THOMAS JEFFERSON UNIVERSITY
Sidney Kimmel Cancer Center

Detection of Sentinel Lymph Nodes in Female Lower Genital Tract Cancer Patients with Contrast-Enhanced Ultrasound Imaging

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IND/IDE Number:	Insert IND or IDE number, if applicable 127,419 (amendment pending)
Study Product:	Sonazoid™ (perfluorobutane) Powder for Injection.
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Signature Page

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable US federal regulations and ICH guidelines.

Principal Investigator:

Signed: _____ Date: _____

Name: Ji-Bin Liu, MD

Title: Professor, Department of Radiology

Statement of Compliance

This study will be conducted in accordance with the International Conference on Harmonisation guidelines for Good Clinical Practice (ICH E6), the Code of Federal Regulations on the Protection of Human Subjects (45 CFR Part 46), and Thomas Jefferson University research policies

List of Abbreviations

AE	Adverse Event
CEUS	Contrast-enhanced Ultrasound
CFR	Code of Federal Regulations
CIOMS	Council for International Organizations of Medical Sciences
CPR	Cardiopulmonary Resuscitation
CRF	Case Report Form
CRO	Clinical Research Organization
CT	Computed Tomographic
CTCAE	Common Terminology Criteria for Adverse Events
FDA	Food and Drug Administration
DSMB	Data Safety Monitoring Board
FWA	Federalwide Assurance
GCP	Good Clinical Practice
HEPS	Hydrogenated Egg Phosphatidyl Serine
HIPAA	Health Insurance Portability and Accountability Act
HSRRB	Human Subjects Research Review Board
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
IND	Investigational New Drug
IRB	Institutional Review Board
IV	Intravenous
LC	Lymphatic channel
LN	Lymph Node
MOP	Manual of Procedures
MRI	Magnetic Resonance Imaging
N	Number (typically refers to participants)
NCI	National Cancer Institute
NIH	National Institutes of Health
OHRP	Office for Human Research Protections
PET	Positron Emission Tomographic
PHI	Protected Health Information
PFB	Perfluorobutane
PI	Principal Investigator

PRC	Protocol Review Committee
QA	Quality Assurance
QC	Quality Control
RES	Reticuloendothelial System
SAE	Serious Adverse Event
SKCC	Sidney Kimmel Cancer Center
SOP	Standard Operating Procedure
SLN	Sentinel Lymph Node
TJU	Thomas Jefferson University
TJUH	Thomas Jefferson University Hospital
UAP	Unanticipated Problem
UCA	Ultrasound Contrast Agent
US	Ultrasound

Study Summary

Title: **Detection of sentinel lymph nodes in female lower genital tract cancer patients with contrast-enhanced ultrasound imaging**

Summary:

This is an open-label, non-randomized trial that will be conducted at Thomas Jefferson University (TJU). This study will compare the use of lymphosonography for sentinel lymph node (SLN) detection to the standard of care for lymphatic mapping that varies depending on the patient's staging (lymph node (LN) dissection and/or sequential PET-CT). The patients will undergo an ultrasound examination to locate the tumor where a baseline grayscale and color Doppler ultrasound scan of the lesion will be obtained. The ultrasound contrast agent Sonazoid (GE Healthcare, Oslo, Norway) will be administrated in 4 aliquots at 12, 3, 6, and 9 o'clock positions around the primary tumor, with 0.25 ml for each aliquot for a total dose of 1.0 ml. Real time contrast enhanced ultrasound imaging (CEUS) will then be performed to identify the number, location and course of the lymphatic channels (LCs) drainage from the tumor to the SLNs. All LNs that demonstrate contrast-enhancement will be considered SLNs and their locations, depth beneath the skin surface and size (measured in three orthogonal dimensions) will be recorded and compared to the standard of care (i.e., to blue dye, pathology or PET-CT). An Aplio i800 scanner (Canon Medical Systems, Tustin, CA) with a curvilinear (8C1), a linear (18L5) and endovaginal (11C3) probes with CEUS capabilities will be used during the study.

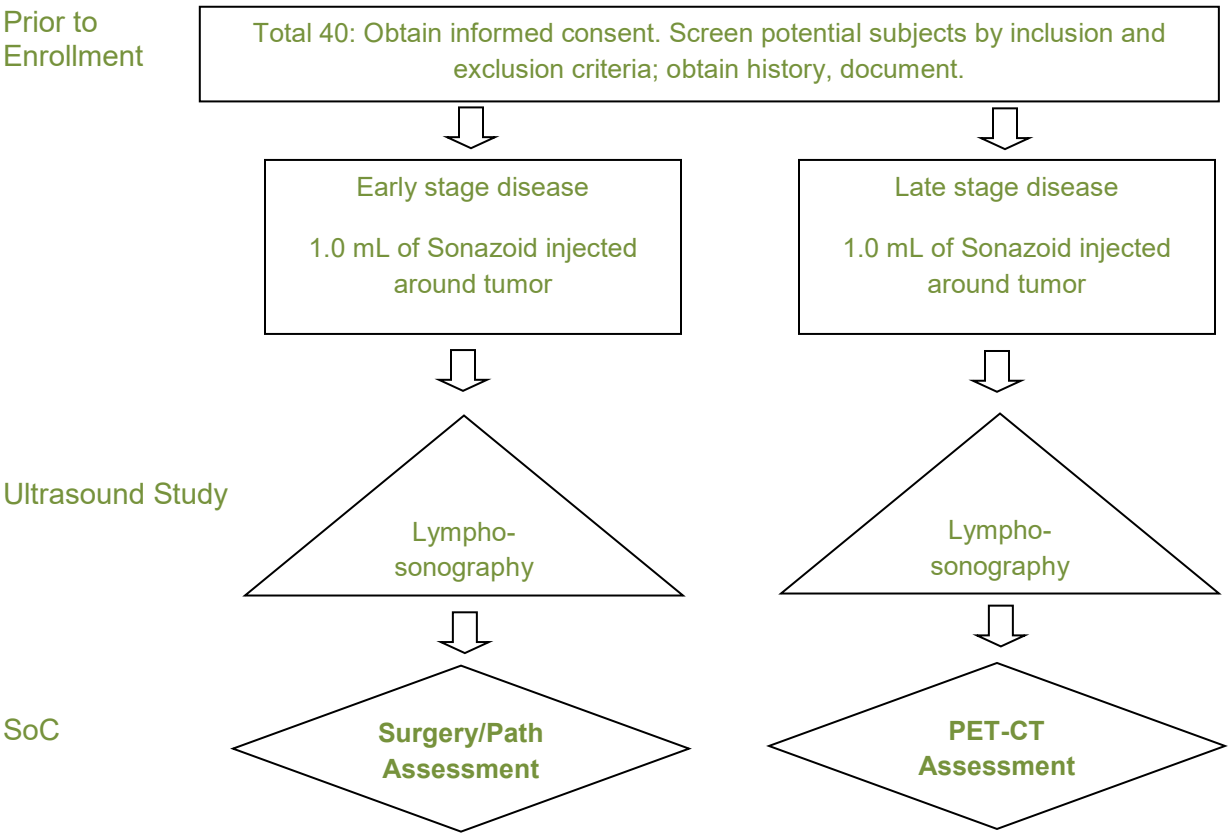
Objectives:

- Primary: To determine the concordance between lymphosonography and the standard of care in the identification SLNs in patients with cervical, vaginal or vulvar cancer.

- Secondary: To determine if lymphosonography can identify more SLNs with metastatic deposits in patients with cervical, vaginal or vulvar cancer when compared to the standard of care.

Population:	40 adult women, who have a diagnosis of cervical, vaginal or vulvar cancer, will be enrolled in this clinical trial at TJU.
Phase:	pilot
Number of Sites:	(1) Thomas Jefferson University (TJU)
Description of Intervention:	A single-site, open-label, non-randomized, pilot study of lymphosonography (i.e., CEUS of subdermal microbubbles) compared to standard of care assessments ((i.e., blue dye, pathology or PET-CT).
Study Duration:	2 years
Participant Participation Duration:	The entire CEUS imaging protocol for the clinical trial will require up to 30 minutes of scanning.
Estimated Time to Complete Enrollment:	Subject recruitment for the clinical trial is expected to last 24 months (January 2024-December 2025).

Schematic of Study Design:



1 Introduction

1.1 Background Information

In the United States there will be an estimated 24,600 new cases of female lower genital tract cancer (cervical, vaginal and vulvar) in 2018 leading to an estimated 6,700 deaths for a mortality rate of 27 % or 21 % of all female genital cancer deaths [Siegel et al. 2016]. The treatment approach varies according to the stage of the disease, and in the majority of the cases, the treatment protocols are similar between all 3 types of cancer. Early stages of disease (stage Ia and Ib for cervical and vulvar cancers, stage I for vaginal cancer) are treated with surgical intervention, and prior to the surgery the patients undergo PET-CT to determine lymph node (LN) metastatic infiltration. This parameter is used to determine the necessity of surgical LN dissection. However, the majority of women are diagnosed with advanced disease (stages 2, 3 and 4), where the treatment of choice is chemotherapy and/or radiotherapy, with the use of PET-CT to determine LN metastatic infiltration pre- and post-treatment.

