

PROTOCOL

TITLE: SUBHARMONIC CONTRAST ULTRASOUND FOR IMPROVED CHARACTERIZATION OF ADNEXAL MASSES – A Pilot Study

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SYNOPSIS

Protocol Title: subharmonic contrast ultrasound for improved characterization of adnexal masses

Trial Objectives: The primary objective of this trial is:

- To develop qualitative subharmonic imaging (SHI) or SHI-derived quantitative biomarkers to generate pilot data for a study to evaluate if they improve the characterization of benign and malignant adnexal masses compared to standard ultrasound or contrast enhanced magnetic resonance imaging (MRI) or the risk of malignancy index (RMI).

and a secondary objective is:

- To compare the SHI depiction of adnexal masses' neovascularity in humans to intra-tumoral microvascular density (iMVD) obtained from CD31 an immunohistochemical marker of angiogenesis.

Trial Design: This is an open-label, non-randomized pilot study that will be conducted at one clinical site, Thomas Jefferson University (TJU). Enrolled patients scheduled for subsequent surgery of an adnexal mass will receive a bolus IV injection followed 15 minutes later by a continuous infusion of the ultrasound contrast agent Definity® (Lantheus Medical Imaging, N Billerica, MA). Ultrasound imaging will be performed using an Logiq E9 scanner with a C10-3v endovaginal (EV) transducer (GE Healthcare, Waukesha, WI) operating in SHI mode. Results will be compared to MRI assessments and pathology obtained as part of the subjects' standard of care.

Trial Population: This trial will consist of up to 45 adult women (21 years of age or older) at TJU, who have an adnexal mass and are scheduled for surgery based on their morphological characteristics (on standard imaging), CA125 levels and menopausal status.

Trial Procedures: Subjects eligible for trial enrollment will be identified by the co-investigator, Dr. Rosenblum, from his patient population of subjects scheduled for surgery of an adnexal mass. The research coordinator for this study will explain the study to the patients. After consenting to participate, a full demographic profile, known drug allergies or intolerances, and review of the subject's medical/surgical

history will be recorded.

Prior to a subject undergoing surgery of an adnexal mass, a baseline grayscale scan of the lesion will be obtained, while power Doppler imaging (PDI) will be performed to select the area with the greatest vascularity. Two contrast injections will be administered. The first injection will involve pulse inversion grayscale EV SHI of the area of greatest vascularity within the adnexal mass (established by baseline PDI). There will be a 15 minute pause between injections to allow for complete contrast clearance. For the second injection, Definity will be infused over 5 minutes and subharmonic destruction/reperfusion SHI imaging performed in multiple imaging planes covering the adnexal mass.

The pre- and then post-contrast US imaging will be read independently by the 2 experienced co-investigators blinded to the surgical results and contrast-enhanced MRI evaluations. Then, sensitivity, specificity and accuracy will be calculated for all imaging modalities (baseline and SHI as well as MRI) and compared to the RMI used to select patients for surgery [Ferrazzi et al. 1997; 2005; Moolthiya & Yuenyao 2009]. Off-line SHI time intensity curves (TICs) will be created on a pixel by pixel basis and TIC parameters will be compared to the lesion characterization by the other imaging modalities with pathology as the reference standard. Finally, the ability of EV SHI to image angiogenesis (specifically fractional tumor vascularity) will be compared to so called “hot spots” within surgical specimens stained for an immunohistochemical predictor of angiogenesis (the endothelial cell marker CD31).

Statistical Methodology: As this project involves a pilot study of a new US imaging mode (SHI) in a completely new application, no power analysis can be performed *a priori*. Nonetheless, our statistical analysis will address the following two major questions:

1. Can EV SHI biomarkers improve the characterization of benign and malignant adnexal masses compared to standard US or contrast enhanced MRI or other parameters?
2. Do SHI ovarian neovascularity measurements correlate with iMVD as a marker of angiogenesis?

