PROTOCOL

TITLE: TWO AND THREE DIMENSIONAL CONTRAST-ENHANCED ULTRASOUND FOR SCREENING OF RENAL CELL CARCINOMA RECURRENCE FOLLOWING CRYOABLATION

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STUDY DURATION: 2 Years

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SYNOPSIS

Protocol Title: Two and three dimensional contrast-enhanced ultrasound for screening of renal cell carcinoma recurrence following cryoablation

Trial Objectives: The primary objective of this trial is to:

- Evaluate if contrast-enhanced ultrasound can be used to successfully detect recurrence of renal cell carcinoma following cryoablation therapy when compared to the gold standards of contrast enhanced computed tomography or magnetic resonance imaging.

The secondary aim of this trial is to:

- Establish whether three-dimensional contrast-enhanced ultrasound provides superior screening of renal cell carcinoma recurrence relative to two-dimensional contrast-enhanced ultrasound.

Trial Design: This is an open-label, non-randomized trial that will be conducted at one clinical site. The subject population will be patients scheduled for follow up contrast enhanced computed tomography (CT) or magnetic resonance imaging (MRI) of a previously cryoablatated renal cell carcinoma (RCC) through Thomas Jefferson University's Urology clinic. Eligible follow up imaging will include patients being seen for an 8, 12, 18, 24, or 36 month CT/MRI follow up. The study will consist of a single ultrasound exam, although patients will be eligible to re-enroll in multiple studies (for example exams at 8, 12 and 18 months), if these exams fit within the study timeframe.

Ultrasound imaging will be performed using a state of the art ultrasound scanner with two and three dimensional curvilinear transducers. If available, image fusion may also be used from patient's previous MR/CT imaging. Patients will first undergo baseline grayscale B-mode and power Doppler imaging (PDI) of the treated mass. Patients will then receive a bolus 1 ml contrast injection of Optison (GE Healthcare, Princeton, NJ), while being imaged with two-dimensional (2D) dual mode B-mode / coded harmonic imaging. Following a 10 minute wait period to allow for full clearance of Optison, patients will receive a second bolus of contrast agent along with three-dimensional (3D) nonlinear imaging. Tumor vascularity and flow kinetics will be determined using both modes and compared to the patient's corresponding contrast-enhanced MRI or CT exams.

Trial Population: This trial will consist of up to 100 adults (18 years of age or older) who have previously undergone cryoablation of RCC at Thomas Jefferson University.

Trial Procedures: Subjects eligible for trial enrollment will be identified by the co-investigators, Drs.

Shaw, Lallas and Trabulsi from their patient population of subjects being seen for follow up of a cryoablated renal mass. These patients will be accrued from the Urology department, as well as from the Small Renal Mass Center at the Genitourinary Multidisciplinary Cancer Center of the Kimmel Cancer Center at Thomas Jefferson University. Current clinically-indicated follow up intervals include 8, 12, 18, 24, or 36 MR/CT post cryoablation. A 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 coordinator will review the consent form with the patient and then the patient will be given the form to review. The patient, coordinator, and a study investigator will all sign the consent form. The patient will be given a copy of the signed consent form for their records.

After consenting to participate in this study, a full demographic profile, known drug allergies or intolerances, and 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).

Study participation will consist of a single ultrasound exam. A baseline ultrasound grayscale scan will be used to identify the tumor and to evaluate the following criteria: size, shape, and orientation of the lesion; echogenicity compared to surrounding tissue. Standard PDI of the lesion will also be performed. When possible, previous MR/CT data will be uploaded onto the scanner in order to perform image fusion for ultrasound guidance during imaging. The distribution of color signals and the overall color content of the tumor will be evaluated by comparing the pattern and amount of color to the normal surrounding tissue. Following baseline scanning, patients will receive a 1 ml intravenous injections of Optison (GE Healthcare, Princeton, NJ) in a peripheral vein followed by renal mass imaging in 2D coded harmonic imaging (CHI; a nonlinear contrast imaging package). Ten minutes after the 2D contrast-enhanced ultrasound (CEUS) exam, patients will receive a second bolus injection of Optison followed by 3D CHI (using a curvilinear 3D array). All contrast injections will be supervised by a board certified physician. Resuscitation equipment and trained personnel will be immediate proximity to the patient during each contrast-enhanced ultrasound exam. Digital clips of the exams will be recorded through the contrast agent wash-in and wash-out phases for at least two minutes and stored for later use. The subject will then be monitored for adverse reactions for 30 minutes. If the patient wishes to continue participating in the study, they will be eligible for CEUS exams during their future clinically scheduled follow up exams as long as these take place within the study timeframe.

Both 2D and 3D CEUS exams and the clinically-indicated contrast-enhanced MRI/CT exam will be

independent and randomly reviewed by two blinded radiologists with experience in both CEUS and body imaging. Readers will evaluate the presence of signal enhancement within the mass, signal distribution and heterogeneity, signal intensity relative to the normal renal parenchyma, and contrast agent wash-in and wash-out kinetics relative to the surrounding parenchyma. The readers will also be asked to evaluate if CEUS findings indicate RCC recurrence. Recurrence will initially be defined as the presence of contrast enhancement within the ablation cavity. Finally, following a one month wait period and randomization, readers will again review cases in order to calculate intra-reader variability.