Detection of subclinical malignancy in draining LNs is important in the management of a variety of malignancies including melanoma, breast, colon and other cancers, where the most important LN to evaluate is the sentinel lymph node (SLN), the first node to receive afferent lymphatic

drainage through lymphatic channels (LCs) from the primary tumor [Morton & Chan 2000]. Various techniques and imaging agents have been developed to map lymphatic drainage from tumors, including injection of blue dye, indocyanine green with near-infrared fluorescence imaging and injection of radiopharmaceuticals (radioisotopes) followed by evaluation with a gamma camera (i.e., lymphoscintigraphy) or intraoperatively with a gamma probe (isotope mapping) [Goldfarb et al. 1998, Gimenez et al. 2001]. These surgical resection techniques and also the use of PET-CT to diagnosis and monitor of female lower genital tract cancers are in general accurate techniques, but pitfalls do exist. For example, the use of blue dye to identify SLNs requires surgical dissection that, in some cases, can be extensive (and multicentric) and all surgical procedures have inherent potential complications [Gimenez er al. 2001]. Radioactive materials make use of small particles and due to their size they may actually pass through the SLNs resulting in drainage into secondary LNs potentially resulting in a more extensive resection than might otherwise be needed [Goldfarb et al. 1998,] or a change in the staging of the disease, upgrading to a more advanced disease than the reality.

PET-CT systems combine positron emission tomographic (PET) scanners and computed tomographic (CT) scanners, which provides images of both anatomic and functional information in a single study. However, this combination result in PET/CT examinations, especially those that include a diagnostic CT study, with increased patient radiation exposure compared to stand-alone CT or PET examinations (as the effective dose is a combination of the dose from PET and the dose from CT). Studies, such as Huang et al. 2009, were conducted to determine the potential risk from radiation exposure, and the risk-benefit ratios were assessed. The conclusion was that total effective dose from each PET/CT study was about five to thirteen times (12-31 mSy) the worldwide average effective dose from background radiation over 1 year, which is estimated to be about 2.4 mSv [Huang et al. 2009]. In addition, patients with cancer often undergo multiple PET/CT examinations for response assessment and treatment monitoring, and given that survival rates are markedly improved nowadays, this increases the concern that in time this cumulative radiation dose can increase the chance of radiation exposure induced cancer. Therefore, the development a non-radiation based imaging alternative is necessary.

Diagnostic ultrasound (US) imaging has been used to evaluate LNs for benign disease as well as metastases, however conventional ultrasound cannot be used for lymphatic mapping, since mapping requires administration of a tracer. This paradigm changed with the development of stable (i.e., encapsulated), gas-filled microbubbles as ultrasound contrast agents (UCAs) [Goldberg et al. 2001]. UCA microbubbles have particle sizes of 2-8 μm that, after intravenous administration, serve as vascular tracers typically providing 20-25 dB of echo enhancement for 3-10 minutes [Goldberg et al. 2001]. Also there are tissue-specific UCAs, which are taken-up by the reticuloendothelial system (RES) [Forsberg et al. 2002]. One such tissue-specific UCA is Sonazoid (GE Healthcare, Oslo, Norway) composed of a lipid stabilized suspension of perfluorobutane microbubbles with a median diameter of 2.4-3.5 μm [Sontum 2008]. This agent is taken up by macrophages of the normal RES in the liver and spleen when injected intravenously [Forsberg et al. 2002].

The initial proof of concept that some UCAs are taken up by LNs (in rabbits) and can be localized using CEUS was reported by investigators at University of California, San Diego [Mattrey et al.

1999]. Our subsequent investigations, using a swine model with naturally occurring melanoma tumors and funded by the NCI, established that the accuracy of SLN detection was 81.8% for lymphosonography, which was significantly higher than the 63.2% achieved with lymphoscintigraphy ($p < 0.0001$) based on imaging 351 SLNs in 63 swine [Goldberg et al. 2011].

The clinical translation of lymphosonography is being actively pursued in two different ongoing, NCI-supported clinical trials, one designed to investigate the diagnostic utility of lymphosonography for SLN identification in women scheduled for surgical excision of a malignant breast mass and the other to investigate the diagnostic utility of lymphosonography for SLN identification in patients undergoing endoscopic ultrasound guided biopsy of esophageal cancer. By injecting a UCA into the peritumoral tissues and following its uptake in the LCs using CEUS, our group can identify the specific location and number of SLNs draining from the primary tumor. The results will be compared to the established techniques currently in use. Lymphosonography used for SLN detection has several potential advantages. Lymphosonography does not require ionizing radiation (a growing concern in medicine) and has markedly better spatial resolution as well as anatomical information, which would allow for more precise SLN localizations; also the portability of US equipment lends itself to use at the patient's bedside, within the surgical suite or elsewhere as necessary. Lymphosonography can be used to clearly delineate adjacent LNs and to identify tumor involvement using conventional US criteria [Goldberg et al. 2011], which is not possible with the use of a gamma probe or by lymphoscintigraphy. Finally, the improvement in SLN detection in patients with breast cancer (from 1.67 to 2.16 SLNs/patient) achieved by using the tissue-specific lymphoscintigraphy agent Tilmanocept [Tokin et al. 2012], indicates that the much larger improvement seen with lymphosonography and the RES-specific UCA Sonazoid (3.2 SLNs/patient) has the potential to change the clinical paradigm for SLN identification. Hence, this project is a natural translation of our experiences with lymphosonography in breast and esophageal cancer patients and it aims to shift the clinical paradigm on mapping and identification of SLNs in women with lower genital tract cancer.

The safety of subdermal injections of Sonazoid was determined in a safety study conducted by our group [Machado et al. 2018]. The study assessed the safety and tolerability of subdermal administration of two dosages Sonazoid (1 and 2 ml) in the breasts of healthy, female volunteers with screening and baseline assessments/procedures that included informed consent, medical history, demography, physical examination, vital signs and a focused physical exam as well as recording of any adverse events (AEs). Only minor local and insignificant adverse experiences confined to the injection site and surrounding area were encountered by the volunteers, local redness was seen in 83% of the cases, local pain in 33% of the cases and local bruising in 25% of the cases [Machado et al. 2018]. All adverse experiences (Table 1) were completely resolved without any intervention by the time the study was completed.

Table 1. Adverse experiences divided by volunteers and by dosage.

Adverse experience	Volunteers (n=12)	1 ml (n=12)	2 ml (n=12)
Redness	10 (83%)	10 (83%)	7 (58%)
Pain	4 (33%)	2 (17%)	3 (25%)
Bruise	3 (25%)	2 (17%)	1 (8%)
Burning sensation	2 (17%)	1 (8%)	2 (17%)

Numbness	1 (8%)	1 (8%)	1 (8%)
Tingling sensation	1 (8%)	0 (0%)	1 (8%)

Sonazoid Intravenous Clinical Safety Trials

Prior to the development of lymphsonography and the subdermal use of UCA, clinical trials were done to evaluate the safety of intravenous use of Sonazoid.

Sonazoid was administered intravenous (IV) in clinical trials in 1699 patients. In these patients 404 (23.8%) reported at least one AE, while 12 of 62 (19.4%) of patients receiving a placebo reported AEs.

Forty-three (2.5%, 43/1699) subjects experienced serious adverse events (SAEs), including 13 deaths. None of these events were considered by the investigators to be related to Sonazoid. All the SAEs were considered to be caused by the underlying disease or related treatment. In addition, there were no clinically significant trends in laboratory tests, vital signs, ECGs, or physical examination findings. The most commonly noted AEs were headache (3.6%, 62/1699), chest pain (2.3%, 39/1699), abdominal pain (1.5%, 25/1699), diarrhea (1.5%, 25/1699), and nausea (1.6%, 28/1699). The majority of AEs were mild to moderate in severity (92.6%, 652/704).

The proposed agent for the current study, Sonazoid® (GE Healthcare, Oslo, Norway), is a sterile non-pyrogenic suspension of lipid stabilized perfluorobutane microbubbles for contrast-enhancement, with a median diameter between 2.4 and 3.5 μm [Sontum 2008]. The FDA has yet to approve Sonazoid for human use in this country. Consequently, we intend to amend our current investigator initiated IND (#127,419) exemption from the FDA covering the subdermal use of Sonazoid to also cover female lower genital cancers. This IND was originally set up for breast cancer and was subsequently amended to also include esophageal cancers.

1.2 Rationale for the Proposed Study

Women with cervical, vaginal or vulvar cancers will undergo surgical resection and/or chemotherapy and/or radiotherapy of their cancer (depending on their staging). The SLN mapping for this group of patients is done as part of the staging process using PET-CT, for patients with stage I that undergo surgical resection there is an additional SLN mapping procedure performed using blue dye. The standard of care for the surgical group of patients is LN dissection usually bilateral given the midline anatomical location of the female lower genital tract. Patients with tumors of higher stages that undergo chemotherapy and/or radiotherapy undergo systematic PET-CT for the continuing SLN staging during treatment. However, each of these methods has potential limitations that can impact the detection of SLNs and the accuracy of disease staging. Our group has demonstrated that CEUS after subdermal administration of a tissue-specific ultrasound contrast agent such as Sonazoid, can be used to noninvasively map lymphatic drainage and localize SLNs (so called “lymphosonography”). Our NIH funded investigations using the Sinclair swine model with naturally occurring melanomas have confirmed that CEUS is superior to radioisotope imaging; detecting almost 20 % more SLNs.

