashed circle and arrow), and nearby muscles. CCA, central carotid artery; IJV, inferior jugular vein.

Participants will be scheduled for the last visit 1 to 2 days after the second ultrasound stimulation to provide another spot urine sample for AKI biomarkers and draw blood for flow cytometry analysis and stimulation of blood cells to assess cytokine production. Furthermore, B mode ultrasonography will be repeated to measure the spleen diameters. No additional ultrasound stimulation will be delivered. A subject experience survey will be performed as above, but no additional visit scheduling will be required at this point. 30 days after the last ultrasound stimulation, all participants will be recontacted via phone to report any new discomforts or health issues that developed within the last month.

6.2.1 Dose Escalation and Regimen

While we will deliver different doses of ultrasound stimulation in this study, it is not technically a dose escalation study since there is no drug regimen. Each visit and/or treatment represents an independent point for end point data collection, so there is not a longitudinal element for determining the effect of a treatment administered over time.

6.2.2 Dose Modifications and Delays

For Group 2, we will monitor data from Group 1 and determine if we still consider an MI of 1.4 as the optimal stimulation for spleen vs. neck targeted US. If the data indicate that a

lower dose would be efficacious, we will evaluate the option of altering this element of Group 2.

6.3 Preparation/Handling/Storage/Accountability

6.3.1 Acquisition and Accountability

The devices have already been obtained for prior research by the Hossack lab at UVA. In the event that new devices are required, these will be obtained from the same manufacturer, Sequoia Ultrasound devices will be programmed by Hossack lab personnel and checked prior to use on subjects for proper functioning. Devices will be stored in secured locations either within the Okusa lab or Hossack lab while not being used for treatments in the study.

6.3.2 Formulation, Appearance, Packaging, and Labeling

The Clinical Acuson Sequoia 512 ultrasound system is manufactured by Siemens Healthcare Diagnostics, Inc, Tarrytown, NY, and is intended for diagnostic ultrasound imaging. It will be programmed by the Hossack lab. The paired Acuson 4C1 and 15L8 probes are intended for diagnostic ultrasound imaging.

6.3.3 Product Storage and Stability

As stated, the devices will be stored in secured lab locations while not in use and tested for proper function prior to use. There are no storage requirements for stability of this device.

6.3.4 Preparation

Device parameters will be programmed and preset by Hossack lab personnel. Parameters will include frequency (MHz), burst MI, burst duration (seconds), burst application interval (seconds) and total ensonification duration (minutes – by watch or timer). The parameters will be double-checked by the ultrasonographer and documented by electronically storing the images prior to the initiation of ultrasound stimulation.

6.4 Study Intervention Compliance

The only protocol requirements for compliance/adherence is the time allowed between ultrasound stimulations. This will be accomplished when scheduling subjects for treatment by verifying the date of their previous ultrasound stimulation and ensuring there is at least a 14-day period between ultrasound stimulation sessions.

6.5 Registration, Randomization and Blinding

Registration: All participants must sign the consent form prior to determination of eligibility for this study. Registration will occur following verification of eligibility by the treating physician. Participants who are consented and accrued to the study should be registered in OnCore in accordance with the Clinical Trial Management System Policy via the UVA OnCore Resources link in Oncore. General guidelines are available in the OnCore User Manual and Data Entry Guide. Participants should receive their first study treatment within 2 months of registration.

Randomization: Participants 1-30 will be assigned to Group 1 (Schema 1.2 on p. 10-11). Once the Group 1 enrollment target is met, participants 31-40 will be assigned to Group 2. Randomization will be used to assign participants to subgroups within Group 1 (1:1) and to an order of treatments within Group 1 subgroups and Group 2 (1:1). Randomization will be stratified by gender and race.

Blinding: This study does not involve any blinding or masking procedures. Subjects will be told which treatment they are receiving.

6.5.1 Emergency Unblinding Procedures
Not applicable.

6.6 Concomitant Therapy
Not applicable.

6.6.1 Rescue Medicine/Supportive Care
Not applicable.

7 STUDY CLOSURE, STUDY INTERVENTION DISCONTINUATION AND PARTICIPANT DISCONTINUATION OR WITHDRAWAL

7.1 Study Discontinuation and Closure
This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. Written notification, documenting the reason for study suspension or termination, will be provided by the suspending or terminating party to regulatory authorities. If the study is prematurely terminated or suspended, the Principal Investigator (PI) will promptly inform the Institutional Review Board (IRB) and sponsor and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule.

Circumstances that would warrant termination or suspension include, but are not limited to

- Determination of unexpected, significant, or unacceptable risk to participants
- Change in funding status

Study may resume once concerns about safety, protocol compliance, and data quality are addressed, and satisfy the sponsor and IRB.

7.2 Participant Discontinuation/Withdrawal
Participants are free to withdraw from participation in the study at any time upon request. A participant's study treatment would be discontinued for the following reasons:

- Pregnancy
- If any clinical 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
- If the participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation
- Participant decision to withdraw from study treatment and/or the study for any reason
- Loss to follow-up
- A patient's substantial inability to follow commends during ultrasound (e.g., inability to remain relatively still for several minutes) or non-compliance (e.g., multiple missed visits, inability and/or unwillingness to adhere to scheduled appointments)
- Initiation of prohibited intervention or medication.

