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LCCC 1922: Contrast-Enhanced Ultrasound for Kidney Cancer Subtyping and Staging

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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 U.S. federal regulations and ICH guidelines.

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Date of Protocol: January 4, 2022

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1.0 BACKGROUND AND RATIONALE

1.1 Study Synopsis

This is a single arm, non-randomized study of 25 adult subjects with suspected kidney cancer and planned surgical nephrectomy that will undergo a contrast enhanced ultrasound (CEUS) of the kidneys to predict kidney mass histologic diagnosis, subtype and stage.

1.2 Kidney Cancer

Kidney cancer incidence has more than doubled over the past 2 decades with nearly 70,000 new cases projected for 2019[1, 2]. This exponential growth is attributable largely to increased detection of incidental small kidney masses[3, 4]. Most of these small masses are resected; however, only 20% harbor aggressive disease. The remaining 80% consist of benign tumors or non-aggressive cancers[5-7] that may not need resection. Unfortunately, current diagnostic techniques do not reliably differentiate aggressive from non-aggressive subtypes. Identifying non-aggressive subtypes pre-resection would reduce unnecessary surgeries and associated risks including kidney function loss, a risk particularly high among individuals with chronic kidney disease (CKD).

Contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI) are the standard tests to diagnose kidney cancer and guide management. However, CT and MRI have key disadvantages that limit their ability to precisely diagnose and stage kidney cancer resulting in costly surgeries and their associated morbidities in patients that may not have needed resection. First, both modalities capture image snapshots over time, and important enhancement characteristics may be missed if the snapshot is not timed correctly. Second, contrast extravasation leads to decreased sensitivity to vascular enhancement, limiting their staging ability. Third, CT and MRI are contraindicated among individuals with moderate and severe CKD, contrast allergies and/or metal implants, relegating these patients to substandard diagnostics. Ultrasound (US) is a safe, inexpensive and accessible imaging tool often used in patients with CKD, but it suffers from a lack of sensitivity. Given the rising incidence of kidney cancer and the increasing number of individuals with CKD who cannot undergo CT/MRI, there is an urgent and unmet need to identify imaging that can safely and accurately differentiate kidney masses needing resection from those that do not. Moreover, among all individuals with kidney masses, including those without CT/MRI contraindications, there is a clear and unmet need to identify imaging that can accurately diagnose subtype and characterize vascular involvement, a key factor in cancer staging.

We propose to investigate an advanced contrast-enhanced ultrasound (CEUS) technique as a novel imaging tool to address these critical gaps in kidney mass diagnosis and staging. We will accomplish the following aims:

1) to predict histologic subtype and stage using 3D CEUS-generated metrics in humans and 2) to compare the predictive capabilities of 2 different CEUS imaging techniques (one using bolus and one using infusion injection) in humans.

1.3 Kidney Cancer Histologic Subtypes

The majority of kidney cancers are renal cell carcinomas (RCC) of which there are three main subtypes: clear cell, papillary and chromophobe. The subtypes differ in incidence, prognosis[8, 9] and vascularity[10]. Accurate subtyping is critical to clinical decision-making as less aggressive subtypes do not require immediate resection. Differentiation of clear cell (a higher risk subtype requiring resection) from other subtypes or benign kidney masses has the potential to reduce unnecessary surgeries[6]. CT/MRI, the predominant imaging modalities used to diagnose RCC, do not reliably predict cancer subtype or grade. Other than surgical resection, biopsy is the only reliable way to preoperatively determine histologic subtype and tumor grade. However, biopsy is not performed at all centers[11] and is subject to sampling error, with as many as 20% of biopsies being non-diagnostic[12].

Differences in tissue vascularity cause differing tissue enhancement[13]. CEUS is highly sensitive for tissue enhancement which can be leveraged to differentiate kidney mass subtype based on enhancement properties. Previous studies have reported accurate diagnosis[14] and subtyping[15] of kidney masses using single plane CEUS with qualitative interpretations. 3D CEUS-generated metrics will capture enhancement in multiple planes and thus produce a more complete picture of the entire mass. Specifically, we will use 3D CEUS-generated quantitative metrics to characterize mass vascularity.

1.4 Tumor staging

For malignant masses requiring resection, treatment decisions are often based on CT/MRI-derived staging. Tumors confined to the kidney are stage 1 or 2. Vascular involvement classifies a tumor as stage 3 or higher. However, CT/MRI often miss subtle vascular involvement, leaving such involvement undetected until pathologic inspection post-resection, making preoperative prognosis estimation inaccurate. Stages 1, 2 and 3 have very different survival rates (81%, 74% and 53%[1], respectively), rendering accurate preoperative staging essential for surgical planning and patient counseling. 3D CEUS' vascular enhancement sensitivity and real-time imaging may better characterize small vessel involvement, aiding surgical planning and patient counseling. In addition, 3D volume rendering of the vessels may be useful for vasculature-based mass staging.

1.5 Chronic Kidney Disease

Individuals with advanced CKD and a kidney mass offer a unique challenge. These patients have an increased risk of kidney cancer, and risk rises with

CKD severity[16]. As CKD severity worsens, contrast CT/MRI risks also increase. If such patients undergo contrast CT, they risk kidney function loss and potential progression to end-stage kidney disease. If they undergo contrast MRI, they risk development of a rare but devastating disease, nephrogenic sclerosing fibrosis. However, without such imaging, they either risk missed detection of a mass needing resection or undergo preemptive resection, potentially of a mass not needing resection and resulting in further loss of kidney function. Identification of a contrast imaging tool that does not pose increased risks to patients with CKD would eliminate these unnecessary risks.

The advanced CEUS techniques of 3D imaging provides a powerful opportunity to fill existing gaps in kidney mass care, including subtype prediction and staging. Additionally, for those with advanced CKD and other contraindications to CT/MRI, CEUS allows for contrast imaging not possible with existing imaging options.