The fundamental hypothesis of this project is that lymphosonography can be used to identify SLNs in patients with cervical, vaginal or vulvar cancers with better success rates than the standard of care. In summary, this study aims to determine the clinical potential of lymphosonography for noninvasive SLN mapping via subdermal injection of tissue-specific UCA in 40 patients with cervical, vaginal or vulvar cancer and compare the use of lymphosonography to the standard of care.

1.3 Potential Risks and Benefits

The known risks from the subdermal administration of Sonazoid are minimal. Only minor local and non-significant adverse experiences confined to the injection site and surrounding area were encountered by the volunteers, local redness was seen in 83% of the cases, local pain in 33% of the cases and local bruising in 25% of the cases.

UCAs in general are very safe with serious anaphylactoid-type reactions reported for intravenous injection at a rate of less than 0.01% [Dietrich et al., 2020].

The use of an intravenous needle and the fluids given through the needle may cause minor discomfort, bleeding under the skin (bruise), and possible infection at the site of needle insertion (although that is extremely unlikely). Clinically significant adverse effects from the administration of Sonazoid are unlikely. Hence, the use of subdermal injection of contrast with the contrast-specific ultrasound imaging technique is expected to provide significantly more information than conventional ultrasound techniques. This may lead to a non-invasive method for identification of SLNs.

The cervical, vaginal or vulvar cancer treatment is being performed as a part of the patient's clinical care and therefore, the risks that are associated with the treatment are not related to the research. The subjects' alternative is not to participate in this study and have only the scheduled standard of care treatment performed.

1.3.1 Potential Risks

To protect patient information, data forms will be completed for all subjects enrolled in the trial. The subject study files will be stored in a secure file cabinet and maintained by the research study coordinator. Study files will be kept for 7 years after the completion of the study. The PI of the study (Dr. Forsberg) and his co-investigators have previous experience running ultrasound clinical trials and the PI will serve as the study sponsor. He will be responsible for ensuring all AEs are properly reported.

The final data will be entered into a database implemented in REDCap (Vanderbilt University, Nashville, TN). The investigators will be responsible for management of the database. The database will be maintained within an organized and secure directory system. Subject identification will be maintained with a study specific alphanumeric code (GYN), patient number (01 and onwards) and the patient's initials.

1.3.2 Benefits

We do not expect any direct benefit for subjects enrolled in this study. The long-term benefits of this study will be the clinical use of a non-radiation based imaging alternative to identify SLNs in patients with cervical, vaginal or vulvar cancers.

1.3.3 **Risk-Benefit Ratio**

The risk benefit ratio is low. Based on the available nonclinical and clinical safety data and the anticipated dose levels of Sonazoid that will be used in this study, safety concerns are minimal. The potential side effects related to Sonazoid administration via subdermal injection are described above and listed in greater detail in the investigator's brochure.

2 Study Objectives

2.1 Objectives

This study aims to determine the clinical potential of lymphosonography for noninvasive SLN mapping in 40 patients with cervical, vaginal or vulvar cancer and compare the use of lymphosonography to the standard of care.

2.1.1 **Hypothesis**

The fundamental hypothesis of this project is that lymphosonography can be used to identify SLNs in patients with cervical, vaginal or vulvar cancers with better success rates than the standard of care.

Primary hypothesis: Does lymphosonography identify more SLNs in patients with cervical, vaginal or vulvar cancer than the standard of care? The fundamental hypothesis is that on average lymphosonography will detect 20 % more SLNs than the standard of care.

Secondary hypothesis: Does lymphosonography identify more SLNs with metastatic deposits in patients with cervical, vaginal or vulvar cancer compared to the standard of care? The fundamental hypothesis is that approximately 20% more cancerous SLNs will be detected with CEUS.

2.1.2 **Primary Objectives**

The primary objectives of this trial are:

1. To determine the concordance between lymphosonography and the standard of care in the identification SLNs in patients with cervical, vaginal or vulvar cancer.

2. To determine if lymphosonography can identify more SLNs with metastatic deposits in patients with cervical, vaginal or vulvar cancer when compared to the standard of care.

2.2 Endpoints/Outcome Measures

The findings of lymphosonography will be compared with other imaging and pathological findings when available; including vascularity as well as the clinical outcome as determined by their

clinician. A full demographic profile, known drug allergies or intolerances, and review of the subject's medical/surgical history will be recorded.

Each case will be read independently by two experienced co-investigators blinded to the clinical outcomes to allow repeatability to be assessed.

2.2.1 Primary Endpoints

The number of SLNs identified by lymphosonography as well as blue dye or PET-CT will be determined for each subject.

2.2.2 Secondary Endpoints

The locations, depth beneath the skin surface and size (measured in three orthogonal dimensions) of each SLN will be recorded

The presence or absence of metastatic deposits in the SLNs will be obtained by pathology (when available).

3 Study Design

This is an open-label, non-randomized trial that will be conducted at TJU. This study will compare the use of lymphosonography for SLN detection to the standard of care lymphatic mapping that varies depending on the patient's staging (LN dissection and/or sequential PET-CT). The patients will undergo an ultrasound examination to locate the tumor where a baseline grayscale and color Doppler ultrasound scan of the lesion will be obtained. Sonazoid will be administrated via subdermal injection in 4 aliquots at 12, 3, 6, and 9 o'clock positions around the primary tumor, with 0.25 ml for each aliquot for a total dose of 1.0 ml. Real time CEUS will then be performed to identify the number, location and course of the LCs drainage from the tumor to the SLNs. All LNs that demonstrate contrast-enhancement will be considered SLNs and their locations, depth beneath the skin surface and size (measured in three orthogonal dimensions) will be recorded and compared to the standard of care (i.e., to blue dye, pathology or PET-CT). An Aplio i800 scanner (Canon Medical Systems, Tustin, CA) with a curvilinear (8C1), a linear (18L5) and endovaginal (11C3) probes with CEUS capabilities will be used during the study.

The findings of lymphosonography will be correlated with other imaging and pathological findings when available; including clinical and surgical outcomes as determined by their clinician. A full demographic profile, known drug allergies or intolerances, and review of the subject's medical/surgical history will be recorded. If the woman is of childbearing potential, she will have a urine pregnancy test (the results of which will be made available to the subject prior to study initiation).

4 Study Enrollment and Withdrawal

This clinical trial will consist of 40 adult women (18 years of age or older), who have been diagnosed with cervical, vaginal or vulvar cancer and are seen in our Department of Gynecology Oncology at TJU.

Subjects eligible for clinical trial enrollment will be identified by the surgical co-investigators, Dr. Rosenblum, Dr. Richard and Dr. Minion from their patient population of women with cervical, vaginal or vulvar cancer seen in the Department of Gynecology Oncology at TJU. The research coordinator for this study will explain the study to the subjects. The subject will be given time to consider the risks and benefits of the study and ask questions about participation. The coordinator will review the informed consent form with the subject and then the subject will be given the form to review. The subject, coordinator, and a study investigator will all sign the consent form. The subject will be given a copy of the signed consent form for their records.

Individual participation in the clinical trial will be limited to one lymphosonography study to be realized during their standard of care cancer treatment determined by their physician according with staging. The entire CEUS imaging protocol for the clinical trial will require up to 30 minutes of scanning.

Subject recruitment for the clinical trial is expected to last 24months (January 2024 - December 2025). Analysis and publication of results are expected to take an additional 6 months.

Subject identification will be maintained with a study specific alphanumeric code (GYN), patient number (01 and onwards) and the patient's initials.

4.1 Eligibility Criteria

4.1.1 Inclusion Criteria

Individuals must meet all of the following inclusion criteria in order to be eligible to participate in the study:

- Be female.
- Be diagnosed with cervical, vaginal or vulvar cancer.
- Be at least 18 years of age.
- If of child-bearing potential, must have a negative pregnancy test
- Be able to comply with study procedures.
- Have read and signed the IRB-approved Informed Consent form for participating in the study.

4.1.2 Exclusion Criteria

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

- Females who are pregnant or nursing.
- Patients who have received an investigational drug in the 30 days before study drug administration, or will receive one within 72 hours afterwards.

- Patients who are medically unstable, patients who are seriously or terminally ill, and patients whose clinical course is unpredictable. For example:
    - Patients on life support or in a critical care unit.
    - Patients with clinically unstable cardiac arrhythmias, such as recurrent ventricular tachycardia.
    - Patients with recent cerebral hemorrhage.
    - Patients who have undergone surgery within 24 hours prior to the study sonographic examination.
- Patients with congenital heart defects.
- Patient with a known allergy to Sonazoid (including an anaphylactic allergy to eggs or egg products).

4.2 Strategies for Recruitment and Retention

Potential research subjects will be identified by a member of the patient's care treatment team, the investigator or a research team member. Investigators will then screen the patient's medical records to determine the subject eligibility for study participation and discuss the study with the patient and their potential for enrolling in the research study. Consenting patients will be screened based on the inclusion/exclusion criteria above.