The reason for participant discontinuation or withdrawal from study treatment will be recorded on the case report form. Participants who sign the informed consent form and subsequently withdraw, or are withdrawn or discontinued from the study, will be replaced. Participants that withdraw from the study (not only from study treatment, but all study follow-up) will not be contacted for any further study visits.

7.3 Dose-Limiting Toxicity
Not applicable.

7.4 Procedures for Discontinuation of Study Intervention
Discontinuation from ultrasound stimulation does not mean discontinuation from the study, and remaining study procedures should be completed as indicated by the study protocol. If a clinically significant finding is identified (including, but not limited to changes from baseline) after enrollment, the investigator or qualified designee will determine if any change in participant management is needed. Any new clinically relevant finding will be reported as an AE.

The data to be collected and procedures to be completed at the time of study intervention discontinuation are included in the schedule of assessments in *section 13.1*.

7.5 Lost to Follow-Up
A participant will be considered lost to follow-up if he or she fails to return for any scheduled visits and is unable to be contacted by the study site staff.
The following actions must be taken if a participant fails to return to the clinic for a required study visit:

- The site will attempt to contact the participant and reschedule the missed visit and ascertain if the participant wishes to and/or should continue in the study.
- Before a participant is deemed lost to follow-up, the investigator or designee will make every effort to regain contact with the participant including regular e-mails and/or phone calls until the participant either responds or the study period is closed.
- These contact attempts should be documented in the 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 Clinical Assessments
For screening purposes, patients will be asked to affirm that they are in good health with no acute or chronic medical conditions or ongoing use of anti-inflammatory medications. This affirmation will be confirmed by a review of the subject's prior medical history and medical record review by a licensed clinician associated with the study and by additional lab tests.

8.1.1 Physical Exam

- **Physical examination** A physical examination will be performed during subject screening to further verify good health and study eligibility. A physical exam will also be performed prior to the final blood draw to verify no new conditions have arisen in the subjects during the course of the study and

ultrasound treatments. These will be completed by a trained physician. Height and weight will also be recorded at the time of physical examination.

- **Vital signs** before and after each ultrasound stimulation (temperature [°C], pulse [beats per min], respiratory rate [breaths per min], systolic and diastolic blood pressure [both in mm Hg]).

8.1.2 Clinical Laboratory Assessments

Laboratory assessments required for screening purposes will include i) a urine pregnancy testing (for women of childbearing potential will require urine pregnancy testing prior to enrollment and, ii) assessment of complete blood count (one EDTA 3.0 ml tube) and serum creatinine, blood urea nitrogen, blood glucose, AST, and ALT (one SST 3.5 ml tube).

8.1.3 Imaging

Ultrasound imaging will be performed during target area acquisition prior to pulsed ultrasound delivery during treatment visits. This is accomplished by placing ultrasound gel on the target area and moving the ultrasound probe over the gel until the tissue target location has been identified. This will be performed by UVA personnel trained in the use of ultrasound.

8.1.4 Assessment of Adverse Events

Each participant will be evaluated by a licensed clinician at each study visit. The NCI Common Terminology Criteria for Adverse Events (CTCAE) version 5 (if applicable) will be used for the characterization and grading of adverse events.

8.1.5 Other Clinical Assessments

Demographics (race/ethnicity) and medical history, smoking status (current, former, never), hypertension, diabetes mellitus, medication (maintenance therapy, vitamins, supplements).

8.2 Research Specimen Collection

8.2.1 Tissue

Not applicable.

8.2.2 Research Blood

~12 ml of blood will be drawn each during the first stimulation visit and both post-stimulation visits using EDTA blood collection tubes. Whole blood will be processed in the Okusa laboratory by centrifugation to pellet cells followed by chemical red blood cell lysis. After RBC lysis, the white blood cells will be pelleted again via centrifugation, counted, and separated into portions for flow cytometry analysis and plating for in vitro stimulation assays. Analyses will also include a complete blood count with differential measured in the UVA central laboratory.

8.2.3 Research Urine

Participants will be asked to provide optional spot urine samples immediately prior to ultrasound stimulation, and ~30 minutes and 24–48 hours after ultrasound stimulation. Urine will be processed by centrifugation. Supernatants will be flash frozen, stored at -80°C, and thawed immediately prior to analysis.

Biomarkers of interest will include neutrophil gelatinase–associated lipocalin (NGAL) and the product of tissue inhibitor of metalloproteinase-2 and insulin-like growth factor binding protein ([TIMP-2]•[IGFBP7]). NGAL is the most extensively investigated biomarker for

diagnosing AKI estimating severity of AKI, and it has been proposed as a distal tubule damage biomarker (27, 28). TIMP-2 and IGFBP7 are 21- and 25-kDa proteins that are secreted in the early phase of tubular damage (e.g., in the context of ischemia (29) or sepsis (30)) by the tubular epithelial cells and are implicated in G1 cell cycle arrest, which is thought to be a part of the protective mechanisms cells use when exposed to stress (31). In contrast to tubular damage biomarkers (e.g., NGAL), TIMP-2 and IGFBP7 can be released in response to non-injurious, noxious stimuli (29). For this reason, both biomarkers are often referred to as kidney stress biomarkers (32). [TIMP-2]•[IGFBP7] have been incorporated in the first diagnostic test for AKI approved by the FDA (Nephrocheck, Astute Medical, San Diego, CA, USA).