1.6 Contrast Enhanced Ultrasound

CEUS has several features that distinguish it from contrast CT/MRI. Unlike contrast CT/MRI, CEUS captures real-time images, enabling dynamic visualization of contrast over time. In addition, the CEUS contrast agent, microbubbles, remains intravascular, and is thus sensitive to lesion enhancement and small vessel involvement, giving CEUS the potential to inform kidney mass staging and surgical planning, capabilities not currently possessed by CT/MRI.

In addition, CEUS microbubbles differ from CT/MRI contrast agents in that they are cleared by the lungs within minutes of administration and have no known nephrotoxicity, making CEUS safe for patients with advanced CKD. For these individuals, CEUS availability would mean access to contrast-enhanced imaging, which would significantly improve the ability to provide a definitive mass diagnosis and increase the chance of avoiding unnecessary surgery. CEUS maintains the advantages of conventional US (lower cost in comparison to CT/MRI and safety).

To-date, most CEUS research has utilized a 2D approach. By adapting routine imaging techniques, we can generate three-dimensional (3D) CEUS images. Our preliminary work suggests that expanding 2D CEUS to a 3D technique will provide deeper data about mass enhancement, facilitating subtype differentiation. Moreover, our group has developed image analysis techniques that can generate 3D renderings of the vasculature (described below).

In 2D or 3D CEUS, microbubbles can be administered by bolus or continuous infusion dosing which correspond with low mechanical index (MI) and flash-replenishment imaging (F-R), respectively. The two approaches yield different information. The optimal approach is unknown.

With bolus administration of undiluted microbubbles, CEUS quantifies measurements like peak intensity, time to peak intensity, and wash-in/wash-out rates from the time intensity curve (TIC), each of which may be used to predict mass subtype. Alternatively, infusion of diluted microbubbles facilitates longer imaging times and uses faster frame rates. Longer imaging time allows for imaging in multiple planes which can capture multiple masses or different planes in a single mass, providing richer information. Faster frame rates can capture rapid wash-in rates with greater granularity than bolus dosing. However, the infusion method TIC does not quantify a wash-out rate, eliminating one metric that may contribute to subtype classification accuracy. Existing data suggest that bolus dosing 2D CEUS has comparable accuracy for malignancy detection compared to contrast-enhanced CT and MRI[17]. The accuracy of infusion dosing is unknown. We will adapt both approaches to generate 3D CEUS images.

1.7 Contrast-Enhanced Ultrasound Diagnostic Imaging of the Kidney

Ultrasound (US) imaging of the kidney is typically performed in the United States without contrast as no US contrast agent is currently approved for this indication. Without contrast, malignant tumors often cannot be definitively differentiated from benign tumors or pseudotumors. For this reason, following the identification of a kidney lesion via US without contrast, contrast-enhanced CT or MRI is then performed to stage and grade the lesion.

CEUS of the kidney for mass or lesion characterization is currently being performed in only certain centers in the United States and is more widespread outside the United States. US contrast agents are gas-filled microbubbles which are intravenously administered in very small volume boluses or slow infusions. These microbubbles typically have mean diameters between 1 to 6 μm, remain intravascular for several minutes, and do not diffuse into the interstitium (pure blood-pool agents)[18]. Three US contrast agents are Food and Drug Administration (FDA) approved for human use: perflutren lipid microspheres (Definity®), sulfur hexafluoride lipid microspheres and perflutren protein-type A microspheres (Optison®). All are FDA indicated for use in cardiac studies. The intent of this study is not to change the labeling of either FDA-approved agent.

1.8 Contrast Agents

Definity® (perflutren lipid) is an FDA-approved US contrast agent indicated to opacify the left ventricular chamber and to improve the delineation of the left ventricular endocardial border in patients with sub-optimal echocardiograms. It is activated by mechanical agitation with a Vialmix® which produces a milky white injectable suspension of perflutren lipid microspheres composed of octafluoropropane. Activated perflutren may be injected by either an intravenous bolus or infusion. See

http://www.accessdata.fda.gov/scripts/cder/drugsatfda/ for full prescribing information. When used in this setting, the maximum dose of perflutren is administered as either bolus doses or one single intravenous infusion.

1.9 Associated Toxicities

Most safety studies have been performed in cardiac patients as this was the only FDA approved indication. Thus far, microbubble agents have been shown to be quite safe[19, 20]. In pre-market clinical trials, 1716 subjects were evaluated with activated perflutren lipid. Of the 1716 subjects, 144 (8.4%) had at least one treatment-related adverse reaction. There were 26 serious adverse events and 15 (0.9%) subjects discontinued because of an adverse event, 1 due to a hypersensitivity reaction and the rest due to dizziness, chest pain, dyspnea or back pain. The events appeared within 1 – 15 minutes of the drug administration and were of moderate intensity resolving usually without treatment within minutes or hours after onset. Of the 11 other serious adverse events, which appeared within 2-15 days of the drug administration, all appeared to be a progression of underlying cardiac and non-cardiac disease. Nineteen subjects (1.1%) suffered serious cardiopulmonary adverse events including 8 deaths. The deaths occurred several days after activated perflutren lipid administration and appear to be related to the course of underlying disease.

For all AEs, there were no differences in the overall incidence based on age, gender, or route of administration. The most common events were (% of patients experiencing): headache (2.3%), back and renal pain (1.2%), flushing (1.1%) and nausea (1.0%).

Initial post-marketing experience, which included over 1 million patients with 5 years surveillance, showed the only medically significant risk was rare allergic events, occurring at a rate of 1 in 10,000[20].

1.10 Cardiopulmonary Reactions

Cardiopulmonary contraindications include cardiac shunts and hypersensitivity to perflutren. The initial mandated 30-minute monitoring period was limited to patients with pulmonary hypertension or unstable cardiopulmonary conditions. The mandatory 30-minute monitoring period was removed in 2011, although a statement remained that most serious cardiopulmonary reactions occur within 30 minutes of administration. For this reason, the label states that cardiopulmonary resuscitation personnel and equipment be readily available prior to perflutren administration, and that all patients be monitored for acute reactions.