The proposed agent for the current study, Optison™ is a sterile non-pyrogenic suspension of encapsulated perflutren microbubbles [Goldberg et al 2001; Miller & Nanda 2004]. The contrast agent is consists of a human serum albumin shell with perflutren core and contains 5.0 - 8.0 x 10^8 microspheres/ ml. The microbubble agent is supplied in a standard-size 3 ml vial and is prepared by manually shaking the vial. Optison will be stored in a secure refrigerator, with only the study investigators and research personnel having access. Optison is currently only approved for use in echocardiography. The agent will be used as an off-label indication for this study.

Statistical Methodology: This project is a pilot study to determine if CEUS can be used for the evaluation renal cell carcinoma recurrence post cryoablation.

Our statistical analysis will address two major questions:

1. Does renal mass vascularity and blood flow kinetics measured on CEUS correlate with findings on contrast-enhanced MRI/CT (and thus, can be used as a possible recurrence screening imaging alternative)?
2. Does the inclusion of volumetric ultrasound improve the sensitivity and specificity of CEUS for monitoring recurrence of RCC post cryotherapy?

Sensitivity, specificity, accuracy, positive predictive value, and negative predictive value will be calculated for the CEUS exams using both the standard of care contrast-enhanced MRI or CT, or pathology when retreatment is indicated as the reference standards. The dichotomous parameter needed for these calculations will be derived by ascertaining whether blinded radiologists would recommend repeated treatment or not based on the degree of residual blood flow observed during the CEUS study. This parameter will be based off of each reader's first read. In the event of discordant readings during this analysis, recurrence will be decided by consensus by the two readers. Correlations between these findings and both contrast-enhanced cross-sectional findings and patient outcomes will be compared using the Fisher exact test. Inter- and intra-observer variability will also be calculated. Quantitative parameters of mass vascularity, changes in perfusion, and changes in contrast fill time will also be

computed and again correlated to the clinical standard.

1. INTRODUCTION

Renal cell carcinoma (RCC) is becoming increasingly common within the US, with over 64,000 new cases and 13,500 deaths expected to occur in 2012 [NCI 2012]. Detection rates of RCC have doubled over the last 50 years, with nearly half of reported cases being detected incidentally during cross sectional imaging [Venkatesan et al. 2011]. While surgical extirpation remains the standard of care, cryoablation therapy is playing a larger role in the treatment of poor surgical candidates and also can preserve renal function [Levinson et al 2008]. Cryoablation is performed at our institution using a Cryocare Surgical System (HealthTronics, Austin TX; formerly EndocareInc, Irvine CA), an FDA approved device for the ablation of malignant tissues (510(k) K003811). Protocols for follow up imaging vary, but mainly rely on contrast-enhanced CT or MRI 1-6 months post cryoablation to evaluate effective ablation based on a lack of vascularity within the mass as well as a reduction in tumor volume [Gervais et al 2008b; Zagoria 2004]. At our institution contrast-enhanced CT or MRI at 3-4 months represents the clinical standard, as earlier imaging has been shown to result in false-positives due to artifacts associated with post-ablation inflammation [Thumar et al. 2010]. Assuming successful treatment, serial follow up imaging is then scheduled in 4 month intervals the first year, 6 month intervals during year 2, and annually three years post treatment. While well validated in RCC recurrence post cryoablation, these contrast materials have been associated with nephro-toxicity (iodinated contrast) and nephrogenic systemic fibrosis (gadolinium-based contrast) [FDA Alert 2006] and are not well suited for a patient population with compromised renal function. Thus, an imaging technique which could effectively screen for recurrence without the associated adverse renal side effects would be beneficial in this patient population.

Ultrasound contrast agents are gas filled microbubbles, encapsulated by a lipid shell for stability. Due to their size (1-8 μm in diameter), these agents are small enough to pass through the pulmonary capillaries, but still restricted to the vascular system [Goldberg et al. 2001]. These microbubbles have been approved for use echocardiography in the United States and also for the characterization of liver lesions throughout Europe and Asia [Bouakaz and de Jong, 2007]. This project will investigate the applicability of CEUS for monitoring RCC recurrence post cryoablation using both 2D and 3D ultrasound. Because ultrasound contrast agents act as a blood pooling agent, enhancement and contrast kinetics on CEUS are expected to correlate well to those observed on MR/CT.

1.1 Background

Kidney and renal pelvis cancers account for 5 and 3% of new cases of cancer diagnosis in US men and women, respectively [ACS 2011]. When clinical intervention is deemed necessary open or laparoscopic surgery represents the first course of treatment [ACS 2011]. In patients not eligible for surgery such as those with multiple renal masses or those with comorbid conditions, ablation treatments are preferred to better preserve renal function [Gervais et al. 2005a]. Ablation of RCC can be performed laparoscopically or percutaneously. However, percutaneous ablation has been associated with fewer probes, shorter anesthesia times, shorter hospital stays, lower hospital charges, and quicker time to total recovery relative to laparoscopic approaches [Bandi et al. 2008; Hinshaw et al. 2008]. Percutaneous ablation can be performed with RF heating (50-60º C for effective cell death) or cryoablation (-40 to -50º C for cell death). The current clinical standard at Thomas Jefferson for renal ablation is cryoablation for two reasons: cryoablation creates an ice ball which can be tracked during the ablation session and is also significantly less painful than heat based techniques which avoids the use of general anesthesia in patients who are frequently suboptimal candidates for general anesthesia [Allaf et al. 2005]. Cryoablation is performed using a Cryocare Surgical System (HealthTronics, Austin TX; formerly EndocareInc, Irvine CA), an FDA approved device for the ablation of malignant tissues (510(k) K003811). Follow up is initially performed by contrast-enhanced CT or MRI at 3-4 months post cryoablation. Assuming successful treatment, serial follow up imaging is then scheduled in 4 month intervals the first year, 6 month intervals during year 2, and annually three years post treatment. Unfortunately contrast administration with both of these imaging modalities has well-described toxicity: iodinated intravenous contrast given with CT is nephrotoxic, especially in patients with renal impairment; and gadolinium-based contrast given with MRI has been associated with nephrogenic systemic fibrosis in patients with advanced kidney disease.