8.2.4 Stool
Not applicable.

8.3 Correlative Studies
White blood cells will be used for flow cytometry and cytokine production assays to be performed in the Okusa lab. A panel of fluorescently tagged antibodies will be developed to identify individual immune cell populations by surface marker expression patterns. Cytokine production analysis will be accomplished by plating $2x10^5$ – $1x10^6$ cells per well and adding inflammatory compounds, such as lipopolysaccharide (LPS), to stimulate cytokine production. After a 24-hour stimulation period, supernatants will be collected from the culture wells and stored at -20 degrees Celsius for future use in Luminex assays to determine the concentration of specific cytokines. Any leftover specimen will be discarded.

8.4 Participant Reported Outcomes
At post-treatment collection visits, subjects will be surveyed to ensure they are not experiencing any unanticipated discomfort or changes in mood or sensation. This will be a simple binary response ("yes" there are noticeable changes post-treatment or "no" there are no discernable changes). If subjects respond that they are experiencing any divergences from normal day-to-day life then they will be asked for additional information in the form of a description of their experience. At enrollment and the baseline visit, subjects will also be informed/reminded that they can send feedback and concerns to study personnel at any time via e-mail and be provided with contact information. 30 +/- 2 days after the last ultrasound stimulation, all participants will be recontacted via phone to report any new discomforts or health issues that may have developed within the last month.

9 DATA AND SAFETY MONITORING PLAN
9.1 Adverse Events and Serious Adverse Events
9.1.1 Definition of Adverse Events (AE)
Adverse event means any untoward medical occurrence associated with the use of an intervention in humans, whether or not considered intervention-related (21 CFR 312.32 (a)).

9.1.2 Definition of Serious Adverse Events (SAE)
An adverse event (AE) or suspected adverse reaction is considered "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 or substantial disruption of the ability to conduct normal life functions, or
- a congenital anomaly/birth defect.

Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse. A planned medical or surgical procedure is not, in itself, an SAE

9.1.3 Definition of a Suspected Adverse Reaction

Any adverse event for which there is a reasonable possibility that the treatment caused the adverse event. Suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by the treatment.

9.1.4 Classification of an Adverse Event

9.1.4.1 Severity of Event

All AEs will be assessed by the study clinician using a protocol defined grading system. AEs will be graded and classified as follows:

- **Grade 1, Neutral** – an event will be considered neutral if there is a noticeable change that may reasonably stem from the treatment protocol, but this change has no impact on the subject's daily life or enjoyment of activities.
- **Grade 2, Mild** – an event will be classified as mild if the detected change has some impact on the subject's daily life, but does not impact overall health or prevent performance of tasks or activities.
- **Grade 3, Significant** – an event will be classified as significant if it results in disruption of a subject's daily life or good-health status.
- **Grade 4, Severe** – an event will be classified as severe if it is considered life-threatening or debilitating to the subject and prevents functioning in daily-life or is deemed a concern to long-term health or physical capability.

9.1.4.2 Relationship to Study Intervention

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

- **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 (dechallenge) should be clinically plausible. The event must be pharmacologically or phenomenologically definitive, with use of a satisfactory rechallenge 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 (dechallenge). Rechallenge information is not required to fulfill this definition.

- **Possibly 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.

9.1.4.3 Expectedness

The research team will be responsible for determining whether an adverse event (AE) is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the study intervention.

9.1.5 Abnormal Laboratory Values

Women with a positive pregnancy testing will be excluded from enrollment or from further participation. Subjects with abnormal laboratory values as the criteria below will be excluded from enrollment or from further participation:

System	Abnormal Laboratory Value
Hematological	
White blood cell count	< 4.00 k/μL
Platelets	< 150 k/μL
Hemoglobin	< 11.0 g/dL
Renal	
Estimated glomerular filtration rate	< 60 mL/min/1.73 m^2
Blood urea nitrogen	> 1.5 x ULN
Hepatic	
AST and ALT	> 2.5 x ULN
Other	
Fasting blood sugar	≥ 126 mg/dL
Hemoglobin A1c	≥ 6.5%

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

The occurrence of an adverse event (AE) or serious adverse event (SAE) may come to the attention of study personnel during study visits and interviews of a study participant presenting for medical care, or upon review by a study monitor.

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

Any medical condition (including a laboratory abnormality) 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 baseline medical condition worsens 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.
Study clinicians will record all reportable events with start dates occurring any time after informed consent is obtained until 30 days after the last day of study treatment. At each study visit, the investigator will inquire about the occurrence of AE/SAEs since the last visit. Events will be followed for outcome information until resolution or stabilization.

9.1.7 Adverse Event Reporting

AEs must be recorded into the University of Virginia OnCore and case report forms per the following guidelines (Table 2).

Table 1

Table C: Low Risk Studies
Reporting requirements for AEs that that occur within 30 days of the last protocol specified treatment/intervention

	Grade 1-2	Grade 1-2		Grade 3	Grade 4
	Expected	Unexpected		Expected or Unexpected	Expected or Unexpected
		Without hospitalization	With hospitalization		
Unrelated Unlikely	Not required	Not required	Not required	Not required	15 days
Possible Probable Definite	Not required	Not required	30 days	15 days	(24-hrs)* 15 days

*Enter into UVA OnCore database within 24 hours if unexpected and definitely related to protocol specified treatment
Hospitalization defined as an inpatient hospital stay or prolongation of a hospital stay equal to or greater than 24 hours

9.1.8 Serious Adverse Event Reporting

The study clinician will report to the sponsor any serious adverse event, whether or not considered study intervention related, including those listed in the protocol or investigator brochure and must include an assessment of whether there is a reasonable possibility that the study intervention caused the event.