Although recent studies have shown no increased risk in patients with pulmonary hypertension[21], patients with a history of known cardiac shunts, pulmonary hypertension or unstable cardiopulmonary conditions

will be excluded from our study. In addition, any patient with a suspected reaction during imaging will be monitored 30-minutes post-contrast agent administration by clinical or research nurse.

1.11 Hypersensitivity Reactions

Anaphylaxis to perflutren is extremely rare, however, in case of a severe reaction, EpiPen® (epinephrine) injections will be kept near the US machine for all patients.

1.12 High Ultrasound Mechanical Index (MI)

While high ultrasound MI of the heart may cause microbubble cavitation and subsequent ventricular arrhythmias, this was seen only at MI>1.6, higher than the typical MI used for microbubble disruption, with an agent no longer in clinical use.[22] No premature ventricular contractions were seen with MI≤1.1. Large clinical trials using MI≤1.0 for expanded cardiac indications have shown no significant concerns for arrhythmias[9, 20, 23].

Nevertheless, the safety of perflutren at MIs >0.8 has not yet been established by the manufacturer. However, real-time measurement of renal blood flow in 19 healthy subjects using perflutren was reported, using a flash replenishment high-MI (MI of 1.0) US technique. The contrast agent was well tolerated with no serious adverse events. One patient each had a 20mmHg increase in systolic blood pressure, a very brief and mild episode of flushing, and mild back pain, but no cardiac arrhythmias were recorded during the study period[24]. A second study using Sonovue® contrast agent with flash-replenishment high-MI to detect changes in kidney perfusion via US in 10 normal volunteers reported the agent was well tolerated, and not associated with any adverse events[25].

Small pre-clinical studies performed in rodents and pigs[26-29] showed conflicting results. One group showed glomerular capillary hemorrhage in exposed animals while other groups showed no sign of damage. Recently published work in a rat model using clinically relevant parameters for CEUS high MI flash-replenishment imaging showed no long-term changes in histology or clinical chemistry measurements[30]. The clinical relevance of these findings has not yet been investigated and is believed to be minimal, if any.

1.13 Preclinical Data

Numerous pre-clinical studies of CEUS have been performed, primarily in rodent kidneys but also larger animals like rabbit and pig, that investigate overall perfusion of diseased kidneys[31-33] or kidneys exposed to various medications[34, 35]. In the field of oncology, many pre-clinical studies have been performed to determine the sensitivity of CEUS for detection of malignancy[36-38]. The use of targeted microbubbles (with antibodies attached to the microbubble surface) has been investigated in this field as well[39, 40].

1.14 Clinical Data to Date

Advancements in imaging technique combined with the nonlinear properties of microbubbles have spurred the development of contrast-specific US techniques such as CPS (Contrast Pulse Sequencing). Such imaging techniques provide high-resolution images of tissue vasculature and allow the assessment of the microcirculation patterns in real-time[41-47]. Outside of the United States, or in the context of clinical trials, other US contrast agents have been used in kidney imaging[43, 44, 47-51]. Levovist® (lipid and galactose microparticle suspension) and Sonovue® (lyophilized sulfur hexafluoride microbubbles), two non-FDA approved similar US contrast agents approved for use in countries outside of the United States, have also been used in the general population to identify and diagnose kidney pseudotumors[52], cystic kidney lesions[44, 48, 50, 53], and solid kidney lesions[47, 51].

In the United States, where there has been significantly less research on using US contrast in humans, the US contrast agent, Definity®, was used in one study to measure renal blood flow in a healthy population[24], effectively reproducing previous animal studies in the human population. Multiple contrast agents were used in a large retrospective study of CEUS in patients with kidney lesions[54] and showed excellent sensitivity (100%) and specificity (99%). Since that study, several smaller studies in the United States have looked at CEUS in the kidney and consistently shown excellent sensitivity[55, 56]. Studies outside the United States show similar sensitivities and specificities[17, 57].

1.15 Dose Rationale and Risk/Benefits

Dosages for Definity are based on package inserts, expert opinion from radiologists who routinely use CEUS for kidney imaging, and our previous experience from clinical trials (IRB 12-2314, 15-1866, 17-1130). Because the kidney is a very vascular organ, use of 0.2mL Definity has been adequate for visualization of lesion enhancement. Our experience has been with both bolus doses of 0.2mL and infusion at set rates based on BMI. The infusion rate or dose can be increased or decreased if visualization is not adequate. Use of either small boluses or infusion allows for multiple injections for patients with multiple lesions. Patients with greater abdominal girth and multiple lesions may require a 2nd vial as their bolus doses or rate of infusion will be higher. All microbubble contrast agents are injected intravenously.

The greatest risk is of an unknown hypersensitivity reaction to the microbubbles as many patients will not have previous exposure to these contrast agents. However, the rate of true allergic reactions is low and similar to that for gadolinium-based contrast agents. Other risks are transient and resolve when the contrast agent is cleared. Overall the risk is minimal. Benefits include improved visualization of kidney lesions compared to non-contrasted ultrasound and the potential reduction of total

lifetime load of gadolinium, exposure to radiation from CT and improvement in cost-effectiveness.

2.0 STUDY OBJECTIVES

2.1 Primary Objectives

2.1.1 To predict kidney mass diagnosis using 3D CEUS generated metrics (see Section 7.2.1).

2.2 Secondary Objectives

2.2.1 To compare diagnostic accuracy of F-R infusion imaging technique to low MI bolus imaging technique.

2.2.2 To predict kidney mass stage using 3D CEUS-generated metrics

2.3 Endpoints

The primary study endpoint will be 3D CEUS generated metric(s) that best predict kidney mass diagnosis based on pathologic examination.