Ultrasound contrast agents are well tolerated and administered intravenously, making them an ideal noninvasive screening tool. Commercially available contrast imaging packages are now available on most ultrasound scanners. Coded harmonic imaging (CHI) represents the current industry standard and improves contrast to tissue ratios by receiving higher frequency harmonics generated by the ultrasound contrast agents [Goldberg et al. 2001]. It has been shown that nonlinear imaging using these agents provides visualization of internal tumor morphology better than PDI [Forsberg et al 2007].

The majority of literature involving CEUS and renal masses involves classification prior to biopsy. Tamai et al. performed CEUS on 29 patients prior to resection and biopsy and found sensitivities of 94.4 and 88.9% for CEUS and contrast CT respectively but specificities of 45.5 and 72.7% [Tamai 2005]. In a larger study involving 143 patients with renal masses, Ignee et al. found that CEUS had a sensitivity of

97% and specificity of 45% when predicting malignancy [Ignee et al. 2010]. Additionally, Williams et al. used CEUS with a flash replenishment imaging mode to measure RCC tumor response to the antiangiogenic drug sunitinib and found a significant drop in fractional vascularity over a two week treatment regime (73.2%, $p < 0.002$), but no correlation with progression free survival.

One of the few studies in the literature using CEUS to evaluate renal ablation was performed by Meloni and colleagues [2008]. Using CEUS as a follow up after percutaneous RF ablation, the group found a sensitivity of 96.6% and specificity of 100% when comparing to either the clinical standard of contrast enhanced MRI or CT. These results are quite convincing, and indicate CEUS should also be useful as a follow up image modality for renal cryoablation. However, this group did not use an FDA approved ultrasound contrast agent and did not use either a standardized ultrasound system or clinical standard. Consequently, our group has proposed the use of CEUS for monitoring the effectiveness of renal mass cryoablation [Eisenbrey et al. 2014]. In one of the first studies using CEUS as an imaging tool before and after cryoablation, our group showed that all masses initially showed heterogeneous enhancement with both subharmonic (an emerging nonlinear imaging technique) and CHI. No adverse events were observed during the study. The optimal dosages of Optison for renal imaging were found to be 1 ml for CHI and 2 ml for subharmonic imaging. In cases where the lesion was adequately visualized at 4 month follow-up subharmonic and harmonic ultrasound showed accuracies of 83% and 75%, respectively in predicting treatment outcome, with a lack of enhancement on CEUS correlating to lack of enhancement on MRI/CT. Additionally, relative to the ablation cavities measured on contrast-enhanced MRI/CT, SHI ablation volumes resulted in a mean error of 3.8 ± 5.4 cm^3 ($p = 0.29$), and HI resulted in a mean error of 1.7 ± 1.2 cm^3 ($p = 0.39$) [Eisenbrey et al. 2014]. However, no cases of recurrence were observed in this study, making it difficult to define the technique's predictive values.

The proposed agent for the current study, Optison is a sterile non-pyrogenic suspension of encapsulated perflutren microbubbles [Goldberg et al 2001; Miller & Nanda 2004]. The contrast agent is consists of a human serum albumin shell with perflutren core and contains $5.0 - 8.0 \times 10^8$ microspheres/ ml. The microbubble agent is supplied in a standard-size 3 ml vial and is prepared by manually shaking the vial. Optison is currently only approved for use in echocardiography. The agent will be used as an off-label indication for this study.

Optison Clinical Safety

Optison is well tolerated and has been used extensively in echocardiography applications [Goldberg et al 2001]. In pre-market clinical trials, Optison was administered to 279 patients. In these patients 47

(16.8%) reported at least one adverse event. Of these events, 1 was classified as serious and required antihistamines for hypersensitivity manifestations of dizziness, nausea, flushing, and temperature elevation. No deaths were reported. Of the reported adverse reactions following the use of Optison the most frequently reported were headache (5.4%), nausea and/or vomiting (4.3%), warm sensation or flushing (3.6%), and dizziness (2.5%). Additional risks associated with the contrast material are described in the attached Optison Product insert (Appendix B). All of the non-serious reported side effects have been transient, usually lasting only a few minutes.

Table 1. Selected Adverse Events Reported in ≥ 0.5% of the Subjects who Received Optison in Controlled Clinical Studies (From Optison Product Insert).