All serious adverse events (SAEs) will be followed until satisfactory resolution or until the site investigator deems the event to be chronic or the participant is stable.

- Internal Event Resulting in death that is deemed DEFINITELY related to (caused by) study participation
    - Report to the UVA IRB-HSR within 24 hours. Report within 24 hours using IRB Online and a phone call.

- Internal, Serious, Unexpected, Probably or Possibly Related
    - Report to the UVA IRB-HSR within 7 days from the time the study team receives knowledge of the event. Timeline includes submission of signed hardcopy of AE form. Report using IRB Online.

9.2 Unanticipated Adverse Device Effects

9.2.1 Definition of Unanticipated Device Effect (UADE)

Unanticipated adverse device effect is any serious adverse effect on health or safety or any life-threatening problem or death caused by, or associated with a device, if that effect, problem, or death was not previously identified in nature, severity, or degree of incidence in the investigational plan or application (including a supplementary plan or application), or any other unanticipated serious problem associated with a device that relates to the rights, safety, or welfare of subjects (21 CFR 812.3(s)).

9.2.2 Reporting of UADE

Single Site Studies (*UVA-IRB-HSR is the IRB of record)*

- Report to the UVA IRB-HSR within 10 calendar days of the study team receiving knowledge of the event. Report using IRB Online.

9.3 Reporting Events to Participants

If there is any new information relevant to the participant's willingness to continue to participate in the study, such as if there is substantial reason to believe new risks of the study treatment have been identified that were not included on the consent form that the participant originally signed, the study team will contact the participant to discuss this information. If the participant is still receiving study treatment, the study team will present the participant with an updated consent form and confirm that he or she wants to continue receiving study treatment. The Sponsor will determine whether new risks are applicable to participants who are in follow-up, whether participants need to be notified, and whether re-consenting is required.

9.4 Events of Special Interest

Not applicable.

9.5 Reporting of Pregnancy

Not applicable.

9.6 Unanticipated Problems

9.6.1 Definition of Unanticipated Problems (UP)

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

- Unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol-related documents, such as the Institutional Review Board (IRB)-approved research protocol and informed

consent document; and (b) the characteristics of the participant population being studied;
- Related or possibly related to participation in the research ("possibly related" means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- Suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.
- This definition could include an unanticipated adverse device effect. Please refer to *section 9.2.1* for the definition of an unanticipated adverse device effect.

9.6.2 Unanticipated Problem Reporting
- Report UPs that are not adverse events, protocol deviations, or data breaches (see *section 9.7* for reporting for data breaches) to the UVA IRB-HSR within 7 calendar days from the time the study team receives knowledge of the event. Report using the Unanticipated Problem Report form.
- Report UPs that are SAEs in accordance with the guidelines for SAE reporting.

9.6.3 Reporting Unanticipated Problems to Participants
If during the course of the study there is an unanticipated problem that affects current or past participants, affected participants will be contacted if needed.

9.7 Data Breach
9.7.1 Definition of Data Breach
An unauthorized acquisition, access, or use of protected health information (PHI) that compromises the security or privacy of such information.

9.7.2 Reporting a Data Breach
- Report to the UVA Corporate Compliance and Privacy Office as soon as possible and no later than 24 hours from the time the incident is identified. Report by telephone.

- Report to InfoSec if the breach involves electronic data. Report as soon as possible and no later than 24 hours from the time the incident is identified. Refer to the following for details: *http://security.virginia.edu/report-information-security-incident*.

- Report to UVA police if the breach includes such things as stolen computers. Report by telephone.

9.8 Protocol Deviation
9.8.1 Definition of Protocol Deviation
A protocol deviation is defined as any change, deviation, or departure from the study design or procedures of a research project that is NOT approved by the institution's IRB prior to its initiation or implementation, OR deviation from standard operating procedures, Good Clinical Practices (GCPs), federal, state or local regulations. Protocol violations may or may not be under the control of the study team or UVA staff. These protocol violations may be major or minor violations.

9.8.2 Reporting of a Protocol Deviation

It is the responsibility of the site investigator to use continuous vigilance to identify and report deviations. All deviations must be addressed in study source documents, reported to the Data Coordinating Center.

- Report to the UVA IRB-HSR major deviations within 7 calendar days from the time the study team received knowledge of the event. Report using the Protocol Deviation and Protocol Exception Reporting Form.

- For minor deviations, please reference the IRB-HSR for tips for recording minor deviations

9.9 Participant Withdrawals/Dropouts Prior to Study Completion

Participants who withdraw consent and those dropping out of the study secondary to an AE will be reported to the IRB of record according to IRB guidelines.

10 STATISTICAL CONSIDERATIONS

The goal of this pilot study is to generate proof-of-concept data as well as identify stimulation parameters that are most effective. Thus, we are not using blinding, arm switching, or other complex study designs that would require additional statistical consideration. The proposed statistical comparisons and data analyses are described in details in the sections below. The proposed tests we are using are appropriate for the type of comparisons we are performing and use of participant immediate baseline values as a normalization method will simplify the group analyses, hopefully limiting the subject-to-subject variability.