As a pilot study this project is not powered to provide definitive answers to the questions presented above, but rather is a first step towards establishing feasibility and to provide data for a future more exhaustive study.

All analyses and computations will be performed using NCSS/PASS 2008 and Stata 12.0 (Stata Corporation, College Station, TX), while the study database will be designed and implemented in REDCap (Vanderbilt University, Nashville, TN). This database will contain all patient information (except names and other identifiers), including results of surgeries, MRI and SHI measurements as well as other clinical variables.

1. INTRODUCTION

In the United States there will be an estimated 22,240 new cases of ovarian cancer in 2013 leading to an estimated 14,030 deaths for a mortality rate of 63 % or 5 % of all female cancer deaths [Siegel et al. 2013]. This makes ovarian cancer one of the ten most common types of cancer in women and an estimated 1 in 71 women in the United States will develop ovarian cancer in their lifetime [Siegel et al. 2013]. World-wide ovarian cancer is the sixth most commonly diagnosed cancer accounting for 4 % of all female cancers. Women diagnosed with disease confined to the ovary (stage I), require less morbid surgical intervention, have a significantly improved quality of life, and most importantly have an overall 5-year survival approximating 90 % [Jinawath & Shih 2011]. Regrettably, almost 75 % of women are diagnosed with advanced disease, which greatly increases mortality (up to 85 %), which clearly indicates the inadequacy of a pelvic examination and conventional endovaginal (EV) ultrasound (US) imaging; as the first-line imaging test for evaluating adnexal masses [Fishman et al. 2005; Mohaghegh & Rockall 2012; Moyle et al. 2010]. Hence, earlier and accurate characterization of adnexal masses is a clinical imperative in order to improve patient's survival and imaging techniques play a critical role in fulfilling this goal [Mohaghegh & Rockall 2012; Moyle et al. 2010].

1.1 Background

Among the early changes that differentiate normal from cancerous tissues is the process by which the tumor develops its blood supply, tumor angiogenesis [Folkman 1990; Li 2000]. In addition, angiogenic vascular morphology, often quantified as the intra-tumoral microvessel density (iMVD), is an independent predictor of malignant disease; including in the ovaries [Brawer et al. 1992; Labiche et al. 2009; Palmer et al. 2007; Weidner et al. 1991; Weind et al. 1998; Wang et al. 2011]. Angiogenesis is a prerequisite for tumor growth greater than 2 mm³ and is also a key factor of metastasis by allowing the ingrowth of vessels into a tumor providing a pathway for systemic dissemination via the blood or lymph system [Holmgren et al. 1995; O'Reilly et al. 1994]. Therefore, tumor angiogenesis is an important marker for imaging adnexal masses. The opportunity for noninvasive imaging of tumor angiogenesis is strengthened by the fact that vessels

form a substantial portion of the mass of malignant tumors, contributing up to 10 % of the total volume of the tumor [Jain 1994]. Currently adnexal masses are imaged with standard EV US, with contrast enhanced MRI or computed tomography (CT) as well as, more rarely, with positron emission tomography (PET) [Bazot et al. 2008; Fleischer et al. 2012; Mohaghegh & Rockall 2012; Moyle et al. 2010]. While overall accuracies of 88 to 93 % have been reported for the latter modalities [Bazot et al. 2008; Mohaghegh & Rockall 2012], they are quite expensive and employ contrast agents which can produce marked adverse reactions, while CT also exposes patients to significant radiation.