An investigator or research coordinator will explain the study to the patient. The patient will be given time to consider the risks and benefits of the study and ask questions about participation. The consent form will be reviewed with the patient and then the patient will be given the form to review. If consent interview is conducted by a coordinator, a study investigator will then discuss the study with the subject and answer any additional questions. The patient, person conducting study interview (if applicable), and a study investigator will all sign the consent form. The patient will be given a copy of the signed consent form for her records.

Trial participants will have the presence of inclusion criteria and absence of exclusion criteria verified by providing a medical history. A full demographic profile, known drug allergies or intolerances, and a review of the subject's medical/surgical history will be recorded. If the subject is a woman of childbearing potential, she will have a urine pregnancy test (the results of which will be made available to the subject prior to study initiation).

4.3 Participant Withdrawal

4.3.1 Reasons for Withdrawal

Participants are free to withdraw from participation in the study at any time upon request.

An investigator may terminate a study participant's participation in the study if:

- Any clinical adverse event (AE), laboratory abnormality, or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the participant.
- The participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation.

4.3.2 Handling of Participant Withdrawals and Participant Discontinuation of Study Intervention

Trial participation will be considered completed if the subject has met all of the following trial requirements:

- Has received one set of subdermal injections of Sonazoid
- Has undergone the complete ultrasound imaging study as described in this protocol

If a subject's participation in the trial is interrupted for any reason (e.g., because of an AE) and the subject has met the criteria described above for completing the trial, the subject's trial participation will be considered completed. If a subject's trial participation is interrupted for any reason by the subject's or investigator's choice and the subject has not met all of the criteria listed above, then the subject will be considered a discontinued subject.

4.4 Premature Termination or Suspension of Study

This study may be 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 the IRB and the FDA. If the study is prematurely terminated or suspended, the principal investigator will promptly inform the IRB and will provide the reason(s) for the termination or suspension.

Circumstances that may warrant termination include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants.
- Insufficient adherence to protocol requirements.
- Data that is not sufficiently complete and/or evaluable.
- Determination of futility.

5 Study Intervention

5.1 Study Product

The UCA Sonazoid consisting of gas-filled microbubbles will be used in this study. Our existing FDA Investigator IND (# 127,419) on subdermal injections of Sonazoid will be amended to include this project. This IND was originally set up for breast cancer and was subsequently amended to also include esophageal cancers. Studies will not commence until regulatory approval has been obtained from the FDA as well as the IRB committee for TJU. The clinical trial will also be registered with ClinicalTrials.gov.

5.2 Study Product Description

Sonazoid is a sterile non-pyrogenic suspension of microspheres of lipid stabilized perfluorobutane (PFB) for contrast-enhancement, with a median diameter between 2.4 and 3.5 μm and less than

0.1 % larger than 7 μm. Sonazoid is formulated as a powder for injection consisting of lyophilized sucrose entrapping hydrogenated egg phosphatidyl serine (HEPS) PFB microspheres under a PFB headspace. Each milliliter of Sonazoid contains roughly 1.2 billion microspheres.

5.2.1 **Acquisition**

Sonazoid will be provided by GE Healthcare (Oslo, Norway) free of charge (similar to our other protocols involving this UCA).

5.2.2 **Formulation, Packaging, and Labeling**

Sonazoid is supplied as a dry powder within 10 mL sealed vials. The headspace of the vials contains PFB. A single vial with 16 μL of Sonazoid microbubbles (2 mL) will be prepared for each subject by resuspending each vial in 2 mL of injection grade water supplied by GE. Each kit will be labeled with the study number as well as vial lot and batch numbers. All materials and supplies used for the subdermal injection procedure will be identical to those described in GE Healthcare's current Sonazoid IND.

Packages will contain the following medical grade, sterile and non-pyrogenic components:

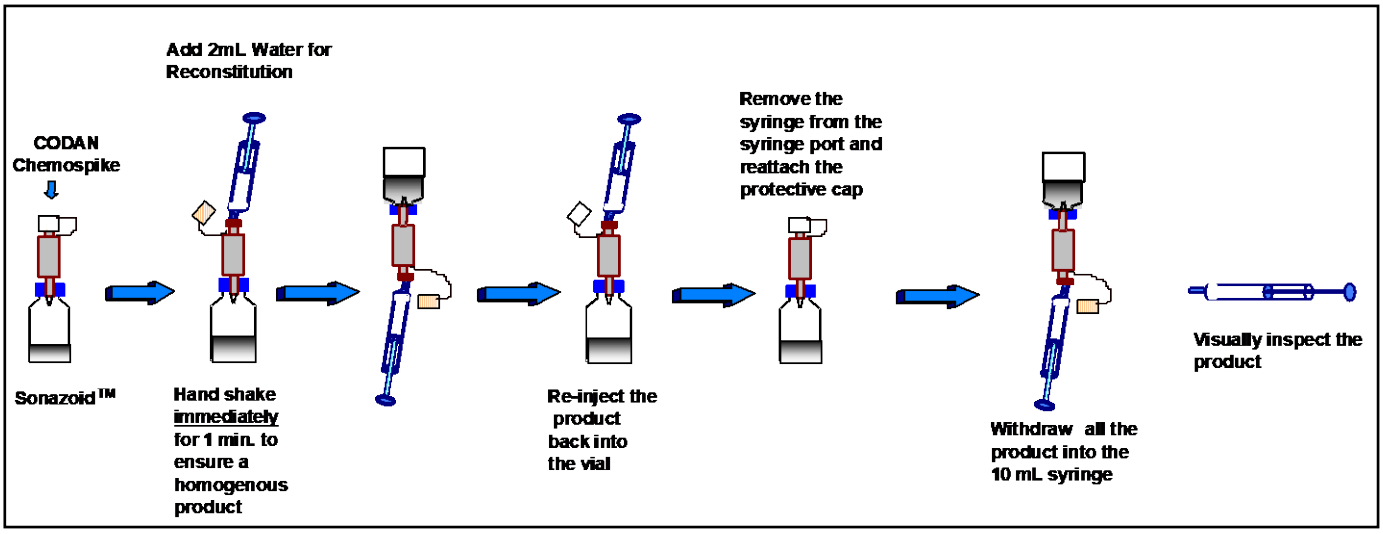
- Sonazoid™ Powder for Injection, 16 μL microbubbles per vial;
- Sterile water for reconstitution of Sonazoid™
- CODAN Chemospike (contains 0.20 μm air filter and 5 μm liquid filter)

5.2.3 **Product Storage and Stability**

Sonazoid will be stored in a secure cabinet, with only the study investigators and research personnel having access. The study researchers will be responsible for drug reconstitution and inventory control. New vials must be prepared for each subject's contrast administration and will be used immediately upon reconstitution. Sonazoid is stable for 4 hours after reconstitution. If the agent is suspended and not used within 4 hours it will be discarded.

5.3 **Dosage, Preparation, and Administration**

- Perforate the stopper of the Sonazoid vial with the provided *CODAN Chemospike.*
- Remove protective cap from the syringe port of the *Chemospike.*
- Using a 2-mL syringe, add 2 mL s*terile water* through the *Chemospike.*
- With the syringe remaining attached to the *Chemospike*, **immediately** shake the product for 1 minute to ensure a homogeneous product.
- Withdraw the product into the syringe and re-inject the product back into the vial again. This is to avoid dilution of the product due to the dead-space volume in the *Chemospike.*
- Remove the syringe from the syringe port and reattach the protective cap. The concentration of the reconstituted product is 8 μL microbubbles/mL.



Each subject will receive a 1 ml subdermal dose of Sonazoid divided into four individual aliquots at four locations (12, 3, 6, and 9 o'clock) around the primary tumor using 20-22 gauge cannula.

5.4 Dose Modifications and Dosing Delays

Not applicable for this study.

5.5 Study Product Accountability

A log of study drug will be kept by the research coordinator on this study. All vials released for studies will be recorded. Unused drug and empty vials will be properly disposed of after reconciling in the log of study drug.

5.6 Dietary Restrictions

No special dietary or "life-style" requirements are applicable.

5.7 Study Procedural Intervention(s) Description

Screening Assessments

Screening assessments will be performed within 4 weeks prior to the administration of Sonazoid. All subjects will receive a written consent form and a verbal explanation of the trial by the investigator and/or the research study coordinator and will be asked to sign the written informed consent. Participants will have the presence of inclusion criteria and absence of exclusion criteria (cf., sections 4.1.1 and 4.1.2) verified by providing a comprehensive medical history, which will include screening assessments for their cervical, vaginal or vulvar cancer diagnosis.

Medical History

A full demographic profile, known drug allergies or intolerances, and a review of the subject's medical/surgical history will be recorded. If the subject is of childbearing potential, she will have

a urine pregnancy test (the results of which will be made available to the subject prior to study initiation).