10.1 Statistical Hypotheses

- Primary Efficacy Endpoint(s):

Null Hypothesis 1: ultrasound treatment does not alter the ability of immune cells to produce cytokines.
Hypothesis 1: ultrasound treatment will reduce the capacity of immune cells to produce inflammatory cytokines
This will be tested by comparing subject immediate baseline data to ultrasound treatment data using 1-way ANOVA tests with all final data from each subject.

Null hypothesis 2: ultrasound treatment does not alter the ability of immune cells to produce cytokines.
Hypothesis 2: There is a ultrasound “dose threshold” that determines the efficacy of ultrasound to limit cytokine production by immune cells.
This will be tested as a dose response effect using all data gathered by the end of the trial.

Null Hypothesis 3: Splenic ultrasound stimulation will have greater efficacy for reducing cytokine production from immune cells than ultrasound targeted to the cervical vagus nerve.
Hypothesis 3: Splenic and cervical vagus-targeted ultrasound will be equally effective at inducing reductions in cytokine production from immune cells.

This will be tested using 1-way ANOVA tests to compare cytokine production between immediate baseline, spleen-targeted, and cervical vagus-targeted data for each subject and across all subjects once all data has been collected.

- Secondary Efficacy Endpoint(s):

Null Hypothesis: ultrasound stimulation has no impact on immune cell distribution in the blood.
Hypothesis: ultrasound stimulation will produce significant changes in the abundance of circulating immune cell populations in the blood.
This will be tested by comparing immediate baseline data (baseline 1 or baseline 2) from each subject to their respective stimulation samples using 1-way ANOVA tests once all data from a subject has been gathered.

10.2 Sample Size Determination

This is a pilot study designed to gather descriptive data that helps characterize the impacts of ultrasound and its potential to reduce inflammatory responses. Given that our comparisons are well-defined across groups and we will be mostly using baseline data as a comparator to treatment data from the same individuals to account for within-subject variation, the data will be considered as independent and analyzed using the traditional methods. Nevertheless, the within-subject correlation will be assessed (see Section 10.4).

We will be collecting 2 types of outcome measures: cytokine concentrations in supernatants and count/percentage of immune cells in blood samples. Each subject's cells will be tested in replicates and the means of the replicate values will be used for evaluating individual subject's responses. The means of each subject's replicate samples will be combined with that from the other subjects of the same group for assessing differences between treatment conditions. If there is too much variation in the absolute values from each subject, we will normalize each measure to its respective subject's baseline as a surrogate test statistic for assessing differences between treatment conditions.

For testing the impact of ultrasound dose on cytokine production and immune cell abundance, the null hypothesis is that there is no difference between immediate baseline and stimulation. We hypothesize that increasing ultrasound MIs will yield increasing anti-inflammation. For testing the efficacy of spleen vs cervical vagus targeting, the null hypothesis is that they will yield differential efficacy for inducing anti-inflammation. We hypothesize that both stimulation sites should produce similar anti-inflammation effects.

For each test, we are using a type I error rate of $\alpha = 0.05$ and a power of 80%. Since we plan to test a broad panel of cytokines and have not finalized our selection of the individual cytokines to include, it is difficult to predict the means for each. However, using our experience with mice and the similarity of much of this analysis to our mouse studies, we are assuming a variance of 20% for our calculations. Thus, using an online sample size calculator for determining ANOVA sample sizes, (*https://homepage.univie.ac.at/robin.ristl/samplesize.php?test=anova*), we have determined that a sample size of 10 subjects per group should be sufficient for this pilot analysis and the identification of variations of interest within the collected data.

Due to the relative simplicity of our treatment protocol, data collection, and smaller sample size for recruitment, we do not anticipate significant impacts from dropout, withdrawal, or missing data on our study power.

10.2.1 Randomization and Measures to Minimize Bias

This study does not include randomization or blinding elements and measurements will all be made by analyzing samples with appropriate equipment. Immune cell population data will be gathered using flow cytometers and cytokine data will be gathered using similar technology (Luminex MagPix). Thus, there is no bias in raw data recording since values will be generated based on the machines' detection and will simply be recorded and compiled by the researchers, bypassing subjectivity in the measurements. All participants and samples will be handled the same throughout the study aside from the differences in the stimulation parameters. Sample collection, processing, and analysis will all be standardized to discourage procedural variations that may impact data values. The fact that there is no separate control group and each participant will be used as their own baseline will also help prevent bias in measurement and analysis.

10.3 Populations for Analyses

Cytokine production analysis, dataset 1: each subject will have their own individual dataset of cytokine measurements that will be analyzed independently.

Cytokine production analysis, dataset 2: all subjects in from Objective 1/Group 1 will be included in the dataset to determine the effects of ultrasound stimulation intensity on inflammation.

Cytokine production analysis dataset 3: all subjects from Objective 2/Group 2 will be included to determine the efficacy of splenic- vs. cervical vagus-targeted ultrasound.

Immune cell population analysis dataset 1: each subject will have their own individual dataset of cytokine measurements that will be analyzed independently.

Immune cell population analysis dataset 2: all subjects in from Objective 1/Group 1 will be included to determine the effects of ultrasound stimulation intensity on inflammation.

Immune cell population analysis dataset 3: all subjects from Objective 2/Group 2 will be included to determine the impact of splenic- vs. cervical vagus-targeted ultrasound.