SELECTED ADVERSE EVENTS REPORTED IN ≥ 0.5% OF THE SUBJECTS WHO RECEIVED OPTISON™ IN CONTROLLED CLINICAL STUDIES	
No. of Patients Exposed to OPTISON™	279
No. of Patients Reporting on Adverse Event	47 (16.8%)
Body as a Whole	38 (13.6%)
Headache	15 (5.4%)
Warm Sensation/Flushing	10 (3.6%)
Chills/fever	4 (1.4%)
Flu-like Symptoms	3 (1.1%)
Malaise/Weakness/Fatigue	3 (1.1%)
Cardiovascular System	12 (4.3%)
Dizziness	7 (2.5%)
Chest Pain	3 (1.1%)
Digestive System	12 (4.3%)
Nausea and/or Vomiting	12 (4.3%)
Nervous System	3 (1.1%)
Respiratory System	5 (1.8%)
Dyspnea	3 (1.1%)
Skin & Appendages	11 (3.9%)
Injection Site Discomfort	3 (1.1%)
Erythema	2 (0.7%)
Special Senses	9 (3.2%)
Altered Taste	5 (1.8%)
(1) Patients are counted separately within each body system. (2) The body system is reported if the aggregate is ≥ 0.5%. Details are not shown if the subsystem is not ≥ 0.5%.	

Additional information concerning pre-clinical and clinical experience with Optison, including the dosing levels and reported subject complaints, can be found in the Optison Package Insert that is included as Appendix B.

1.2 Rationale

The fundamental theory behind this study is that 2D and 3D CEUS, which uses a blood pooling agent for contrast, will provide a good indicator of changes in vascularity post-ablation over time, and that these findings will correlate with MRI/CT recurrence findings. If CEUS correlates with the current clinical standard of a contrast-enhanced MRI or CT, this alternative will lead to substantial cost savings and a non-nephrotoxic follow up imaging alternative in patients with renal compromise.

We propose a clinical trial to determine the feasibility of using 2D and 3D CEUS for the evaluation of RCC recurrence post cryoablation. The purpose of this study is to compare ultrasound derived vascularity and blood flow measurements post ablation to both the clinical evaluation standard and patient outcomes (when retreatment is indicated and pathology becomes available).

2. TRIAL OBJECTIVES

Trial Objectives: The primary objective of this trial is to:

- Evaluate if CEUS can be used to successfully detect RCC recurrence in patients who are up to three years post cryotherapy.

The secondary aim of this trial is to:

- Establish whether 3D CEUS provides an improvement over 2D CEUS in monitoring cryoablation therapy and detecting recurrence.

The tertiary aim of this trial is to:

- Add to the emerging literature on the safety of Optison in patients with renal impairment.

3. TRIAL DESIGN

This is an open-label, non-randomized trial that will be conducted at one clinical site. The subject population will be patients scheduled for follow up contrast enhanced CT or MRI of a previously cryoablatated RCC through Thomas Jefferson University's Urology clinic. Eligible follow up imaging will include patients being seen for an 8, 12, 18, 24, or 36 month CT/MRI follow up. The study will consist of a single ultrasound exam, although patients will be eligible to re-enroll in multiple studies (for example exams at 8, 12 and 18 months), if these exams fit within the study timeframe. Study participation will consist of a single ultrasound exam. During each exam patients will undergo a grayscale B-mode, power Doppler, and 2 CEUS exams of the tumor. The CEUS will involve 1 ml bolus injections of Optison through an 18- to 20-gauge angiocatheter placed in a peripheral arm vein, preferably an

antecubital vein. Each bolus injection of Optison will be followed with a slow flush of 5 ml of normal saline. CEUS will first be performed using a 2D curvilinear transducer at a low mechanical index in CHI mode. Data will be stored over the contrast wash-in and wash-out cycle and compared to MRI / CT follow up results and patient outcomes. When available, image fusion may also be incorporated to aid in scanning and lesion identification. The second CEUS exam will then use a 3D curvilinear probe to provide volumetric CHI of the mass. At a minimum, volumes will be acquired at baseline, contrast wash-in, peak enhancement, and during contrast wash-out. Volumes will again be compared to MRI / CT follow up results and patient outcomes.

3.1 Trial Duration

Individual participation for the majority of patients will be limited to 1 ultrasound exam during their cryoablation follow up in Thomas Jefferson University's Urology clinic. However, patients will be eligible for serial imaging (for example exams at 8, 12 and 18 months), if these exams fit within the study timeframe. Exams will take place the day of the patient's MRI/CT follow up or during their Urology clinic follow up (in cases where MR/CT imaging is performed outside of Jefferson). The entire ultrasound imaging protocol will require approximately one hour including a 30 min observation period. Patients will be monitored for at least 30 minutes before being discharged.

Subject recruitment is expected to last 22 months (January 2015 – November, 2016). Analysis and publication of results are expected to take an additional 2 months (November 2016– January, 2016). Volunteer Registry Database forms will be submitted to the National Institute of Health's Office of Regulatory Compliance and Quality at the completion of the research study.

4. TRIAL POPULATION

Trial Population: This trial will consist of up to 100 adults (18 years of age or older) undergoing MRI/CT imaging to monitor recurrence of RCC following cryoablation therapy.