5.8 Administration of Procedural Intervention

Administration of Sonazoid will be performed under direct supervision of a medical doctor. Emergency services (e.g., a crash cart) will be available within the hospital in case of any acute adverse reactions. Additionally, resuscitation equipment will be in immediate proximity to the patient. Patients will be monitored for AEs post Sonazoid treatment for a minimum of one hour. Trained CPR personnel and resuscitation equipment will be in attendance during this monitoring period. A dose of one vial with 16 µl of Sonazoid microbubbles (2 ml total after resuspension) will be prepared for each subject by resuspending each vial in 2 ml of injection grade water supplied by GE (as described in section 5.3). All materials and supplies used for the infusion procedure will be identical to those described in GE Healthcare's current Sonazoid IND. Each subject will receive 1 ml subdermal dose of Sonazoid divided into four individual aliquots of 0.25ml at four locations (12, 3, 6, and 9 o'clock) around the primary tumor using a 20-22 gauge cannula.

Ultrasound Imaging (lymphosonography)

The ultrasound examinations will be performed by a qualified sonographer or physician. Procedures and equipment for this trial will be used in accordance with typical clinical procedures. All trial procedures will be conducted in accordance with Good Clinical Practice. An Aplio i800 scanner with a curvilinear (8C1), a linear (18L5) and an endovaginal (11C3) probe CEUS will be used during the study. The patients will undergo an ultrasound examination to locate the tumor where a baseline grayscale and color Doppler ultrasound scan of the lesion will be obtained. Sonazoid will be administrated via subdermal injection in 4 aliquots at 12, 3, 6, and 9 o'clock positions around the primary tumor, with 0.25 ml for each aliquot adding to a total dose of 1.0 ml. Real time CEUS will then be performed to identify the number, location and course of the LCs drainage from the tumor to the SLNs. All LNs that demonstrate contrast-enhancement will be considered SLNs and their locations, depth beneath the skin surface and size (measured in three orthogonal dimensions) will be recorded and compared to the standard of care (i.e., to pathology or PET-CT). Digital clips of the ultrasound study will be acquired.

Contraindications

Sonazoid should not be administered to patients with known or suspected hypersensistivity to egg phosphatidyl serine. Patients with a history of anaphylactic allergy to eggs or egg products will be excluded from the study.

Safety Assessments

Emergency services (e.g., a crash cart) will be available within the hospital in case of any acute adverse reactions. Adverse events will be monitored during the entire procedure.

5.9 Administration of Procedural Intervention

Each subject will receive a 1 ml subdermal dose of Sonazoid divided into four individual aliquots at four locations (12, 3, 6, and 9 o'clock) around the primary tumor using 20-22 gauge cannula by the Ultrasound Research team (consisting of MDs (radiologists and gynecological oncologists),

sonographers, RNs, basic scientists and research coordinators). We anticipate each session will take approximately 45 min out of which around 25 min will be the actual scanning time.

5.10 Procedures for Training of Clinicians on Procedural Intervention

The personnel performing the lymphosonography procedures are all from the Ultrasound Research lab or Gynecological Oncology and have extensive experience with injections as well as CEUS and ultrasound imaging procedures. These individuals will all receive training by the PI in this specific protocol.

6 Study Schedule

6.1 Screening

Assess patient eligibility, complete appropriate screening procedures and evaluations prior to study entry.

- Obtain informed consent
- A qualitative urine pregnancy test, for female patients of childbearing potential.

6.2 On Study Period

The ultrasound examinations will be performed by a qualified sonographer or physician. Procedures and equipment for this trial will be used in accordance with typical clinical procedures. All trial procedures will be conducted in accordance with Good Clinical Practice. An Aplio i800 scanner with a curvilinear (8C1), a linear (18L5) and an endovaginal (11C3) probe CEUS will be used during the study. The patients will undergo an ultrasound examination to locate the tumor where a baseline grayscale and color Doppler ultrasound scan of the lesion will be obtained. Sonazoid will be administrated in 4 aliquots at 12, 3, 6, and 9 o'clock positions around the primary tumor, with 0.25 ml for each aliquot adding to a total dose of 1.0 ml. Real time CEUS will then be performed to identify the number, location and course of the LCs drainage from the tumor to the SLNs. All LNs that demonstrate contrast-enhancement will be considered SLNs and their locations, depth beneath the skin surface and size (measured in three orthogonal dimensions) will be recorded and compared to the standard of care (i.e., to pathology or PET-CT). Digital clips of the ultrasound study will be acquired.

6.3 End of Treatment Study Procedures

Trial participation will be considered completed if the subject has met all of the following trial requirements:

- Has received one set of subdermal injections of Sonazoid
- Has undergone the complete ultrasound imaging study as described in this protocol

6.4 Withdrawal Visit/Discontinuation of Therapy

If a subject's participation in the trial is interrupted for any reason (e.g., because of an AE) and the subject has met the criteria described above for completing the trial, the subject's trial

participation will be considered completed. If a subject's trial participation is interrupted for any reason by the subject's or investigator's choice and the subject has not met all of the criteria listed above, then the subject will be considered a discontinued subject.

7 Study Procedures and Evaluations

7.1 Study Procedures/Evaluations

As outlined in section 5.7:

- Medical history
- Lymphosonography

8 Evaluation of Safety

8.1 Specification of Safety Parameters

8.1.1 Unanticipated Problems

Unanticipated problems (UAPs) include, in general, any incident, experience, or outcome that meets 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 IRB-approved research protocol and informed consent document; and (b) the characteristics of the participant population being studied;

UAPs are considered to pose risk to participants or others when they suggest 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.

8.1.2 Adverse Events

An adverse event (AE) is any untoward or unfavorable medical occurrence in a human participant, including any abnormal sign (for example, abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the participant’s participation in the research, whether or not considered related to the participant’s participation in the research.

The safety of subdermal injections of Sonazoid was determined in a safety study conducted by our group [Machado et al. 2018]. The study assessed the safety and tolerability of subdermal administration of two dosages Sonazoid (1 and 2 ml) in the breasts of healthy, female volunteers with screening and baseline assessments/procedures that included informed consent, medical history, demography, physical examination, vital signs and a focused physical exam as well as recording of any adverse events (AEs). Only minor local and insignificant adverse experiences confined to the injection site and surrounding area were encountered by the volunteers, local redness was seen in 83% of the cases, local pain in 33% of the cases and local bruising in 25% of the cases [Machado et al. 2018]. All adverse experiences were completely resolved without

any intervention by the time the study was completed as shown in Table 1 (repeated below for convenience).

Sonazoid Intravenous Clinical Safety Trials

Prior to the development of lymphsonography and the subdermal use of UCA, clinical trials were done to evaluate the safety of intravenous use of Sonazoid.

Table 1. Adverse experiences divided by volunteers and by dosage.

Adverse experience	Volunteers (n=12)	1 ml (n=12)	2 ml (n=12)
Redness	10 (83%)	10 (83%)	7 (58%)
Pain	4 (33%)	2 (17%)	3 (25%)
Bruise	3 (25%)	2 (17%)	1 (8%)
Burning sensation	2 (17%)	1 (8%)	2 (17%)
Numbness	1 (8%)	1 (8%)	1 (8%)
Tingling sensation	1 (8%)	0 (0%)	1 (8%)

Sonazoid was administered intravenous (IV) in clinical trials in 1699 patients. In these patients 404 (23.8%) reported at least one AE, while 12 of 62 (19.4%) of patients receiving a placebo reported AEs.

Forty-three (2.5%, 43/1699) subjects experienced serious adverse events (SAEs), including 13 deaths. None of these events were considered by the investigators to be related to Sonazoid. All the SAEs were considered to be caused by the underlying disease or related treatment. In addition, there were no clinically significant trends in laboratory tests, vital signs, ECGs, or physical examination findings. The most commonly noted AEs were headache (3.6%, 62/1699), chest pain (2.3%, 39/1699), abdominal pain (1.5%, 25/1699), diarrhea (1.5%, 25/1699), and nausea (1.6%, 28/1699). The majority of AEs were mild to moderate in severity (92.6%, 652/704).

8.1.3 **Serious Adverse Events**

A serious adverse event (SAE) is one that meets one or more of the following criteria:

- Results in death
- Is life-threatening (places the participant at immediate risk of death from the event as it occurred)
- Is disabling or incapacitating
- Results in inpatient hospitalization or prolongation of existing hospitalization
- Results in a persistent or significant disability or incapacity
- Results in a congenital anomaly or birth defect

- An important medical event that may not result in death, be life threatening, or require hospitalization may be considered an SAE when, based upon appropriate medical judgment, the event may jeopardize the participant or may require intervention to prevent one of the outcomes listed in this definition.

8.2 Safety Assessment and Follow-Up

The PI will follow AEs with start dates occurring any time after informed consent is obtained until 7 (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation. Events will be followed for outcome information until resolution or stabilization.