10.4 Statistical Analyses

10.4.1 General Approach

Data will be presented as individual points for each measurement and calculation performed. Each value obtained from an individual's samples will be plotted as a discrete, independent data point when displayed in graphical formats. Means, medians, and standard deviations will be calculated and presented in table format. The response will be defined as the difference between post-ultrasound intervention (stimulation 1 or stimulation 2, respectively) and immediate baseline (baseline 1 or baseline 2, respectively). The potential within-subject correlation in the response from two sets of ultrasound stimulations will be evaluated in the linear mixed effects model and quantified using the intra-class correlation (ICC). If ICC is significantly different from zero, then the clustering effect at the subject level will be accounts for using the linear mixed effects model.

Additional details regarding the statistical approach are found in the sections above and below. Briefly, all statistical tests will use $p \leq 0.05$ as the significance cut-off level unless multiple comparison testing requires this be adjusted. All tests will be two-tailed. Given our smaller sample sizes, the normality of each group will be interrogated using the Lilliefors

test. The outcome of this test will then determine the details of the subsequent analysis and tests performed. If there are points that we suspect to be outliers, we will consider using the Grubbs test to verify or assuage our suspicions and ensure the highest quality data is included in our analyses.

10.4.2 Analysis of the Primary Efficacy Endpoint(s)

For both primary endpoint objectives 1 & 2: Cytokine production levels from ex vivo supernatants will be quantified as pg/ml concentrations. Each participant will have their own individual concentration data sets. Each of the 4 blood samples from a participant (baseline 1, stimulation 1, baseline 2, stimulation 2) will be assessed for cytokine production. The cytokine concentrations will be determined by Luminex analysis. Thus, an individual's data set will consist of presenting the individual data points and the means and/or medians of the values from each sample. To compare baseline and ultrasound stimulation conditions for each participant, ANOVA or non-parametric equivalent statistical tests will be performed. In addition, the means of the ultrasound stimulation conditions will be normalized to the respective immediate baseline data from the same participant (e.g., fold change from baseline or percent of baseline). Normalized data will be compiled by experimental group to assess the magnitude of divergence from baseline for each stimulation. The experimental groups are: Objective 1 - 1) spleen, MI = 0.6; 2) spleen, MI = 1.0; 3) spleen, MI = 1.4; 4) spleen, MI = 1.8; Objective 2 - 5) spleen, MI = 1.4; 6) neck, MI = 1.4. All groups will be compared to each other in an ANOVA/non-parametric analysis and multiple comparison tests will be performed to identify where any differences lie. The appropriate tests will be chosen once we have the data and can properly assess the statistical assumptions that can be made.

10.4.3 Analysis of the Secondary Endpoint(s)

The secondary endpoint analysis is fully independent from analysis of the primary endpoint. The analyses will be performed in parallel and are meant to assess completely separate immunological characteristics of circulating immune cells. Immune cell flow cytometric data will be obtained by isolating cells from the blood samples and staining them with fluorescent antibodies to identify individual immune cell populations prior to running through a flow cytometer. The population characteristics will be presented as cell numbers per ml of blood (calculated from # of cells detected via flow cytometry and volume of blood run through the cytometer), percent of total events detected, and percent of total CD45+ cells detected. For each participant, they will have their own individual data sets, as above for the primary endpoint. Each blood sample from a participant (baseline 1, stimulation 1, baseline 2, stimulation 2) will be assessed for immune cell populations. Thus, an individual's data set will consist of presenting the individual data points and the means and/or medians of the values from each sample. To compare baseline and ultrasound stimulation conditions for each participant, ANOVA or non-parametric equivalent statistical tests will be performed. In addition, the means of the ultrasound stimulation conditions will be normalized to the respective baseline data from the same participant (e.g., fold change from baseline or percent of baseline). Normalized data will be compiled by experimental group to assess the magnitude of divergence from baseline for each treatment. The experimental groups are: Objective 1 - 1) spleen, MI = 0.6; 2) spleen, MI = 1.0; 3) spleen, MI = 1.4; 4) spleen, MI = 1.8; Objective 2 - 5) spleen, MI = 1.4; 6) neck, MI = 1.4. All groups will be compared to each other in an ANOVA/non-parametric analysis and multiple comparison tests will be performed to identify where any differences lie. The appropriate tests will be chosen once we have the data and can properly assess the statistical assumptions that can be made.

10.4.4 Safety Analyses and Safety Evaluation
The only intervention in this study is a brief, non-invasive ultrasound stimulation that we do not anticipate to have appreciable detrimental impacts on subject health or day-to-day life. Tolerability and safety will be assessed based on patient experience responses during visits and voluntary communication from subjects in the event they experience an event they are concerned about and measurement of urinary AKI biomarkers. The safety evaluation involved in this study is collection of subject experience responses and spot urine collection. Urinary biomarker levels before and after ultrasound stimulation will be compared using the Wilcoxon Signed-Rank test.

10.4.5 Baseline Descriptive Statistics
The entire study is descriptive in nature and baseline data constitutes a significant portion of the analysis datasets.

10.4.6 Planned Interim Analyses
Not applicable.

10.4.7 Sub-Group Analyses
This is a pilot trial to gather descriptive data. Sub-group implications that are present in the datasets will be used to inform future analysis and study designs, but is not a primary concern in this study.

10.4.8 Tabulation of Individual Participant Data
Individual participant data will be assessed independently for part of the analysis, but will not necessarily be tabulated.