3.0 PATIENT ELIGIBILITY

3.1 Inclusion Criteria

3.1.1 A suspected diagnosis of kidney cancer with a solid or partially solid lesion and planned surgical nephrectomy within 3 months before surgery

3.1.2 Able to provide written informed consent

3.1.3 Willing to comply with protocol requirements

3.1.4 At least 18 years of age

3.2 Exclusion Criteria

3.2.1 Critically ill or medically unstable or in an intensive care setting and whose critical course during a potential observation period would be unpredictable

3.2.2 Known hypersensitivity to sulfur hexafluoride or to any component of perflutren lipid (Definity®)

3.2.3 Right to left shunt, severe pulmonary hypertension (Pulmonary artery pressure >90mmHg), or adult respiratory distress syndrome

3.2.4 Has any other medical condition or other circumstances that would significantly decrease the chances of obtaining reliable data or of achieving the study objectives

3.2.5 Unstable cardiopulmonary disease including any of the following:

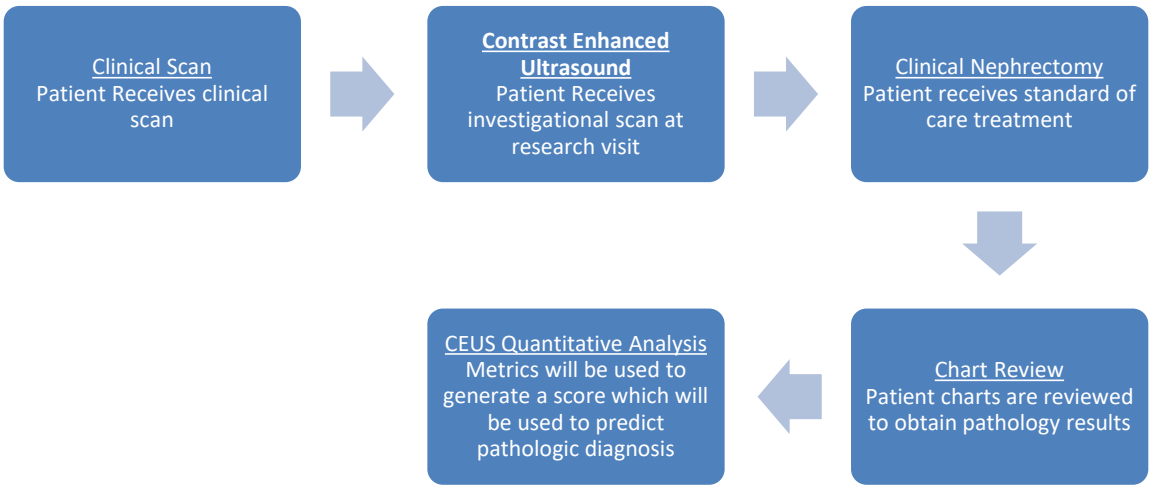
- Severe congestive heart failure (class IV in accordance with the classification of the New York Heart Association)
- Unstable angina
- Symptomatic arrhythmia (i.e. tachycardia, bradycardia, supraventricular tachycardia, ventricular fibrillation, ventricular tachycardia, atrial flutter or fibrillation)
- Myocardial infarction within 14 days prior to the date of proposed microbubble administration.

3.2.6 Any woman who is pregnant or has reason to believe she is pregnant (the possibility of pregnancy has to be excluded by negative urine β-HCG results, obtained the same day as the CEUS, or on the basis of patient history, e.g.: tubal ligation, hysterectomy or a minimum of 1 year without menses)

3.2.7 Obesity that limits obtainment of acceptable images

4.0 STUDY PLAN

4.1 STUDY SCHEMA



4.2 Study Procedures

4.2.1 Enrollment/Recruitment

We will recruit subjects from UNC urology and urologic oncology clinics. Patients ≥18 years of age who have at least one suspected kidney cancer with a solid or partially solid lesion and planned surgical resection will be eligible. Pregnant patients and patients with known adverse microbubble reactions will be excluded. We will approach patients during clinic visits or by telephone and schedule consenting individuals for 3D CEUS within 3 months before planned surgery. If surgery is delayed, lesions will be reimaged prior to surgery to meet the 3-month window. The goal is to minimize changes that may occur in tumor pathology between the time of imaging and time of resection. Subjects may also be asked to return for an additional imaging visit if they are determined to have more than one eligible lesion.

4.2.2 Imaging Procedures

4.2.2.1 Low MI imaging with sweeps

Upon subject arrival, a urine pregnancy test will be conducted for subjects with child bearing potential. Once confirmed that a subject is eligible, a nurse will place an intravenous (IV) catheter, and a sonographer will determine mass location and approach with a baseline B-mode US. The nurse will prepare the CEUS contrast agent, Definity®, to be administered via infusion pump according to package insert instructions. We will use a Siemens ACUSON Sequoia S512 with a 4C1 curved array transducer equipped with cadence pulse sequencing technology for contrast-enhanced imaging.

Each subject will be injected with a bolus of 0.2-0.4 mL undiluted microbubbles placed on an infusion pump for consistent injection rate and followed by 5mL sterile saline. The sonographer will capture sweeps through the kidney in 2-3 different planes (i.e. x-y, y-z, or x-z) of imaging during contrast infusion. Each imaging plane will require a new bolus. During imaging, the subject will be instructed to take shallow breaths to minimize movement artifact.We will capture a B-mode image perpendicular to each sweep direction to determine the total length of each sweep. The subject will be instructed to take shallow breaths to minimize movement artifact. Imaging time will be 15-20 minutes.

4.2.2.2 Flash-replenishment infusion imaging

Following low MI bolus imaging, we will use the F-R infusion protocol and infuse diluted microbubbles with a calibrated syringe pump. We will translate the transducer across the mass in 0.5-1 cm steps to image the entire mass, executing a F-R pulse at each step. Imaging time will be 3-15 minutes depending on mass size. We will monitor the subject for adverse

reactions during the procedure. Images will be coded and stored for later analysis.

4.2.3 Medical Record Abstraction

We will perform chart review to obtain histologic diagnosis of subtype as determined by standard of care pathologic examination. Routine formalin fixed specimens will be examined with standard light microscopy. An experienced UNC pathologist will document size, gross description, and tumor subtype, grade and stage per standard-of-care clinical pathology processes using the American Joint Committee on Cancer TNM staging system and the World Health Organization/International Society of Urologic Pathologists grading system. All pathological studies will be conducted per institutional standards.