8.3 Recording Adverse Events

The following subsections detail what information must be documented for each AE occurring during the time period specified in Section 8.2 Safety Assessment and Follow-Up. If the patient has experienced AE(s), the investigator will record the following information in the AE log:

- The nature of the event(s) will be described by the investigator in precise standard medical terminology (i.e. not necessarily the exact words used by the patient).
- The duration of the event will be described in terms of event onset date and event ended data.
- The intensity of the AE will be described according to Common Terminology Criteria for Adverse Events version 5.0 (CTCAE), for details see National Cancer Institute home page http:\evs.nci.nih.gov\ftp1\CTCAE\About.html of the AE. The CTCAE displays Grades 1 through 5 with unique clinical descriptions of severity for each AE based on this general guideline:
    - **Grade 1**: Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
    - **Grade 2:** Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living.
    - **Grade 3**: Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care activities of daily living.
    - **Grade 4**: Life-threatening consequences; urgent intervention indicated.
    - **Grade 5**: Death related to AE.
- The Causal relationship of the event to the study medication will be assessed as one of the following:
    - **Unrelated**: There is not a temporal relationship to investigational product administration (too early, or late, or investigational product not taken), or there is a reasonable causal relationship between noninvestigational product, concurrent disease, or circumstance and the AE.
    - **Unlikely**: There is a temporal relationship to investigational product administration, but there is not a reasonable causal relationship between the investigational product and the AE.
    - **Possible**: There is reasonable causal relationship between the investigational product and the AE. Dechallenge information is lacking or unclear.
    - **Probable**: There is a reasonable causal relationship between the investigational product and the AE. The event responds to dechallenge. Rechallenge is not required.
    - **Definite**: There is a reasonable causal relationship between the investigational product and the AE.

- The Expectedness of the AE
- Action taken
- The outcome of the AE – whether the event is resolved or still ongoing.

8.3.1 Relationship to Study Intervention

The relationship to study intervention or study participation must be assessed and documented for all AEs. Evaluation of relatedness must consider etiologies such as natural history of the underlying disease, concurrent illness, concomitant therapy, study-related procedures, accidents, and other external factors.

The following guidelines are used to assess relationship of an event to study intervention:

1. Related (Possible, Probable, Definite)
    a. The event is known to occur with the study intervention.
    b. There is a temporal relationship between the intervention and event onset.
    c. The event abates when the intervention is discontinued.
    d. The event reappears upon a re-challenge with the intervention.
2. Not Related (Unlikely, Not Related)
    a. There is no temporal relationship between the intervention and event onset.
    b. An alternate etiology has been established.

8.3.2 Expectedness

The PI is responsible for determining whether an 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 intervention. Risk information to assess expectedness can be obtained from preclinical studies, the investigator’s brochure, published medical literature, the protocol, or the informed consent document.

8.3.3 Severity of Event

Adverse events will be graded for severity according to the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0.

8.3.4 Intervention

Any intervention implemented to treat the AE must be documented for all AEs.

8.4 Safety Reporting

All adverse events and serious adverse events that should be reported as defined in section 8.1.1 will be recorded in the patient's CRF. All adverse reaction will be reported according to the CT-3; detailed Guidance on the collection, verification and presentation of adverse event/reaction reports arising from clinical trials on medicinal products for human use. Link:

https://ec.europa.eu/health//sites/health/files/files/eudralex/vol-10/2011_c172_01/2011_c172_01_en.pdf

All events will be graded by the Investigator according to the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0. A grading (severity) scale is provided for each AE term. SAEs must be reported within 24 hours after the site has gained knowledge of the SAE to the IRB, and the FDA. Every SAE must be documented by the investigator on the SAE pages (to be found as part of the CRF). The SAE Report Form must be completed and signed. SAEs and possible SAEs will be reported to the regulatory authorities and IEC according to local regulations and followed-up until the resolution of the event. The initial report shall promptly be followed by detailed, written reports if necessary. The initial and follow-up reports shall identify the trial subjects by unique code numbers assigned to the latter.

The sponsor keeps detailed records of all SAEs reported by the investigators and performs an evaluation with respect to causality and expectedness. Based on, among other, SAE reports the sponsor will evaluate whether the risk/benefit ratio associated with study is changed. We will in all cases follow the ICH Harmonised Guideline Integraded Addendum to ICH E6(R1): Guideline for Good Clinical Practise ICH E6(R2) ICH Consensus Guideline. Link: https://ichgcp.net/411-safety-reporting/

8.4.1 Reporting to IRB

8.4.1.1 *Unanticipated Problems*

All incidents or events that meet criteria for unanticipated problems (UAPs) as defined in Section 8.1.1 Unanticipated Problems require the creation and completion of an unanticipated problem report form (OHR-20).

UAPs that pose risk to participants or others, and that are not AEs, will be submitted to the IRB on an OHR-20 form via the eazUP system within 10 working days of the investigator becoming aware of the event.

UAPs that do not pose risk to participants or others will be submitted to the IRB at the next continuing review.

8.4.1.2 *Adverse Events*

Grade 1 AEs will be reported to the IRB at continuing review.

Grade 2 AEs will be reported to the IRB at the time of continuing review.

8.4.1.3 *Serious Adverse Events*

SAEs will be reported to the IRB on OHR-10 forms via the electronic reporting system (eSAEy) according to the required time frames described below.

Grade 3-4 AEs that are unexpected and deemed to be at least possibly related to the study will be reported to the IRB within 2 working days of knowledge of the event.

Grade 3-4 AEs that are deemed unrelated to the study will be reported to the IRB within 5 working days.

Grade 5 AEs will be reported to the IRB within one working day of knowledge of the event.

All SAEs will be submitted to the IRB at continuing review, including those that were reported previously.

8.4.2 Reporting to SKCC DSMC

All AEs and SAEs, safety and toxicity data, and any corrective actions will be submitted to the DSMC per the frequency described in the SKCC DSMP. The report to the SKCC DSMC will also include any unanticipated problems that in the opinion of the PI should be reported to the DSMC.

For expedited reporting requirements, see **Table 2** below:

8.4.1 Reporting to Funding Sponsor

Once a year throughout the clinical trial, the sponsor will provide the Competent Authority with a listing of all suspected serious adverse reactions which have occurred over this period and a report of the subjects' safety. Annual reports are submitted in accordance with ICH guideline E2F - Note for guidance on development safety update reports.

8.4.1 Reporting to FDA

The PI and the SKCC regulatory team will be responsible for submitting annual reports to the FDA on the progress of the study.

8.4.1 Reporting of Pregnancy

Pregnancy is an exclusion criteria.

Table 2: DSMC AE/SAE Reporting Requirements

	Grade 1	Grade 2		Grade 3				Grades 4 and 5
				Unexpected		Expected		
	Unexpected and Expected	Unexpected	Expected	With Hospitalization	Without Hospitalization	With Hospitalization	Without Hospitalization	Unexpected and Expected
Unrelated Unlikely	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	5 Working Days	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	5 Working Days	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Phase I - 48 Hours (Death: 24 Hours) Phase II - 5 working days
Possible Probably Definite	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	48 Hours (Death: 24 Hours)	Phase I - 48 Hours Phase II - 5 working days	48 Hours (Death: 24 Hours)	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Phase I and Phase II - 48 Hours (Death: 24 Hours)

8.5 Halting Rules

The whole trial may be discontinued at the discretion of the PI or the sponsor in the event of any of the following:

- Occurrence of AEs unknown to date in respect of their nature, severity and duration
- Medical or ethical reasons affecting the continued performance of the trial

9 Study Oversight

In addition to the PI's responsibility for oversight, study oversight will be under the direction of the SKCC's Data and Safety Monitoring Committee (DSMC). The SKCC DSMC operates in compliance with a Data and Safety Monitoring Plan (DSMP) that is approved by the NCI.

10 Clinical Site Monitoring and Auditing

Clinical site monitoring and auditing is conducted to ensure that the rights of human participants are protected, that the study is implemented in accordance with the protocol and/or other operating procedures, and that the quality and integrity of study data and data collection methods are maintained. Monitoring and auditing for this study will be performed in accordance with the SKCC's Data and Safety Monitoring Plan (DSMP) developed by the SKCC Data and Safety Monitoring Committee (DSMC). The DSMP specifies the frequency of monitoring, monitoring procedures, the level of clinical site monitoring activities (e.g., the percentage of participant data to be reviewed), and the distribution of monitoring reports. Some monitoring activities may be performed remotely, while others will take place at the study site(s). Appropriate staff will conduct monitoring activities and provide reports of the findings and associated action items in accordance with the details described in the SKCC DSMP.

11 Statistical Considerations

The fundamental hypothesis of this project is that lymphosonography can be used to identify SLNs in patients with cervical, vaginal or vulvar cancers with greater success than the standard of care. For this project the standard of care is either blue dye injections (it the subject goes to surgery) or PET-CT results (if the subject goes to chemotherapy) as part of their management.

In this study we do not focus on whether an SLN is correctly identified, as there is no way of knowing that (unless the subject goes for surgery and the SLN has metastatic deposits but his will only be the case for a small subset) and the number of SLNS not identified by lymphosonography or by the standard of care will, for obvious reasons, be unknown. Hence, we presume all identified SLN are correctly identified.

11.1 Study Hypotheses

Primary hypothesis: Does lymphosonography identify more SLNs in patients with cervical, vaginal or vulvar cancer than the standard of care? The fundamental hypothesis is that on average lymphosonography will detect 20% more SLNs than the standard of care.

Secondary hypothesis: Does lymphosonography identify more SLNs with metastatic deposits in patients with cervical, vaginal or vulvar cancer compared to the standard of care? The fundamental hypothesis is that approximately 20% more cancerous SLNs will be detected with CEUS.

H1: On average lymphosonography will detect 20% more SLN than the standard of care (blue dye or PET-CT).

$H1_0$ The mean number of SLNs per subject identified by lymphosonography is less than 120% of thoset identified by standard of care.