10.4.1 Safety evaluation
The safety evaluation involved in this study is collection of subject experience responses for identification of AEs and verification of ultrasound stimulation safety and feasibility.

11 REGULATORY AND OPERATIONAL CONSIDERATIONS

11.1 Regulatory and Ethical Considerations
11.1.1 Informed Consent Document
Consent forms will be written in accord with federal regulations and will be reviewed and approved by the UVA IRB-HSR prior to use. Signed consent forms and other research records will be retained in a confidential manner.

11.1.2 Consent Procedures and Documentation
Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continues throughout the individual's study participation. Consent forms will be Institutional Review Board (IRB)-approved and the participant will be asked to read and review the document. A member of the study team will explain the research study to the participant and answer any questions that may arise. A verbal explanation will be provided in terms suited to the participant's comprehension of the purposes, procedures, and potential risks of the study and of their rights as research participants. Participants will have the opportunity to carefully review the written consent form and ask questions prior to signing. The participants should have the opportunity to discuss the study with their family or surrogates or think about it prior to agreeing to

participate. The participant will sign the informed consent document prior to any procedures being done specifically for the study. Results from procedures completed prior to consent for standard of care purposes may be used for research purposes. Participants must be informed that participation is voluntary and that they may withdraw from the study at any time, without prejudice. A copy of the informed consent document will be given to the participants for their records. The informed consent process will be conducted and documented in the source document (including the date), and the form signed, before the participant undergoes any study-specific procedures. The rights and welfare of the participants will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

11.1.3 Confidentiality and Privacy

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, and the sponsor(s) and their interventions. 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. Consents will be maintained in a confidential manner in accordance with the code of federal regulations and HIPAA. When possible, specimens will be coded with study-specific IDs (not MRN or name). No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor. All research activities will be conducted in as private a setting as possible.

The study monitor, other authorized representatives of the sponsor, representatives of the Institutional Review Board (IRB), or regulatory agencies may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records.

The study participant's contact information will be securely stored at each clinical site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IRB, Institutional policies, or sponsor requirements.

Study participant research data, which is for purposes of statistical analysis and scientific reporting, will be transmitted to and stored at the UVA School of Medicine (SOM). Individual participants and their research data will be identified by a unique study identification number. The study data entry and study management systems used by clinical sites and by UVA SOM research staff will be secured and password protected.
To further protect the privacy of study participants, the research team may apply for a Certificate of Confidentiality. 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 proceeding, 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.

11.1.4 Future Use of Stored Specimens and Data

This study does not include plans to store any subject specimens.

11.1.5 Safety Oversight

The principal investigator, Prof. Mark D. Okusa, MD, FASN, will serve as the safety monitor for this study. Any study under the purview of the University of Virginia HSR-IRB is subject to review of UVA documents. Studies are chosen for Post-approval Monitoring (PAM) either a) at random or b) requested by a study team member or any member of the IRB-HSR.

The purpose of Post-approval Monitoring audits is to ensure that documentation of clinical research studies is of the highest quality, verify protocol adherence, and ensure that all Federal and local rules concerning clinical research are being fulfilled. Post-approval monitoring is done by staff within the office of the Vice President for Research (VPR) in accordance with their Standard Operating Procedures. The conduct of an on-site review may include but is not limited to:

- requests for progress reports from investigators,
- examinations of research records, including signed informed consent documents, protocol modifications, and unexpected, serious, and/or related adverse experience reports,
- contacts with research subjects, or
- observation of the consent process and/or research procedures. Examples of when observation of the consent process could occur are:
    - Full board IRB determines during review of a project that a conflict of interest exists such that the informed consent process should be observed by a neutral party;
    - IRB is made aware of a complaint or concern with regard to the informed consent process; or
    - IRB determines as a result of the monitoring process that the consent process is insufficient and education/training is required for conduct of consent.

11.1.6 Site Monitoring

Any study under the purview of the University of Virginia HSR-IRB or HSR-SBS is subject to review. Studies are chosen either a) at random or b) requested by a study team member or any member of the IRB-HSR and the DSMC.
The purpose of audits is to ensure that documentation of clinical research studies is of the highest quality, verify protocol adherence, and ensure that all Federal and local rules concerning clinical research are being fulfilled. A study will be triggered for an audit once 3 patients have been registered in OnCore.
Semi-annual auditing is required for all High-risk studies. For high-risk studies, if findings are satisfactory after two reviews, protocols will be audited once a year. Any time findings are unsatisfactory, auditing will return to the original schedule.

11.1.7 Quality Assurance and Quality Control

Each clinical site will perform internal quality management of study conduct, data and biological specimen collection, documentation and completion according to institutional policies.

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 site(s) for clarification/resolution.

Following written Standard Operating Procedures (SOPs), the monitors will verify that the clinical trial is conducted and data are generated and biological specimens are collected, documented (recorded), and reported in compliance with the protocol, International Conference on Harmonisation Good Clinical Practice (ICH GCP), and applicable regulatory requirements (e.g., Good Laboratory Practices (GLP), Good Manufacturing Practices (GMP)).

11.2 Data Handling and Record Keeping

11.2.1 Data Collection and Management Responsibilities

Data collection is the responsibility of the clinical trial staff at the site under the supervision of the site investigator. The investigator is responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported.

All source documents should be completed in a neat, legible manner to ensure accurate interpretation of data.