Ultrasound imaging has great potential in providing clinically relevant information related to angiogenesis by measuring ovarian tumor flow and vascular volume in real time and when combined with gas microbubbles to perform contrast-enhanced ultrasound (CEUS) results improve even further [Fleischer et al. 2010; 2012; Goldberg et al. 2001]. Moreover, microbubble-based ultrasound contrast agents not only enhance the backscattered signals, but at higher acoustic pressures they also act as nonlinear oscillators producing significant energy components in the received echo signals [Goldberg et al. 2001; Lauterborn 1976]. These nonlinear bubble echoes can be separated from tissue echoes and used to create contrast specific ultrasound imaging modalities. Several previous studies have addressed the use of CEUS for benign and malignant ovarian tumors [Fleischer et al. 2008; 2010; 2012; Orden et al. 2003; Marret et al. 2004; Testa et al. 2005; Wang et al. 2011]. Results show that CEUS can characterize blood flow kinetics in ovarian tumors, but suffers from reduced blood-to-tissue contrast due to second harmonic generation in tissue, which will inevitably degrade the final outcomes [Hamilton & Blackstock 1998].

Alternatively, our group has been developing a new contrast-specific imaging modality: subharmonic imaging (SHI), transmitting at double the resonance frequency ($2f_0$) and receiving at the subharmonic (f_0), because of the excellent suppression of tissue echoes (i.e., much better SNR) relative to other US contrast modes [Forsberg et al. 2000]. Feasibility studies of SHI have been conducted *in vitro* and *in vivo* by our group [Bhagavattheeshwaran et al. 2004; Dave & Forsberg 2009; Dave et al. 2010; 2011; 2012a; 2012b;

2012c; 2012d; Eisenbrey et al. 2011a; 2011b; 2011c; 2012; Forsberg et al. 2000; 2006; 2007; Shi et al. 1999; Sridharan et al. 2013; 2015] and by others [Chomas et al. 2002; Faez et al. 2011a; 2011b; Goertz et al. 2005; 2006; 2007; Helfield et al. 2012; Needles et al. 2008; Shankar et al. 1998]. We have demonstrated, in a first-in-humans study of 14 women with 16 breast lesions that SHI can detect the slow, small volume blood flow associated with tumor angiogenesis [Dave et al. 2010; Eisenbrey et al. 2011b; 2011c; Forsberg et al. 2007]. Moreover, unlike the gadolineum chelates used as MRI contrast agents, gas-filled contrast bubbles are pure intravascular tracers and flow through the body differently, which enables CEUS to depict tumor vascularity differently from MRI. Moreover, SHI can be used for quantitative perfusion estimation (in ml/min/g), which has been a long sought after clinical goal and is potentially useful for the characterization of malignant vs. benign lesions [Forsberg et al. 2006; Sridharan et al. 2013]. We have created SHI parametric images of ultrasound contrast kinetic data acquired in our breast pilot study [Eisenbrey et al 2011b]. This innovative technique will be expanded to adnexal masses in this project.

Based on our previous *in vitro* and *in vivo* studies and since it is one of only three ultrasound contrast agents, which are commercially available and approved by the FDA for use in humans (in echocardiography) in the United States, we have selected the contrast agent Definity (Lantheus Medical Imaging) for use in this project. The route of administration and the dosages employed in the proposed clinical trial follow the recommendations issued by the manufacturer and the FDA. However, the FDA has yet to approve Definity for use in gynecological imaging and, as such this constitutes an off-label use of the agent. Consequently, we intend to apply to the FDA for an investigator initiated IND (investigational new drug) exemption to cover the use of Definity in patients with adnexal masses. Definity is a sterile, non-pyrogenic suspension of liposome-encapsulated perfluoropropane microbubbles [Goldberg et al. 2001; Miller & Nanda 2004]. Definity is well tolerated by patients. The largest safety studies published to date on the use of US contrast agents in humans (involving up to 4,300,966 subjects) concluded that these agents have a good safety profile in both cardiac and abdominal US applications [Dolan 2009; Main et al. 2008; 2009; Wei et al. 2008].