4.3 Time and Events Table

	Screening	Pre-CEUS	CEUS study	Early Termination[1]	Post-CEUS study
Inclusion/ Exclusion Criteria	X				
Comprehensive Medical History	X	X[2]			
Informed Consent	X[1]	X[1]			
Education of potential side effects		X			
Pregnancy test (urine), if applicable		X[3]			
Contrast-Enhanced US (CEUS)			X		
AE Assessment			X[4]	X	
Review medical record for pathology results				X	X

[1]Consent can be obtained and signed at either screening visit or during the pre-study visit. It will always be reviewed at pre-study visit, even if signed during screening visit.
[2]Comprehensive medical history obtained at time of enrollment; thereafter history focused on symptoms and assessments.
[3]Women of childbearing potential must have negative urine pregnancy test on the day of CEUS study, prior to the study.
[4]Adverse event assessment will happen during the CEUS study when they will be monitored by nursing staff with vital signs (blood pressure and heart rate) recorded in the hospital's electronic monitoring system. Adverse events will be recorded on an adverse events CRF.

5.0 INVESTIGATIONAL CONTRAST AGENT

5.1 Description

5.1.1 Perflutren Lipid Microspheres (Lantheus Medical Imaging)

The Definity® vial contains components that upon activation yield perflutren lipid microspheres composed of octafluoropropane. Perflutren is a diagnostic drug that is intended to be used for contrast enhancement. The vial contains a clear, colorless, sterile, non-pyrogenic, hypertonic solution which is activated by mechanical agitation with Vialmix®.

Vialmix® is the activation device used in the preparation of US contrast imaging agents, including Definity®. Prior to activation, each Definity® vial contains 6.52 mg/mL octafluoropropane in the headspace and 0.75 mg lipid blend (0.045 mg DPPA, 0.401 mg DPPC, and 0.304 mg MPEG5000 DPPE), 103.5 mg propylene glycol, 126.2 mg glycerin, 2.34 mg sodium phosphate monobasic monohydrate, 2.16 mg sodium phosphate dibasic heptahydrate and 4.87 mg sodium chloride in water in the clear liquid. Upon activation, each mL of the milky white suspension contains a maximum of 1.2×10^{10} perflutren lipid microspheres with approximately 150 µL/mL octafluoropropane.

5.2 IND Exemption

The investigators feel that this study meets the regulatory definition of a study that is exempt from obtaining an IND.

5.3 Treatment Regimen

The contrast agent will be dosed undiluted (0.2-0.4 mL Definity) for up to 7 intravenous injections, depending on the number of lesions (2-3 bolus injections per lesion and one infusion injection). The activated undiluted dose will be placed in the tubing and attached to an infusion pump loaded with a syringe of sterile saline. The pump will be programmed to a standard rate for all patients and set to administer a total of 5 mL saline flush after the contrast agent is fully injected. Infusion dosing will be used after completion of bolus dosing.

5.4 Preparation and Administration of Study Drug

The contrast agent, Definity will be dispensed from the UNC Investigational Drug Service (IDS). It will be dispensed in inactivated form to a study team member. The contrast agent will be activated just prior to administration (ideally to be used within 5 minutes of activation). Contrast agent will be activated by nursing staff trained in the activation of Definity and according to package insert instructions, including use of VialMix®, the device used to activate Definity. Administration will occur IV in coordination with sonography staff trained specifically in contrast ultrasound imaging.

5.5 Subject Compliance Monitoring

Since the study only involves a single visit for most subjects, there will be no issues with compliance and no role for compliance monitoring.

5.6 Prior and Concomitant Therapy

Comparison imaging studies (ultrasound and MRI) completed prior, concomitantly or post-CEUS will also be collected.

5.7 Packaging

- Definity comes in single use 2-mL clear glass vials.

5.8 Receiving, Storage, Dispensing and Return

5.8.1 Receipt of Drug Supplies

Upon receipt of the of the study treatment supplies at the Investigational Drug Service (IDS), an inventory must be performed and a drug receipt log filled out and signed by the person accepting the shipment. It is important that the designated study staff counts and verifies that the shipment contains all the items noted in the shipment inventory. Any damaged or unusable study drug in a given shipment (active drug or comparator) will be documented in the study files. The investigator must notify study sponsor of any damaged or unusable study treatments that were supplied to the investigator's site.

5.8.2 Storage

- Definity is stored between 2-8°C.

5.8.3 Dispensing of Study Drug

Study drug (contrast agent) is not dispensed to the subject. It is used one-time, at the study visit and administered by study members.

5.8.4 Return or Destruction of Study Drug

At the completion of the study, there will be a final reconciliation of drug shipped, drug consumed, and drug remaining. This reconciliation will be logged on the drug reconciliation form, signed and dated. Any discrepancies noted will be investigated, resolved, and documented prior to return or destruction of unused study drug. Drug destroyed on site will be documented in the study files.

6.0 ADVERSE EVENTS

6.1 Definitions

6.1.1 Adverse Event (AE)

An adverse event (AE) is any untoward medical occurrence (e.g., an abnormal laboratory finding, symptom, or disease temporally associated with the use of a drug) in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal product, whether or not related to the medicinal product.

Hospitalization for elective surgery or routine clinical procedures that are not the result of an AE (e.g., surgical insertion of central line) need not be considered AEs and should not be recorded as an AE. Disease progression

should not be recorded as an AE, unless it is attributable by the investigator to the study therapy.

6.1.2 Suspected Adverse Reaction (SAR)

A suspected adverse reaction (SAR) is any AE for which there is a *reasonable possibility* that the drug is the cause. *Reasonable possibility* means that there is evidence to suggest a causal relationship between the drug and the AE. A suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a drug.