Subjects eligible for trial enrollment will be identified by the co-investigators, Drs. Shaw, Lallas and Trabulsi from their patient population of subjects who have previously undergone cryoablation of RCC and are being monitored for recurrence. These patients will be accrued from the Urology department, as well as from the Small Renal Mass Center at the Genitourinary Multidisciplinary Cancer Center of the Kimmel Cancer Center at Thomas Jefferson University Hospital. A 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 coordinator will review the consent form with the patient and then the patient will be given the form to review. The patient, coordinator, and a study investigator will all sign the consent form. The patient will be given a copy of the signed consent form for their records.

4.1 Inclusion Criteria

All subjects accepted for this trial must:

- Previously received cryotherapy of RCC.
- Be scheduled for contrast-enhanced MRI/CT to monitoring of RCC recurrence as part of their 8, 12, 18, 24, or 36 month CT/MRI follow up.
- Be at least 18 years of age.
- Be medically stable.
- If a female of child-bearing age, must have a negative pregnancy test.
- Have signed Informed Consent to participate in the study.

4.2 Exclusion Criteria

Subjects with any of the following conditions or who have had the following procedures will be excluded from this trial:

- Females who are pregnant or nursing.
- 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 unstable occlusive disease (eg, crescendo angina)
  - Patients with clinically unstable cardiac arrhythmias, such as recurrent ventricular tachycardia.
  - Patients with uncontrolled congestive heart failure (NYHA Class IV)
- Patients with recent cerebral hemorrhage.
- Patients with known sensitivities to albumin, blood, or blood products
- Patients who have undergone surgery within 24 hours prior to the study sonographic examination.
- Patients with known hypersensitivity to perflutren
- Patients with cardiac shunts.
- Patients with congenital heart defects.
- Patients with severe emphysema, pulmonary vasculitis, or a history of pulmonary emboli.
- Patients with respiratory distress syndrome.
- Patients with renal insufficiency such that they cannot get intravenous contrast as part of screening or follow-up.

Subject identification will be maintained with a study specific alphanumeric code including the subject number (01-100).

5. MEDICATIONS

Optison will be provided by GE Healthcare, Princeton, NJ.

Optison is a sterile non-pyrogenic suspension of encapsulated perflutren microbubbles [Goldberg et al 2001; Miller & Nanda 2004]. The contrast agent is consists of a human serum albumin shell with perflutren core and contains 5.0 - 8.0 x 10^8 microspheres/ ml. The microbubble agent is supplied in a standard-size 3 ml vial and is prepared by manually shaking the vial. Detailed instructions are provided in the Optison Product Insert, found in Appendix B.

Optison will be stored in a secure refrigerator, with only the study investigators and research personnel having access. Unused drug and empty vials will be properly disposed of after reconciling the log of study drug kept at Thomas Jefferson University.

5.1 Administration

All contrast injections will be supervised by a board certified physician. Resuscitation equipment and trained personnel will be immediate proximity to the patient during each contrast-enhanced ultrasound exam. Optison will be administered by bolus IV injection through an 18- to 20-gauge angiocatheter placed in a peripheral arm vein, preferably an antecubital vein. Subjects will be instructed not to move their arm during the administration of the contrast agent.

5.2 Contraindications

Optison should not be administered to patients with known or suspected hypersensitivity to perflutren, blood, or blood products. The safety of Optison in patients with 1) right-to-left, bi-directional or transient right-to-left cardiac shunts; 2) severe emphysema, pulmonary vasculitis or a history of pulmonary emboli; 3) respirator distress syndrome has not been studied. Therefore, patients with any of these conditions will be excluded from participation.

5.3 Randomization

This is a non-randomized trial; therefore, no randomization procedure is required.

5.4 Blinding and Unblinding Methods

This is an open-label trial; therefore, no blinding or unblinding procedures for the trial drug are required.

5.5 Storage

Optison vials will be stored in a secure refrigerator, with only the study investigators and research personnel having access. The study research nurse will be responsible for drug suspension and inventory control.

6. TRIAL PROCEDURES

6.1 Patient Enrollment

Trial Procedures: Subjects eligible for trial enrollment will be identified by the co-investigators, Drs. Shaw, Lallas and Trabulsi from their patient population of subjects under active surveillance for recurrence of a previously cryoablated RCC. A 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 coordinator will review the consent form with the patient and then the patient will be given the form to review. The patient, coordinator, and a study investigator will all sign the consent form. The patient will be given a copy of the signed consent form for their records.

6.2 Screening Assessments

Screening assessments will be performed prior to CEUS imaging. 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 age, she will have a urine pregnancy test (the results of which will be made available to the subject prior to study initiation).

6.3 Ultrasound Imaging

All contrast injections will be supervised by a board certified physician. Resuscitation equipment and trained personnel will be in immediate proximity to the patient during each CEUS exam. The ultrasound examinations will be performed by a qualified sonographer. Ultrasound exams will take place the morning prior to the patient's 8, 12, 18, 24, or 36 month CT/MRI follow up (scheduled as part of their clinical care) or during their consultation in Jefferson's Urology clinic (for patients who have imaging performed outside Jefferson). 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. For the ultrasound examination, the patient will be asked to lie in the supine position and a catheter will be placed in a superficial vein (preferably an antecubital vein). Acoustic coupling gel will be applied to the area of interest. A state of the art ultrasound scanner with 2D and 3D curvilinear probes will be used.