$H1_a$ The mean number of SLNs per subject identified by lymphosonography is greater than 120% of those identified by standard of care.

H2: On average 20% more cancerous SLNs will be detected by lymphosonography than by standard of care (i.e., blue dye) using pathology as the reference standard.

$H2_0$ The % of metastatic SLNs identified by lymphosonography is less than 120% of that identified by standard of care.

$H2_a$ The % of metastatic SLNs identified by lymphosonography is greater than 120% of that identified by standard of care.

11.2 **Analysis Plans**

Thomas Jefferson University Hospital cares for approximately 200 patients with female lower genital tract cancer per year. The patients studied in this project will be women over the age of 18 who have a diagnosis of cervical, vaginal or vulvar cancer. The patient population of this project will reflect the population demographics found at major American urban academic health centers. The overall TJUH demographics include 60 % Caucasian, 16 % African American, 13 % Hispanic, 5 % Asian, 1 % Other, and 5 % unknown patients. The goal of this pilot study is to enroll 40 patients over 2 years. Given that these women have been diagnosed with cancer, they are usually very motivated to participate in research studies that include additional evaluations and, therefore, we do not expect recruitment to be a major issue.

For the primary hypothesis the number of SLN detected by CEUS will be compared to the number of SLN detected by the standard of care (i.e., by blue dye or by PET-CT) using paired t-test or paired Wilcoxon test (depending on whether the data is normal distributed or not).

For the secondary hypothesis the percentage of SLNs with metastatic deposits identified by lymphosonography and by blue dye will be pairwise compared after eliminating the SLNs identified by both methods using two-sample t-test or Wilcoxon test (again after testing for normalcy). The histopathological assessment of the surgically removed LNs will serve as the reference standard.

11.3 **Interim Analyses and Stopping Rules**

None.

11.3.1 **Safety Review**

The investigator is responsible for the detection and documentation of events meeting the criteria and definition of an AE or an SAE.

11.3.2 **Efficacy Review**

See section 11.2

11.4 **Sample Size Considerations**

The sample size analysis is based on the primary aim of this study (i.e., on Aim 1) and assumes that on average lymphosonography will detect 20% more SLN than the standard of care (blue dye or PET-CT]. Hence, a power analysis was performed using NCSS/PASS 2019 (NCSS, East Kaysville, UT) and a sample size of 40 patients achieves 86.9% power to detect the effect size of

0.5 (i.e., assuming the 20% more detected SLN with CEUS versus the standard of care techniques, and twice of standard deviation of paired difference) at 5% significance level using a two-sided paired t-test.

11.4.1 **Replacement Policy**

Subjects that withdraw from the study will not be replaced. If a sufficiently high number of subjects withdraw, the protocol will be amended to allow for greater enrollment.

11.4.2 **Accrual Estimates**

40 adult women, who have a diagnosis of cervical, vaginal or vulvar cancer, will be enrolled in this clinical trial at TJU.

11.5 **Exploratory Analysis**

The statistical analyses above will be repeated split by cancer type, but no firm conclusions are expected given this is a pilot study with a limited sample size.

11.6 **Handling Screen Failure/Subject Discontinuation**

Subjects will be free to discontinue trial participation at any time. The investigator will also discontinue any subject from the trial if, in the investigator's opinion, it is not safe for the subject to continue. The date the subject is withdrawn from a treatment and/or from the trial and the reason for discontinuation will be recorded on the CRF.

If a subject's participation in the trial is interrupted for any reason (e.g., because of an AE) and the subject has met the criteria described above for completing the trial, the subject's trial participation will be considered completed. If a subject's trial participation is interrupted for any reason by the subject's or investigator's choice and the subject has not met all of the criteria listed above, then the subject will be considered a discontinued subject.

12 **Source Documents and Access to Source Data/Documents**

Study staff will maintain appropriate medical and research records for this study, in compliance with ICH E6, and regulatory and institutional requirements for the protection of confidentiality of participant information. Study staff will permit authorized representatives of SKCC and regulatory agencies to examine (and when required by applicable law, to copy) research records for the purposes of quality assurance reviews, audits, and evaluation of the study safety, progress and data validity.

Source Data Verification

The investigator will be visited on a regular basis by the Study Monitor. Monitoring will include source data verification (SDV) and discuss the progress of the study. SDV is confirmed by comparing completed CRFs with matched source documentation in subject’s research binder. Monitors will also review drug usage logs according to European GCP. The PI will perform spot checks to verify CRFs and database entries match source documents. Verified data is entered

into a REDCap computer database by the study coordinator from the completed CRF's for statistical evaluation at Thomas Jefferson University.

The monitor and/or regulatory authorities will be allowed audits at the investigation site and source data verification in which case a review of those parts of the hospital records relevant to the study may be required.

13 Quality Control and Quality Assurance

Case report forms (CRF) will be provided for the recording of all data. Data will be recorded directly and legibly onto the record forms, in blue/black ink. The signature of the investigator will attest the accuracy of the data on each CRF. If any assessments are omitted, the reason for such omissions will be noted on the CRFs. Corrections, with the reason for the corrections, should be made legibly, dated and initialled. Correction fluid is not allowed. All original data collected with paper and pen will immediately be recorded electronically by the study coordinator within the RedCap database.

Data collection and accurate documentation are the responsibility of the study staff under the supervision of the investigator. All source documents and laboratory reports must be reviewed by the study team and data entry staff, who will ensure that they are accurate and complete. UAPs and AEs must be reviewed by the investigator or designee. The monitor and/or regulatory authorities will be allowed audits at the investigation site and source data verification in which case a review of those parts of the hospital records relevant to the study may be required.

14 Ethics/Protection of Human Participants

14.1 Ethical Standard

The investigator will ensure that this study is conducted in full conformity with the principles set forth in The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research, as drafted by the US National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research (April 18, 1979) and codified in 45 CFR Part 46 and/or the ICH E6.

14.2 Institutional Review Board

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. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented in the study.

14.3 Informed Consent Process

Informed consent is a process that is initiated prior to the individual agreeing to participate in the study and continues throughout study participation. Extensive discussion of risks and possible benefits of study participation will be provided to participants and their families, if applicable. A consent form describing in detail the study procedures and risks will be given to the participant. Consent forms will be IRB-approved, and the participant is required to read and review the document or have the document read to him or her. The investigator or designee will explain the research study to the participant and answer any questions that may arise. The participant will sign the informed consent document prior to any study-related assessments or procedures. Participants will be given the opportunity to discuss the study with their surrogates or think about it prior to agreeing to participate. They may withdraw consent at any time throughout the course of the study. A copy of the signed informed consent document will be given to participants for their records. The rights and welfare of the participants will be protected by emphasizing to them that the quality of their clinical care will not be adversely affected if they decline to participate in this study. The consent process will be documented in the esearch record.

14.4 **Exclusion of Women, Minorities, and Children (Special Populations)**

The subject population of this study will be 100% women over 18 years old. Children, and pregnant women will be excluded based on the benefit and risk assessment. No patient will be excluded based on race. We include only female 18 years of age or older regardless of race and speaking language. Since this study does not provide direct benefit to subjects, we determined the age limit of 18 so no state law requires parent’s consent for our subject to participate in the study.

14.5 **Participant Confidentiality**

Participant confidentiality is strictly held in trust by the investigators, study staff, and the sponsor(s) and their agents. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to any study information relating to participants.

The study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor.

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

Data forms will be completed for all subjects enrolled in the trial. The subject study files will be stored in a secure file cabinet and maintained by the research study coordinator. Study files will be kept for 7 years after the completion of the study. The PI of the study (Dr. Forsberg) and his co-investigators have previous experience running ultrasound clinical trials and the PI will serve as the study sponsor. He will be responsible for ensuring all AEs are properly reported.

The final data will be entered into a database implemented in REDCap (Vanderbilt University, Nashville, TN). The investigators will be responsible for management of the database. The database will be maintained within an organized and secure directory system.

15 Data Handling and Record Keeping

The investigators are responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported. All source documents must be completed in a neat, legible manner to ensure accurate interpretation of data. The investigators will maintain adequate case histories of study participants, including accurate case report forms (CRFs), and source documentation.

All analyses and computations will be performed using NCSS/PASS 2008 and Stata 15.1 (Stata Corporation, College Station, TX), while the study database will be designed and implemented in REDCap (Vanderbilt University, Nashville, TN). This database will contain all patient information (except names and other identifiers), including results of the cancer treatment, pathology, CEUS and PET-CT as well as the clinical variables.

15.1 Data Management Responsibilities

Data collection and accurate documentation are the responsibility of the study staff under the supervision of the investigator. All source documents and laboratory reports must be reviewed by the study team and data entry staff, who will ensure that they are accurate and complete. The investigator or designee must review unanticipated problems and deviations.

15.2 Data Capture Methods

Data reflecting participant's experiences with the study will be recorded on CRFs by the investigator.

All patient data will be captured electronically via REDCap (Vanderbilt University, Nashville, TN) in a password protected computer and image data will be stored in the secured computer hard drive. Image data will be de-identified. Copies of study documents will have personal identifying information retracted and replaced with study patient ID number. Originals of the study documents with personal identifying information will be retained at TJU. All this information will be available for audit.