1.1.1 Definity Clinical Safety

Definity is well tolerated and has been used extensively in echocardiography applications [Goldberg et al 2001]. In pre-market clinical trials, Definity was administered to 1716 patients. In these patients 269 (8.4%) reported at least one adverse event (AE). Of these events, 26 were classified as serious including 19 (1.1%) patients experiencing serious cardiopulmonary symptoms including eight deaths. The deaths occurred several days after activated Definity administration and appear to be related to the course of underlying disease. Of the 11 other serious AEs, which appeared within days of the drug administration (2-15 days), all appeared to be a progression of underlying cardiac and non-cardiac disease. However, a role for Definity in the initiation or course of these AEs cannot be ruled out. Of the reported adverse reactions following the use of Definity the most frequently reported were headache (2.3%), back and renal pain (2.1%), flushing (1.1%), and nausea (1.0%). Additional risks associated with the contrast material are described in the attached Definity 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 $\geq 0.5\%$ of the Subjects who Received Definity in Controlled Clinical Studies

<u>No. of Patients Exposed to Definity</u>	1716	
<u>No. of Patients Reporting an Adverse Event</u>	269 (8.8%)	
Central and peripheral nervous system	54	(3.2%)
Headache	40	(2.3%)
Dizziness	11	(0.6%)
Body as a Whole	41	(2.4%)
Back/Renal Pain	20	(1.2%)
Chest Pain	13	(0.8%)
Digestive System	31	(1.8%)
Nausea	17	(1.0%)
Vascular (extracardiac) disorders	19	(1.1%)
Flushing	19	(1.1%)
Application Site Disorders	11	(0.6%)
Injection Site Reactions	11	(0.6%)

Additional information concerning pre-clinical and clinical experience with Definity, including the dosing levels and reported subject complaints, can be found in section 6.5 and the Definity Package Insert that is included as Appendix B.

1.2 Rationale

Our fundamental hypothesis is that subharmonic signals from ultrasound contrast microbubbles can be employed to improve the characterization of adnexal masses as benign or malignant (either through direct imaging or through quantitative SHI biomarkers). Hence, we propose the development of a novel and innovative ultrasound based technology for the noninvasive evaluation of adnexal masses (i.e., contrast enhanced SHI) in order to improve the management of ovarian cancer.

2. TRIAL OBJECTIVES

Trial Objectives: The primary objective of this trial is:

- To develop qualitative subharmonic imaging (SHI) or SHI-derived quantitative biomarkers and **to generate pilot data for a study to evaluate if they improve the characterization of benign and malignant adnexal masses compared to standard ultrasound or contrast enhanced magnetic resonance imaging (MRI) or the risk of malignancy index (RMI).**

and a secondary objective is:

- To compare the SHI depiction of adnexal masses' neovascularity in humans to intra-tumoral microvascular density (iMVD) obtained from CD31 an immunohistochemical marker of angiogenesis.

3. TRIAL DESIGN

This is an open-label, non-randomized pilot study that will be conducted at one clinical site, Thomas Jefferson University (TJU). Enrolled patients scheduled for subsequent surgery of an adnexal mass will receive a bolus IV injection followed 30 minutes later by a continuous infusion of the ultrasound contrast agent Definity. Contrast ultrasound imaging will be performed using an EPIQ scanner with a broad

bandwidth C10-3v EV transducer operating in SHI mode. Results will be compared to MRI assessments and pathology obtained as part of the subjects' standard of care.

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 of childbearing potential, she will have a urine pregnancy test (the results of which will be made available to the woman prior to study initiation).

3.1 Trial Duration

Individual participation in this trial will be limited to one ultrasound imaging study prior to their surgery for removal of an adnexal mass (as part of the standard of care at TJU). The entire ultrasound imaging protocol will require approximately one hour.

Subject recruitment is expected to last 18 months (February, 2016 – July, 2017). Analysis and publication of results are expected to take an additional month (August, 2017).

4. TRIAL POPULATION

Trial Population: This trial will consist of up to 45 adult women (21 years of age or older) at TJU, who have an adnexal mass and are scheduled for surgery based on their morphological characteristics (on standard imaging), CA125 levels and menopausal status.