A baseline ultrasound grayscale scan will be used to identify the tumor and to evaluate the following criteria: size, shape, and orientation of the lesion; echogenicity compared to surrounding tissue. Standard power Doppler of the lesion will also be performed. When possible, previous MR/CT data will be uploaded onto the scanner in order to perform image fusion for ultrasound guidance during imaging. The distribution of color signals and the overall color content of the tumor will be evaluated by comparing the pattern and amount of color to the normal surrounding tissue. Following baseline scanning, patients will receive a 1 ml intravenous injection of Optison (GE Healthcare, Princeton, NJ) in a peripheral vein followed by renal mass imaging in 2D CHI (a nonlinear contrast imaging package). Ten minutes after the 2D CEUS exam, patients will receive a second bolus injection of Optison followed by 3D CHI (using a curvilinear 3D array). All contrast injections will be supervised by a board certified physician. Resuscitation equipment and trained personnel will be immediate proximity to the patient during each contrast-enhanced ultrasound exam. Digital clips of the exams will be recorded through the contrast agent wash-in and wash-out phases for at least two minutes and stored for later use. The subject will then be monitored for adverse reactions for 30 minutes. If the patient wishes to continue participating in the study, they will be eligible for CEUS exams during their future clinically scheduled follow up exams as long as these take place within the study timeframe.

6.4 Safety Monitoring

Patients will be monitored for AEs during and 30 minutes after contrast administration. All other procedures will be performed according to standard of care. No further patient monitoring is required as side effects of Optison are acute in nature and no renal toxicity has been reported (See Table 1, section 2.1).

6.5 Efficacy Assessments

Both 2D and 3D CEUS exams and the clinically-indicated contrast-enhanced MRI/CT exam will be independent and randomly reviewed by two blinded radiologists with experience in both CEUS and body imaging. Readers will evaluate the presence of signal enhancement within the mass, signal distribution and heterogeneity, signal intensity relative to the normal renal parenchyma, and contrast agent wash-in and wash-out kinetics relative to the surrounding parenchyma. The readers will also be asked to evaluate if CEUS findings indicate RCC recurrence. Recurrence will initially be defined as the presence of contrast enhancement within the ablation cavity. Finally, following a one month wait period and randomization, readers will again review cases in order to calculate intra-reader variability. Both qualitative and quantitative findings will be obtained for the ultrasound study. Sensitivity, specificity,

accuracy, positive predictive value, and negative predictive value will be calculated for the contrast-enhanced ultrasound exam using both the standard of care contrast-enhanced CT or MRI, and pathology (if retreated) as the reference standards. The dichotomous parameter needed for these calculations will be derived by ascertaining whether blinded radiologists would recommend further treatment or not based on the degree of residual blood flow observed during the CEUS study. Correlations between these assessments and both contrast-enhanced MRI or CT findings will be compared using the Fisher exact test. Inter- and intra-observer variability will also be calculated. Quantitative parameters of mass vascularity, perfusion, and contrast fill time will be computed relative to the surrounding renal parenchyma and again correlated to the clinical standard. Finally, the patient's billed amount for their contrast-enhanced MRI/CT exam (cost of exam prior to insurance reimbursements) will be recorded and compared to the cost of a real ultrasound exam and one vial of ultrasound contrast agent to compare potential cost savings to the patient. Quantitative parameters will be compared between recurrent and disease free groups using a Student's t-test.

6.6 Safety Assessments

Adverse events will be monitored during the entire procedure. Specifically, the patient will be monitored with non-leading questions to monitor the patient for the transient side effects that are described below.

6.6.1 Risks/Benefits Assessment.

Serious cardiopulmonary and allergic reactions including fatalities have occurred during or following administration of Optison, causing the FDA to place a black box warning on the agent. However these occurrences have been rare (less than 1 in 5,000 patients). As a result, patients with cardiac shunts or unstable cardiopulmonary conditions will be excluded. All contrast injections will be supervised by a board certified physician. Resuscitation equipment and trained personnel will be immediate proximity to the patient during each contrast-enhanced ultrasound exam. Patients will also be monitored for 30 minutes after contrast administration for any adverse reactions. The majority of adverse events from Optison were transient and mild in severity. Of the reported adverse reactions following the use of Optison the most frequently reported were headache (5.4%), nausea and/or vomiting (4.3%), warm sensation or flushing (3.6%), and dizziness (2.5%).

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.

Clinically significant adverse effects from the administration of Optison are unlikely. The validation of an imaging technique which could replace a clinical standard that uses in a nephrotoxic contrast media in renally compromised patients would be of immense benefit.

To minimize and/or eliminate risks a nurse will be present during the entire procedure. Adverse events will be monitored during the entire procedure and for 30 minutes post injection.

The risk benefit ratio is low. Based on the available non-clinical and clinical safety data and the dosage levels of Optison that will be used in this study, safety concerns are minimal. The potential side effects related to Optison administration are described in Table 1 above.

6.6.2 Adverse Events

An AE includes any condition that was not present prior to trial treatment, but appeared following initiation of trial medication; any condition that was present prior to trial treatment, but worsened during trial medication; or any condition, of which the subject has a history, that was not present prior to trial medication initiation but reappeared following administration of Optison. This would include conditions that are likely to be associated with an underlying or intermittent disease (e.g., angina, flu, etc.).