Causality assessment to a study drug is a medical judgment made in consideration of the following factors: temporal relationship of the AE to study drug exposure, known mechanism of action or side effect profile of study treatment, other recent or concomitant drug exposures, normal clinical course of the disease under investigation, and any other underlying or concurrent medical conditions. Other factors to consider in considering drug as the cause of the AE:

- Single occurrence of an uncommon event known to be strongly associated with drug exposure (e.g., angioedema, hepatic injury, Stevens-Johnson Syndrome)
- One or more occurrences of an event not commonly associated with drug exposure, but otherwise uncommon in the population (e.g., tendon rupture); often more than once occurrence from one or multiple studies would be needed before the sponsor could determine that there is *reasonable possibility* that the drug caused the event.
- An aggregate analysis of specific events observed in a clinical trial that indicates the events occur more frequently in the drug treatment group than in a concurrent or historical control group

6.1.3 Unexpected AE or SAR

An AE or SAR is considered unexpected if the specificity or severity of it is not consistent with the applicable product information (e.g., Investigator's Brochure (IB) for an unapproved investigational product or package insert/summary of product characteristics for an approved product). Unexpected also refers to AEs or SARs that are mentioned in the IB as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but are not specifically mentioned as occurring with the particular drug under investigation.

6.1.4 Serious AE or SAR

An AE or SAR is considered serious if, in the view of either the investigator or sponsor, it results in any of the following outcomes:

- Death;

- Is life-threatening (places the subject at immediate risk of death from the event as it occurred);
- Requires inpatient hospitalization (>24 hours) or prolongation of existing hospitalization;*
- Results in congenital anomaly/birth defect;
- Results in a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions;
- Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse drug experience when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in the definition. For reporting purposes, also consider the occurrences of pregnancy as an event which must be reported as an important medical event.

*Hospitalization for anticipated or protocol specified procedures such as administration of chemotherapy, central line insertion, metastasis interventional therapy, resection of primary tumor, or elective surgery, will not be considered serious adverse events.

Pregnancy that occurs during the study must also be reported as an SAE.

6.2 Documentation of non-serious AEs or SARs

For non-serious AEs or SARs, documentation must begin from day 1 of study treatment and continue through the 30-day follow-up period after treatment is discontinued.

Collected information should be recorded in the Case Report Forms (CRF) for that patient. Please include a description of the event, its severity or toxicity grade, onset and resolved dates (if applicable), and the relationship to the study drug. Documentation should occur at least monthly.

6.3 SAEs or Serious SARs

6.3.1 Timing

After informed consent but prior to initiation of study medications, only SAEs caused by a protocol-mandated intervention will be collected (e.g. SAEs related to invasive procedures such as biopsies, medication washout).

For any other experience or condition that meets the definition of an SAE or a serious SAR, recording of the event must begin from day 1 of study treatment and continue through the 30-day follow-up period after treatment is discontinued.

6.3.2 Documentation and Notification

These events (SAEs or Serious SARs) must be recorded in the SAE console within Oncore™ for that patient within 24 hours of learning of its occurrence.

6.3.3 Reporting

IRB Reporting Requirements:

- The UNC-IRB will be notified of all SAEs that qualify as an Unanticipated Problem as per the UNC IRB Policies using the IRB's web-based reporting system within 7 days of the Investigator becoming aware of the problem.

Pregnancy

Pregnancies and suspected pregnancies (including a positive pregnancy test regardless of age or disease state) of a female subject occurring while the subject is on study should be recorded as SAEs. The patient is to be discontinued immediately from the study. The female subject should be referred to an obstetrician-gynecologist, preferably one experienced in reproductive toxicity for further evaluation and counseling.

The Investigator will follow the female subject until completion of the pregnancy, and must document the outcome of the pregnancy (either normal or abnormal outcome). If the outcome of the pregnancy was abnormal (e.g., spontaneous or therapeutic abortion), the Investigator should report the abnormal outcome as an AE. If the abnormal outcome meets any of the serious criteria, it must be reported as an SAE.

6.4 Data and Safety Monitoring Plan

The Principal Investigator will provide continuous monitoring of patient safety in this trial with periodic reporting to the Data and Safety Monitoring Committee (DSMC).

Meetings/teleconferences will be held at a frequency dependent on study accrual, and in consultation with the study Biostatistician. These meetings will include the investigators as well as protocol nurses, clinical research associates, regulatory associates, data managers, biostatisticians, and any other relevant personnel the principal investigators may deem appropriate. At these meetings, the research team will discuss all issues relevant to study progress, including enrollment, safety, regulatory, data collection, etc.

The team will produce summaries or minutes of these meetings. These summaries will be available for inspection when requested by any of the regulatory bodies charged with the safety of human subjects and the integrity of data including, but not limited to, the oversight (Office of Human Research Ethics (OHRE) Biomedical IRB, the Oncology Protocol Review

Committee (PRC) or the North Carolina TraCS Institute Data and Safety Monitoring Board (DSMB).

The UNC LCCC Data and Safety Monitoring Committee (DSMC) will review the study on a regular (quarterly to annually) basis, with the frequency of review based on risk and complexity as determined by the UNC Protocol Review Committee. The UNC PI will be responsible for submitting the following information for review: 1) safety and accrual data including the number of study participants treated; 2) significant developments reported in the literature that may affect the safety of participants or the ethics of the study; 3) preliminary response data; and 4) summaries of team meetings that have occurred since the last report. Findings of the DSMC review will be disseminated by memo to the UNC PI, PRC, and the UNC IRB and DSMB.

7.0 STATISTICAL CONSIDERATIONS

7.1 Sample Size and Accrual

We will enroll 25 adult subjects with suspected with suspected kidney cancer and planned surgical nephrectomy. We have deep recruitment experience for kidney CEUS imaging. Approximately 150 nephrectomies for localized disease are performed at UNC yearly. Based on our previous recruitment rate of ~75% and a strong partnership with our urology colleagues, we anticipate no problems meeting our recruitment goal of 25 subjects.

7.1.1 Primary Objective

7.1.1.1 Predicting kidney mass diagnosis using 3D CEUS generated metrics.

The proposed sample of 25, assuming 60% clear cell legions, achieves 63.2% power to detect a difference of 0.1000 between a diagnostic test with an area under the ROC curve (AUC) of 0.8000 and another diagnostic test with an AUC of 0.9000 using a two-sided z-test at a significance level of 0.050. The correlation between the two diagnostic tests is assumed to be 0.9 for the positive group and 0.9 for the negative group.