Subjects eligible for trial enrollment will be identified by the co-investigator, Dr. Rosenblum, from his patient population of subjects scheduled for surgery of an adnexal mass. The research coordinator for this study will explain the study to the patients. 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 woman 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 fulfill all the following criteria:

- Be diagnosed with an adnexal mass.
- Be scheduled for surgery to remove the adnexal mass.
- Be at least 21 years of age.
- Be clinically stable.
- If a female of child-bearing potential, must have a negative pregnancy test.
- Be conscious and able to comply with study procedures.
- Have read and signed the IRB-approved Informed Consent form for participating in the study.

4.2 Exclusion Criteria

Subjects who fulfill 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 have received an investigational drug in the 30 days before study drug administration, or will receive one within 72 h afterwards.
- Patients with known or suspected right-to-left, bi-directional, or transient right-to-left cardiac shunts.
- Patients with pulmonary hypertension or unstable cardiopulmonary conditions.
- Patients currently on chemotherapy or with other primary cancers requiring systemic or hepatic loco-regional treatment.
- Patients who are clinically unstable, patients who are seriously or terminally ill with a life expectancy of less than 1 month, and patients whose clinical course are unpredictable. For example:
 - Patients on life support or in a critical care unit.
 - Patients with unstable occlusive disease (e.g., 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 who have undergone surgery within 24 hours prior to the study sonographic examination.
- Patients with a history of anaphylactic allergy to Definity, manifested by one or more of the following symptoms: generalized urticaria, difficulty in breathing, swelling of the mouth and throat, hypotension, or shock.
- Patients with congenital heart defects.
- Patients with severe emphysema, pulmonary vasculitis, or a history of pulmonary emboli.
- Patients with respiratory distress syndrome.
- Patients with thrombosis within the splenic vein.

Subject identification will be maintained with a study specific alphanumeric code including the institution (TJU), patient number (001 and onwards) and the patient's initials.

5. MEDICATIONS

Definity will be provided by Lantheus Medical Imaging (N Billerica, MA). An investigator initiated IND (investigational new drug) application will be submitted to the FDA to cover the use of Definity in patients with adnexal masses (since Definity is currently only approved for use in echocardiography). Studies will not commence until regulatory approval has been obtained from the FDA as well as the IRB committee for TJU.

Definity is a sterile, non-pyrogenic suspension of liposome-encapsulated perfluoropropane microbubbles [Goldberg et al. 2001; Miller & Nanda 2004]. The contrast agent is composed of a blend of three phospholipids contained in a matrix of sodium chloride, propylene glycol, and glycerin in water. The contrast agent is supplied in a vial that contains the phospholipids and perfluoropropane gas. The microbubble agent is supplied in a standard-size 2 ml vial and is prepared by shaking the vial with the aid of a shaking device (Vialmix: ESPE, Seefeld, Germany). The vial contains a clear, colorless, sterile, non-pyrogenic, hypertonic liquid, which upon activation with the aid of a Vialmix, provides a homogeneous, opaque, milky white injectable suspension of perflutren lipid microspheres. After activating the contents of the vial in a Vialmix, each ml of the milky white suspension contains a maximum of 1.2×10^{10} perflutren lipid microspheres, and about 150 $\mu\text{l}/\text{ml}$ (1.1 mg/ml) octafluoropropane. The mean diameter range is 1.1 μm to 3.3 μm with 98% microspheres having size less than 10 μm . The maximum microsphere diameter is 20 μm . Detailed resuspension instructions are provided in the Definity Product Insert, found in Appendix B.