The subjects will be monitored for AEs during the entire procedure. All AEs, including both observed or volunteered problems, complaints, signs or symptoms, and diagnoses, occurring from the initiation of Optison dosing until the completion of the Optison administration will be recorded on a serious or non-serious AE data form, whether or not associated with the use of the trial medication. All adverse events are reported to the Clinical Trial Support Office (CTSO) via the password protected Kimmel Cancer Center Adverse Event Reporting System. In addition all unexpected and serious adverse events (SAEs) are reported to the TJU IRB and to the Food and Drug Administration (FDA) if applicable. The investigator is required to submit all unexpected and serious adverse events to the TJU IRB and the DSMB within 48 hours. Fatal adverse events related to treatment which are unexpected must be reported within 24 hours to the TJU IRB and the DSMB through the CTSO. Fatalities not related to the study drug/device must be reported within 5 days.

The AE forms will include: subject identification number and initials; subject's date of birth, gender, and ethnicity; date of Optison administration; signs/symptoms and severity; date of onset; date of resolution or death; relationship to the study drug; action taken; concomitant medication(s) including dose, and route and duration of treatment.

Whenever possible, the AE will be evaluated and reported as a diagnosis rather than individual signs and symptoms. If a definitive diagnosis is not possible, the individual signs and symptoms will be recorded. The investigator will evaluate and note the duration, intensity, and relationship to (association with) the Optison administration, the action taken, and the determination of seriousness for each AE.

INTENSITY OF AES

The intensity of the AE will be characterized as mild, moderate, or severe. Mild AEs are usually transient, require no special treatment, and do not interfere with the subject's daily activities. Moderate AEs traditionally introduce a low level of inconvenience or concern to the subject and may interfere with daily activities but are usually ameliorated by simple therapeutic measures. Severe AEs interrupt a subject's usual daily activity and typically require systemic drag therapy or other treatment. When the intensity of the AE changes over time, the maximum intensity will be recorded.

RELATIONSHIP TO OPTISON ADMINISTRATION

The relationship or association of the AE to the Optison 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 Optison and will be recorded on a data form. The investigator will refer to the Optison Package Insert for assistance in determining AE relationship.

An "unlikely" relationship indicates that there is little or no chance that Optison 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 Optison 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 Optison administration and, based upon the investigator's clinical experience, the association of the event with the trial medication seems likely.

SERIOUS ADVERSE EVENTS

A "serious" AE (SAE) is defined as a significant clinical hazard, contraindication, or precaution that:
Results in death

- Is life-threatening (In the opinion of the investigator, there is an immediate risk of death from the AE as it occurred. This does not include an AE that had it occurred in a more serious form may have caused death.)
- Results in a persistent or significant temporary disability/incapacity defined as a substantial disruption of a person's ability to conduct normal life functions
- Results in or prolongs an existing in-patient hospitalization (an overnight stay in the hospital, regardless of length) [Note: A hospitalization for an elective procedure or treatment which is not associated with an AE, hospitalization for a pre-existing condition which did not worsen, and hospitalization for reasons of convenience or observation, do not constitute an SAE.]
- Is a congenital anomaly/birth defect (in offspring of a subject taking the trial medication, regardless of time to diagnosis)
- Is an important medical event that may not result in death, be life-threatening, or require hospitalization but based upon the appropriate medical judgment, the event may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed for the definition of a serious adverse experience.

All unexpected and serious adverse events are reported to the TJU IRB and to FDA if applicable. The investigator is required to submit all unexpected and serious adverse events to the TJU IRB and the data safety monitoring board within 48 hours. Fatal adverse events related to treatment which are unexpected must be reported within 24 hours to the TJU IRB and the DSMB through the CTSO.

The written report for any SAEs that occur during the study, whether or not related to the Optison administration will be submitted immediately (within 24 hours) to the TJU IRB.

The designated medical monitor will review all serious and unexpected adverse events associated with the protocol and provide an unbiased written report of the event within 10 calendar days of the initial report. At a minimum, the medical monitor will comment on the outcomes of the adverse event and relationship of the event to the Optison administration.

A copy of the SAE will be retained on file with the respective subject's data forms.

6.7 End-of-Treatment and End-of-Trial Evaluations

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.

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

- Has received two injections of Optison combined with 2D and 3D imaging of the area of interest

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.

Findings from this study will be submitted in abstract form to the annual meetings of the American Institute of Ultrasound in Medicine, the Radiological Society of North American, and the American Urological Association. A manuscript detailing study findings will also be submitted to either a Radiology of Urology journal.

7. DATA MANAGEMENT AND STATISTICAL ANALYSES

7.1 Data Management

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. Subject study files will be kept for 7 years after the completion of the study. The final data will be entered into a database. The investigator will be responsible for management of the database. The database will be maintained within an organized and secure directory system.

Findings from this study will be submitted in abstract form to the annual meetings of the American Institute of Ultrasound in Medicine, the Radiological Society of North American, and the American Urological Association. A manuscript detailing study findings will also be submitted to either a Radiology of Urology journal.

7.2 Statistical Analyses

Qualitative findings will be generated from Radiologist interpretations of exams. Both 2D and 3D CEUS exams and the clinically-indicated contrast-enhanced MRI/CT exam will be independent and randomly

reviewed by two blinded radiologists with experience in both CEUS and body imaging. Readers will evaluate the presence of signal enhancement within the mass, signal distribution and heterogeneity, signal intensity relative to the normal renal parenchyma, and contrast agent wash-in and wash-out kinetics relative to the surrounding parenchyma. The readers will also be asked to evaluate if CEUS findings indicate RCC recurrence. This parameter will be based off of each reader's first read. In the event of discordant readings during this analysis, recurrence will be decided by consensus by the two readers. Finally, following a one month wait period and randomization, readers will again review cases in order to calculate intra-reader variability.