15.3 Types of Data

We are committed to respecting patient’s privacy and to keeping their personal health information confidential. The personal health information includes the information in Health care records and information that can identify the patient. For example, personal information may include name, address, phone number, social security number, and medical information.

The personal health information that may be collected, used, and shared for this research includes:

- Information from medical records
- Demographic information such as name, gender, birth date, ethnicity, medical history and health care providers
- Physical examinations, procedures, imaging tests, labs, medical conditions and medications
- Information collected about any research related injury

15.4 Study Records Retention

All records pertaining to the conduct of the clinical study, including CRFs, informed consent forms, source documents, and other study documentation must be retained for seven (7) years after the end of the study.

Other study documentation includes all protocols and amendments, drug supply receipt, dispensing and final disposition records, IRB correspondence and approvals, signed consent forms, a blank copy of study consent forms, Form 1572, curriculum vitae or biosketches of members of the research team including the medical monitor, HSRRB correspondence and approval, and Statement of Investigator forms.

Source documents include all original records of observations, results, and activities necessary to reconstruct and evaluate the study. Source documents include but are not limited to surgical reports, PET-CT studies, ultrasound images, subject progress notes, hospital charts, radiologic reports or pharmacy records, and any other records or reports of procedures performed during the study. Source documents also may include copies of the CRF or sponsor supplied worksheets when original information is recorded directly onto these forms.

Whenever possible, an original recording of an observation should be retained as the source document. However, a photocopy of a record is acceptable provided it is legible and is a verified copy of the original document.

15.5 **Protocol Deviations**

A protocol deviation is any noncompliance with the protocol, International Conference on Harmonization (ICH), Good Clinical Practice (GCP) and protocol-specific guidelines. The deviation (any activity conducted outside the parameters established by the protocol) may be either on the part of the participant, the investigator, or the study site staff and may or may not pose a risk to participants or others or may affect the integrity of the data obtained from the study.

The risk posed by the deviation, to the study or the study participant gives rise to an Unanticipated problem (UAP). It is crucial to document the deviation/unanticipated problem in the protocol deviation log (Appendix D) and submitted to the IRB as per the sites regulations. As a result of deviations, corrective actions are to be developed and implemented promptly.

UAPs and protocol deviations that pose risk to participants or others, and that are not AEs, or that affect study integrity will be submitted to the IRB via the <eazUP system> within 5 working days of the investigator becoming aware of the event.

UAPs and protocol deviations that do not pose risk to participants or others and do or do not affect study integrity must be entered in the deviation log (Appendix D) and submitted to the IRB at the next continuing review.

GE Healthcare will be informed of all AEs within 48 hours. In addition all unexpected SAEs are reported to the TJU IRB, the SKCC DSMB and to the FDA.

Relationship to Sonazoid Administration

The relationship or association of the AE to the Sonazoid administration will be characterized as "unlikely," "possible," or "probable." A relationship assessment will be performed by the investigator to determine if an AE is attributable to Sonazoid and will be recorded on a data form. The investigator will refer to the Sonazoid investigator brochure for assistance in determining AE relationship.

An "unlikely" relationship indicates that there is little or no chance that Sonazoid caused the reported AE; other conditions, including concurrent illnesses, progression or expression of the disease state, or a reaction to a concurrent medication, appear to explain the reported AE.

A "possible" relationship indicates that the association of the AE with Sonazoid is unknown. However, the AE is not reasonably attributed to any other condition.

A "probable" relationship indicates that a reasonable temporal association exists between the AE and Sonazoid administration and, based upon the investigator's clinical experience, the association of the event with the trial medication seems likely.

16 Study Finances

16.1 Funding Source

This study is financed through a grant from the U.S. National Institute of Health (NIH): R21 CA249870.

16.2 Conflict of Interest

Any investigator who has a conflict of interest with this study (patent ownership, royalties, or financial gain greater than the minimum allowable by their institution, etc.) must have the conflict reviewed by a properly constituted Conflict of Interest Committee with a Committee-sanctioned conflict management plan that has been reviewed and approved by the study sponsor prior to participation in this study. All Jefferson University Investigators will follow the TJU Conflicts of Interest Policy for Employees (107.03).

16.3 Participant Stipends or Payments

Participants will not receive payment for participating in this study.

17 Publication and Data Sharing Policy

Upon study completion and finalization of the study report the results of this study will either be submitted for publication and/or posted in a publicly assessable database of clinical study results. The results of this study will also be submitted to the Competent Authority and the Ethics Committee according to USA national regulations.

All personnel who have contributed significantly with the planning and performance of the study (Vancouver convention 1988) may be included in the list of authors.

18 Literature References

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Appendices

The following documents are officially affiliated with the protocol and will be submitted to the IRB as a part of the protocol. As such, changes to these items require a protocol amendment.

Appendix A: Schedule of Events

Investigator Obligations

A. Institutional Review Board (IRB) and Human Subjects Research Review Board (HSRRB) Review/Approval

The protocol and informed consent for this study must be reviewed and approved by an appropriate IRB and HSRRB prior to enrollment of participants in the study. It is the responsibility of the investigator to assure that all aspects of the ethical review are conducted in accordance with FDA Regulations 21 CFR Part 56. A letter documenting the IRB and HSRRB approval which specifically identifies the study/protocol must be obtained by the investigator prior to initiation of the study. Amendments to the protocol will be subject to the same requirements as the original protocol. The HSRRB must review and approve each modification to the study prior to implementation.

A progress report with a request for re-evaluation and re-approval will be submitted by the investigator to the IRB and HSRRB at intervals required by the IRB, and not less than annually.

After completion or termination of the study, the investigator will submit a final report to the IRB. This report should include: deviations from the protocol, the number and types of participants evaluated, the number of participants who discontinued (with reasons), results of the study, if known, and all AEs, including deaths.

B. Informed Consent

Signed, written informed consent which conforms to FDA Regulation 21 CFR Part 50, must be obtained from each participant prior to entering the study. Each participant will be provided a written consent form and verbal information in an understandable manner which describes the nature and duration of the study. The research study coordinator or

the investigator will conduct the informed consent interview in a private examination room. The potential subject will be allowed to discuss the study with the investigator, research study coordinator, or any persons who may have accompanied the potential subject. Additionally, the participant must be allowed adequate time to consider the potential risks and benefits associated with his participation in the study. The research study coordinator will sign the informed consent as the person conducting the consent interview.

C. Data Reporting and Data Forms

Data reflecting participant's experiences with the study will be recorded on CRFs by the investigator.

D. Records Retention

All records pertaining to the conduct of the clinical study, including CRFs, informed consent forms, source documents, and other study documentation must be retained for seven (7) years after the end of the study.

Other study documentation includes all protocols and amendments, drug supply receipt, dispensing and final disposition records, IRB correspondence and approvals, signed consent forms, a blank copy of study consent forms, Form 1572, curriculum vitae or biosketches of members of the research team including the medical monitor, HSRRB correspondence and approval, and Statement of Investigator forms.

Source documents include all original records of observations, results, and activities necessary to reconstruct and evaluate the study. Source documents include but are not limited to UEA procedure reports, MRI studies, ultrasound images, subject progress notes, hospital charts, radiologic reports or pharmacy records, and any other records or reports of procedures performed during the study. Source documents also may include copies of the CRF or sponsor supplied worksheets when original information is recorded directly onto these forms.

Whenever possible, an original recording of an observation should be retained as the source document. However, a photocopy of a record is acceptable provided it is legible and is a verified copy of the original document.

E. Deviation from the Protocol

The investigator will not deviate from the protocol without prior written approval from the IRB and the HSRRB. In medical emergencies, the investigator will use medical judgment and remove the participant from immediate hazard. The HSRRB and the IRB will be notified regarding the type of emergency and course of action taken. Any other changes to or deviations from the protocol will be made as an amendment to the protocol. The amendment must be submitted for review and approval to the local IRB and the HSRRB for review and approval.

F. Roles and Responsibilities of Study Personnel

Flemming Forsberg, PhD, Professor of Radiology and Director of Ultrasound Physics, will serve as Principal Investigator on this project. He will be responsible for the scientific goals of the project. Dr. Forsberg will oversee patient recruitment, informed consent, ultrasound studies, and the data entry and statistical analyses. He will also supervise the CEUS data acquisition from patients. Dr. Forsberg will also prepare any manuscript(s) resulting from this grant.

Norman G. Rosenblum, MD, PhD Professor of Obstetrics and Gynecology will assist with the patient recruitment, UCA injections and advise on clinical issues.

Scott D. Richard, MD, Associate Professor of Obstetrics and Gynecology will assist with the patient recruitment, UCA injections and advise on clinical issues.

Lindsey E. Minion, MD, Associate Professor of Obstetrics and Gynecology will assist with the patient recruitment, UCA injections and advise on clinical issues.

Andrej Lyshchik, M.D., Ph.D, Assistant Professor of Radiology will aid in patient recruitment, informed consent, ultrasound studies, and advise on clinical issues.

Ji-Bin Liu, M.D., Research Professor of Radiology will assist with the patient recruitment, interpret ultrasound images and advise on clinical issues

Priscilla Machado, MD, Project Manager will perform ultrasound studies and will also prepare any manuscript(s) resulting from this project.