The proposed sample of 25, assuming 60% clear cell legions, achieves 88.7% power to detect a difference of 0.1500 between a diagnostic test with an area under the ROC curve (AUC) of 0.8000 and another diagnostic test with an AUC of 0.9500 using a two-sided z-test at a significance level of 0.050. The correlation between the two diagnostic tests is assumed to be 0.9 for the positive group and 0.9 for the negative group.

7.1.2 Secondary Objectives

7.1.2.1 To compare diagnostic accuracy of F-R infusion imaging technique to low MI bolus imaging technique.

The proposed tests with sample size 25 have 63.2% power to detect a difference of 0.1 in the AUCs (.8 vs .9) and 88.7% power to detect a difference of 0.15 (0.8 vs 0.95).

7.1.2.2 To predict kidney mass stage using 3D CEUS-generated metrics

Based on our clinical experience, we estimate 15 patients will have a T2a category or lower lesion and 25% of those will have a change from clinical to pathological staging. A sample size of 15 patients will not have sufficient power to detect areasonable effect size but computation of parameter estimates and standard errors will inform larger future studies.

7.2 Data Analysis Plans

7.2.1 Predicting kidney mass diagnosis using 3D CEUS generated metrics.

Image Analysis (Low MI TIC analysis): We will use Matlab (Mathworks, Natick, MA) for time intensity curve (TIC) analysis and 3D volume rendering. For each imaging plane, we will create a region of interest (ROI) by delineating the entire mass in the image slice. We will produce a TIC by taking the mean signal intensity in the ROI at each time point and calculate the metrics of wash-in rate (WI), wash-out rate (WO), mean transit time (MTT), time to peak (TTP) and peak enhancement relative to the surrounding parenchyma (PE) for each x-sweep position. A weighted average based on the ROI area at each position will be used to produce single values for the 3D volume for each TIC metric (WI, WO, MTT, TTP and PE). We will repeat this process for each plane of imaging.

3D volume rendering: We will also create 3D renderings of the tumor vasculature from the CEUS images. We will sort the image data collected over the 3-minute period after a bolus injection by the x- (or y-) sweep position, then by capture time in relation to the start of bolus injection through the wash-out period. We will produce a cumulative contrast image (CCI) at each position with the time-dependent image data by summing all time-dependent image data at each position. The images show where the microbubbles have travelled throughout the acquisition period, and since microbubbles are confined to the vascular spaces, the images provide excellent delineation of the vasculature. We will stack the CCIs together to create a 3D rendering.

Multiparametric score and statistical analysis: We will combine the quantitative data derived from CEUS image analyses into a single score. For the primary analysis using the gold

Table 1. Factors to be used in multiparametric score

CEUS factors	B-mode factors	Patient factors
WI, WO, MTT, TTP, PE, total volume	Length, echogenicity, shape	Age, sex, race

standard of histologic diagnosis to differentiate clear cell from non-clear cell tumors, we will use logistic regression to build predictive models based on multiple parameters from CEUS (WI, WO, MTT, TTP, PE and tumor volume), B-mode US images (length, echogenicity, shape), as well as patient factors (age, sex, race) (Table 1). We will assess the effects of individual parameters using hypothesis tests based on parameter estimates from the fitted logistic regression model. This approach facilitates assessment of the added value of the CEUS measurements over other patient factors. We will calculate nonparametric estimates and 95% confidence intervals for area under the curve (AUC) using the fitted logistic regression models, both with and without non-CEUS patient factors. We will use model selection procedures, including AUC, to identify a final model, with a one-sided test of AUC ≤.8 vs AUC ≥.8 at alpha=.05 for the final model. We will use model selection procedures based on optimizing AUC to identify a final model, with a one-sided test of AUC<=0.8 vs AUC>=.8 at alpha=.05 for the final model.

Gold Standard Comparator: Histologic diagnosis from pathologic tissue.

7.2.2 Comparing diagnostic accuracy of F-R infusion imaging technique to low MI bolus imaging technique.

Image Analysis (Flash-Replenishment TIC analysis): For each 0.5-1 cm step, we will produce a TIC by delineating an ROI of the entire mass and measuring the mean contrast signal intensity over the time course of the F-R. We will model TIC using a mono-exponential equation, $UI=A(1 - e^{-\beta t})$, where UI is ultrasound intensity and β, the slope of the tangent to the curve, represents the rate of perfusion. The height of the curve, A, represents the perfusion volume. To produce a single value for β for the entire tumor volume, we will calculate a weighted average based on the ROI area of the mass at each step. This value will serve as the WI rate.

Gold Standard Comparator: Histologic diagnosis from pathologic tissue.

Statistical Analysis: We will follow the model building and evaluation strategy used for the primary objective. This will be done separately for bolus and infusion imaging modalities, as well as combining the two multiparametric scores. We will compare AUCs from final logistic regression models obtained for bolus and infusion using a Z-test at alpha=0.05, with standard errors obtained by bootstrapping correctly adjusting for the fact that the two AUCs are calculated using the same data and hence correlated. We will conduct similar tests comparing the AUC based on the combined model to those based on bolus and infusion multiparametric scores separately. The proposed tests with sample size 25 have 80% power to detect a difference of .1 in the AUCs (0.8 vs 0.9) and 90% power to detect a difference of 0.15 (0.8 vs 0.95).

7.2.3 Predicting kidney mass stage using 3D CEUS-generated metrics

Image Eligibility: CEUS mass images from individuals with clinical stage T2a or lower tumors will be eligible for this sub-analysis. We estimate that 15 tumors will meet these criteria since over 50% of kidney cancer resections at UNC are for T2a or lower clinical stage tumors.

Imaging Procedure and Analysis: We will use Matlab (Mathworks, Natick, MA) to measure WI and WO rates from TIC analyses. We will apply the 3D volume rendering exploratory analyses described in the primary outcome to this outcome measure. Qualitative interpretation of 3D volume renderings will be assessed and compared to the clinical outcome.

Gold Standard Comparator: We will compare the gold standard of post-surgical pathologic stage to WI/WO rates generated from CEUS TICs.