Quantitative findings we be generated by calculating of mass vascularity, perfusion, and contrast fill time. This will be done using a commercial contrast quantification package, which fits wash-in and wash-out curves to signal intensity as a function of time to quantify key parameters. Contrast parameters in the area of the previously treated mass will be computed relative to the surrounding renal parenchyma and again correlated to the clinical standard. Finally, the patient's billed amount for their contrast-enhanced MRI/CT exam (cost of exam prior to insurance reimbursements) will be recorded and compared to the cost of a real ultrasound exam and one vial of ultrasound contrast agent to compare potential cost savings to the patient.

Data will first be analyzed on a per case basis (treating each case as an independent data point). However, analysis may also be performed on a patient by patient basis if a large number of enrolled patients undergo serial imaging (potentially required to ensure adequate enrollment). Sensitivity, specificity, accuracy, positive predictive value, and negative predictive value will be calculated for the qualitative data using both the standard of care contrast-enhanced CT or MRI, and pathology (if retreated) as the reference standard. The dichotomous parameter needed for these calculations will be derived by ascertaining whether blinded radiologists would recommend further treatment or not based on the degree of residual blood flow observed during the contrast-enhanced ultrasound. Correlations between these findings and both contrast-enhanced MRI or CT findings will be compared using the Fisher exact test. Inter-rater variability will be calculated using each reader's first set of reads, while intra-rater variability will be calculated by comparing each reader's initial and 1 month analysis. Intra-rater and inter-rater variability will be calculated using a Shrout and Fleiss Interclass Correlation [Shrout and Fleiss 1979]. Quantitative parameters will be compared between groups using a Student's t-test.

7.2.1 Sample Size Calculation

We hope to enroll up to 100 patients within 22 months as part of this study. This should be achievable,

given that our Urology clinic sees approximately 200 cryoablation follow up patients annually. As this is a pilot feasibility study it is difficult to estimate statistical power *a priori.* However, a power analysis was run in PASS12 (NCSS LLC, Kaysville, UT) to estimate the size of the confidence interval when estimating the test's overall level of specificity. A two sided confidence interval of one proportion was simulated using a Clopper-Pearson exact solution, assuming 100 patients and a total study recurrence rate of 5%. The relationship between the confidence level and 95% confidence interval and confidence interval width is shown below.

Confidence Level	Size (N)	Target Width	Actual Width	Proportion (P)	Lower Limit	Upper Limit	Width if P = 0.5
0.800	95		0.089	0.900	0.849	0.938	0.141
0.850	95		0.099	0.900	0.842	0.941	0.157
0.900	95		0.112	0.900	0.834	0.946	0.177
0.950	95		0.131	0.900	0.821	0.952	0.209

Thus, we expect 100 patients will enable us to estimate the specificity of the test to within 11% with 90% confidence. While multiple time points of the same patients cannot strictly be treated as independent samples (required to achieve adequate sample size within the two year window), data will also be analyzed on a per patient basis. Additionally, data from this study will also be used for future grant submissions to run a fully powered study in which conclusive hypotheses comparing 2D and 3D, as well as quantifiable parameters can be answered.

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APPENDIX A - 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 laboratory reports, electrocardiogram tracings, X-ray films, ultrasound images, subject diaries, subject progress notes, hospital charts, appointment books, 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

John Eisenbrey, PhD, Research Assistant Professor of Radiology, will serve as Principal Investigator on this project. He will be responsible for the scientific goals of the project. Dr. Eisenbrey will oversee patient recruitment, informed consent, ultrasound studies, and the data entry and statistical analyses. He will also supervise the data acquisition from patients.

Flemming Forserbg, PhD, Professor of Radiology and Director of Ultrasound Research will assist in data collection, data analysis, and publication of results.

Colette Shaw, MD, Assistant Professor of Radiology will assist with the patient recruitment, interpret ultrasound images and advise on clinical issues.

Edouard Trabulsi, MD Associate Professor of Urology will assist with the patient identification and recruitment, interpret clinical results and advise on clinical issues.

Costas D. Lallas, MD, Assistant Professor of Urology will assist with the patient identification and recruitment, interpret clinical results and advise on clinical issues.

Ji-Bin Liu, MD, Research Professor of Radiology will assist with interpretation of results and advise on clinical issues.

Andrej Lyshchik, MD, Assistant Professor of Radiology will assist with interpretation of results and advise on clinical issues

Maria Stanczak, MS, Research Sonographer in the Department of Radiology will be responsible for performing the ultrasound examinations and interpreting results.

Colleen Dascenzo, CCRC, will be responsible for screening, recruiting, and scheduling patients and will explain the study to them. In addition, she/he will perform data entry.

Patrick O'Kane, M.D., Assistant Professor of Radiology, will act as the medical monitor for this project.

Signature of PI: _____
John Eisenbrey, PhD

APPENDIX B – OPTISON PRODUCT INSERT

APPENDIX C – CASE REPORT FORMS

APPENDIX D – THOMAS JEFFERSON UNIVERSITY IRB APPROVAL