Studies using Oncore: Data will be collected using a password-protected, centralized electronic case report form called **ON**-line **C**linical **O**ncology **R**esearch **E**nvironment = **Oncore**.] Data recorded in the electronic case report form (eCRF) derived from source documents should be consistent with the data recorded on the source documents.

Cellular data will be collected using high dimensional spectral cytometers, such as the Cytek Norhtern Lights or Aurora, and cytokine data will be collected with the Magpix analyzer housed in the Flow Cytometry Core Facility (FCCF). These machines output data in the .fcs format which can be analyzed by flow cytometry analysis software, for example FlowJo. Cytokine data will be processed by the FCCF and returned to investigators in a spreadsheet format. Data will be analyzed using a combination of computational spreadsheet software such as Microsoft Excel, statistical software such as Prism, and/or statistical programming languages such as R. All samples will be immediately deidentified following collection and referred to by their experimental identifiers during processing, data collection, and analysis. A classified and secured record key that associates subjects with their experimental identifiers will be maintained in a single location that is firewalled, encrypted, and password protected. There should be no need to reference this document aside from quality control to ensure that the proper baseline control samples are associated with the proper treatment samples for statistical analysis purposes.

11.2.2 Study Records Retention

Study documents should be retained for a minimum of 2 years after the last approval of a marketing application in an International Conference on Harmonisation (ICH) region and until there are no pending or contemplated marketing applications in an ICH region or until at least 2 years have elapsed since the formal discontinuation of clinical development of the study intervention. These documents should be retained for a longer period, however, if required by local regulations. No records will be destroyed without the written consent of the sponsor, if applicable. It is the responsibility of the sponsor to inform the investigator when these documents no longer need to be retained. Record retention will be in accord with device and HIPAA regulations.

11.3 Publication and Data Sharing Policy

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

The research team for this study is small and unified in vision. All investigators and personnel with a significant role in the design, completion, and/or analysis of the study will be included as authors in the event that results are published.

This study will comply with the NIH Data Sharing Policy and Policy on the Dissemination of NIH-Funded Clinical Trial Information and the Clinical Trials Registration and Results Information Submission rule. As such, this trial will be registered at ClinicalTrials.gov, and results information from this trial will be submitted in accordance with regulations and any active contractual obligations. In addition, every attempt will be made to publish results in peer-reviewed journals.

11.4 Conflict of Interest Policy

Although this is a pilot study designed to collect proof of principle data for future use and study design (if the results support the hypotheses), the independence of this study from any actual or perceived influence is still 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 in the design and conduct of this trial. The study leadership in conjunction with the institution 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.

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13 APPENDICES

13.1 Schedule of Activities (SoA)

Procedures	Screening/Baseline 1, Day -2 to -1	Study Visit 1, Day 1	Study Visit 2 Day 2 (+1) day	Study Visit 3/ Baseline 2 Day 14	Study Visit 4 Day 15 (+1) day	Final Study Visit 5 (via phone) Day 30 +/-2 days
Informed consent	X					
Demographics	X					
Medical history	X					
Group assignment	X					
Administer study intervention (ultrasound stimulation) or sham treatment		X				
Administer study intervention (ultrasound stimulation)				X		
Diagnostic ultrasonography		X	X	X	X	
Concomitant medication review	X	X----------X				
Physical exam	X			X		
Vital signs	X	X		X		
Height	X					
Weight	X	X	X	X	X	
Hematology [a]	X	X[b]	X[b]	X[b]	X[b]	
Serum chemistry [c]	X					
Pregnancy test [d]	X					
Survey form		X----------X				X
Adverse event review and evaluation		X----------X				X
Urinary AKI biomarkers [e,f]		X	X	X	X	
Other assessments (flow cytometry analysis of immune cell populations, ex vivo stimulation assays to measure cytokine production)		X	X	X	X	

a: Complete blood count.
b: Complete blood count with differential.
c: Serum creatinine, BUN, AST, ALT, fasting blood sugar, hemoglobin, A1c.
d: Urine pregnancy test (women of childbearing potential).
e: Spot urine sample (optional).
f: Optional urine samples will be collected at Visits 1 to 4 (i.e., prior to ultrasound stimulation, and ~30 minutes and 24 hours after ultrasound stimulation).

13.2 Reporting Table

Single-site study; section not applicable.

13.4 Protocol Amendment History

Version	Date	Description of Change	Brief Rationale
1.1	September 2, 2022	Refinement of exclusion criteria	To improve quality and participant safety
1.2	January 29, 2023	Incorporation of AKI biomarkers in urine, complete blood count with differential sent to the UVA laboratory prior to ultrasound stimulation and post-treatment visit, respectively; incorporation of B mode ultrasonography performed at post-treatment visits	To gain additional insights on safety of ultrasound stimulation
1.3	May 15, 2024	a) Added additional control subgroup to Objective 1; b) Refinement of criteria required before ultrasound stimulation can be delivered; c) weight data collection at Visits 1 to 4; d) timepoints of urine collection as noted in 8.2.3 (Research Urine) were not consistent with the ones given in 13.1 (Schedule of Activities)	a) Review of the data collected so far indicates a sham treatment would be useful for increasing the confidence in our analysis and conclusions b) To improve participant safety and interpretability of results; c) to see whether ultrasound stimulation has any effects on weight; d) correction of urine collection timepoints in “Schedule of Activities”