Statistical Analysis: We will use the continuous variables of WI and WO to predict the binary outcome measure of pathologic stage T3 disease or greater. We will calculate means (standard deviations) and 95% confidence intervals for WI and WO for those ≥ stage T3 vs. < stage T3, and compare via t-tests at alpha=.05. We will use logistic regression to assess ability of WI and WO to predict stage T3 disease or greater, both separately and together. We will obtain nonparametric estimates of AUC along with 95% confidence intervals for WI alone, WO alone, and WI and WO combined using the fitted logistic regression models. We will also add patient specific factors to the logistic regression models, to assess the added value of the WI and WO measurements, with analyses to include tests of the effect parameters from the fitted logistic regression models and estimates and confidence intervals for AUCs from those models.

8.0 STUDY MANAGEMENT

8.1 Institutional Review Board (IRB) Approval and Consent

It is expected that the IRB will have the proper representation and function in accordance with federally mandated regulations. The IRB should approve the consent form and protocol.

In obtaining and documenting informed consent, the investigator should comply with the applicable regulatory requirement(s), and should adhere to Good Clinical Practice (GCP) and to ethical principles that have their origin in the Declaration of Helsinki.

Before recruitment and enrollment onto this study, the patient will be given a full explanation of the study and will be given the opportunity to review the consent form. Each consent form must include all the relevant elements currently required by the FDA Regulations and local or state regulations. Once this essential information has been provided to the patient and the investigator is assured that the patient understands the implications of

participating in the study, the patient will be asked to give consent to participate in the study by signing an IRB-approved consent form.

Prior to a patient's participation in the trial, the written informed consent form should be signed and personally dated by the patient and by the person who conducted the informed consent discussion.

8.2 Registration Procedures

Study participants will be registered into OnCore®, a web based clinical research platform by one of the Study Coordinators.

8.3 Data Management and Monitoring/Auditing

Copies of completed CRFs with subject IDs will stored within subjects' binders and data will be entered in the secure REDCap database. Study personnel will have access to enter data in the study database. Data should be entered in the study database within 5 business days to ensure timely entry.

The coordinator will complete the first CRF together with the PI to verify that it is completed correctly. We will verify a randomly selected 25% of all source documents. The randomization for this verification will be generated using a random number generator in Excel. Source document verification and monitoring will be documented and stored with the regulatory files.

8.4 Adherence to the Protocol

Except for an emergency situation in which proper care for the protection, safety, and well-being of the study patient requires alternative treatment, the study shall be conducted exactly as described in the approved protocol.

8.4.1 Emergency Modifications

UNC investigators may implement a deviation from, or a change of, the protocol to eliminate an immediate hazard(s) to trial subjects without prior UNC's IRB/IEC approval/favorable opinion.

For any such emergency modification implemented, an IRB modification form must be completed by UNC Research Personnel within five (5) business days of making the change.

8.4.2 Single Subject Exceptions

8.4.3 Eligibility single subject exceptions are not permitted for Lineberger Comprehensive Cancer Center Investigator Initiated Trials under any circumstances. Other types of single subject exceptions may be allowed if proper regulatory review has been completed in

accordance with Lineberger Comprehensive Cancer Center's Single Subject Exceptions Policy. Other Protocol Deviations/Violations

According to UNC's IRB, a protocol deviation is any unplanned variance from an IRB approved protocol that:

- Is generally noted or recognized after it occurs
- Has no substantive effect on the risks to research participants
- Has no substantive effect on the scientific integrity of the research plan or the value of the data collected
- Did not result from willful or knowing misconduct on the part of the investigator(s).

An unplanned protocol variance is considered a violation if the variance meets any of the following criteria:

- Has harmed or increased the risk of harm to one or more research participants.
- Has damaged the scientific integrity of the data collected for the study.
- Results from willful or knowing misconduct on the part of the investigator(s).
- Demonstrates serious or continuing noncompliance with federal regulations, State laws, or University policies.

If a deviation or violation occurs please follow the guidelines below:

Protocol Deviations: UNC personnel will record the deviation in OnCore®, and report to any sponsor or data and safety monitoring committee in accordance with their policies. Deviations should be summarized and reported to the IRB at the time of continuing review.

Protocol Violations: Violations should be reported by UNC personnel within one (1) week of the investigator becoming aware of the event using the same IRB online mechanism used to report Unanticipated Problems.

Unanticipated Problems:
Any events that meet the criteria for "Unanticipated Problems" as defined by UNC's IRB must be reported by the study team using the IRB's web-based reporting system.

8.5 Amendments to the Protocol

Should amendments to the protocol be required, the amendments will be originated and documented by the Principal Investigator at UNC. It should also be noted that when an amendment to the protocol substantially alters the study design or the potential risk to the patient, a revised consent form might be required.

The written amendment, and if required the amended consent form, must be sent to UNC's IRB for approval prior to implementation.

8.6 Record Retention

Study documentation includes all eCRFs, data correction forms or queries, source documents, Sponsor-Investigator correspondence, monitoring logs/letters, and regulatory documents (e.g., protocol and amendments, IRB correspondence and approval, signed patient consent forms).

Source documents include all recordings of observations or notations of clinical activities and all reports and records necessary for the evaluation and reconstruction of the clinical research study.

Government agency regulations and directives require that all study documentation pertaining to the conduct of a clinical trial must be retained by the study investigator. In the case of a study with a drug seeking regulatory approval and marketing, these documents shall be retained for at least two years after the last approval of marketing application in an International Conference on Harmonization (ICH) region. In all other cases, study documents should be kept on file until three years after the completion and final study report of this investigational study.

8.7 Obligations of Investigators

The Principal Investigator is responsible for the conduct of the clinical trial at the site in accordance with Title 21 of the Code of Federal Regulations and/or the Declaration of Helsinki. The Principal Investigator is responsible for personally overseeing the treatment of all study participants. The Principal Investigator must assure that all study site personnel, including sub-investigators and other study staff members, adhere to the study protocol and all FDA/GCP/NCI regulations and guidelines regarding clinical trials both during and after study completion.

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