Definity is well tolerated by patients. The largest safety studies published to date on the use of ultrasound contrast agents in humans (involving up to 4,300,966 subjects) concluded that these agents have a good safety profile in both cardiac and abdominal ultrasound applications [Dolan 2009; Main et al. 2008; 2009; Wei et al. 2008]. However, as rare but serious cardiopulmonary reactions have been reported

following the intravenous injection of Definity, all subjects will be closely monitored by a physician during the entire imaging period [FDA Alert 2007; 2008].

Briefly, Definity 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. All subjects will receive an initial bolus injection at a steady rate not to exceed 1 ml/s with doses of 1.3 to 1.5 ml. Each bolus injection of Definity will be followed with a flush of 10 ml of normal saline. There will be a 15 minute pause between injections to allow for complete contrast clearance. For the second injection, we will infuse up to 1.5 ml of Definity mixed in 25 ml of saline over 5 minutes (which is the approved dose and concentration for infusion of Definity).

Definity will be stored in a secure cabinet, with only the study investigators and research personnel having access. New vials must be prepared for each subject's contrast administration. Unused drug and empty vials will be properly disposed of after reconciling in the log of study drug.

5.1 Contraindications

Definity should not be administered to patients with known or suspected right-to-left, bi-directional or transient right-to-left cardiac shunts, by intra-arterial injection, or to patients with known hypersensitivity to perflutren. Patients with any of these characteristics will be excluded from the study. Additional exclusion criteria are listed in section 4.2.

5.2 Randomization

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

5.3 Blinding and Unblinding Methods

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

5.4 Storage

Definity vials will be stored in a locked refrigerator, with only the study investigators and research personnel having access. The study researchers will be responsible for drug reconstitution and inventory control. Activated Definity should be used within 5 minutes, but once activated the vials can be carried around to be used again for a period of 12 hours by reactivating with 10 seconds of hand agitation. After a period of 12 hours from the initial activated time, unused activated vials will be discarded.

6. TRIAL PROCEDURES

6.1 Screening Assessments

All subjects will receive a written consent form and a verbal explanation of the study by an investigator and/or the research study coordinator and will be asked to sign the written informed consent. Study participants will have the presence of inclusion criteria (section 4.1) and absence of exclusion criteria (section 4.2) verified by providing a comprehensive medical history (and a urine pregnancy test in the case of women of child bearing potential).

6.2 Treatment Administration

Administration of Definity will be performed under direct supervision of a medical doctor. Emergency services (e.g., a crash cart) will be available within the hospital in case of any acute adverse reactions. Additionally, resuscitation equipment and trained CPR personnel will be in immediate proximity to the patient during Definity administration.

Briefly, Definity microbubbles will be activated with a Vialmix device using a full 45-second activation cycle to ensure consistent microbubble size, as per manufacturer's recommendation. A needle will be

inserted into an activated Definity vial to be used as an air vent. An 18 to 20 gauge needle will be attached to 3 or 5 cc syringe and inserted into the vial to withdraw 1.3 to 1.5 ml of activated Definity. All subjects will receive an initial bolus injection at a steady rate not to exceed 1 ml/s with doses of 1.3 to 1.5 ml. Each bolus injection of Definity will be followed with a flush of 10 ml of normal saline. Following a 15 minutes interval a dose of one vial of activated Definity, mixed in 25 ml of saline, will be prepared for each subject and infused through a peripheral vein at a rate sufficient to provide diagnostic intra-tumoral contrast visibility. Infusions will be performed as described in the “Instructions for Use” provided by the manufacturer. All materials and supplies used for the infusion procedure will be identical to those described by the manufacturer. Up to 1.5 ml of activated Definity per vial will be extracted (as described above) and added to 25 ml of preservative-free saline. The IV bag will then be gently squeezed to evenly distribute the microbubbles and an infusion will be initiated. As per the manufacturer’s recommendation the infusion rate may vary between 4 ml/min to 10 ml/min and in this study the infusion rate will be maintained within this range but will be adjusted to provide diagnostic intra-tumoral contrast visibility.

6.3 Trial Assessments

6.3.1 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 woman is of childbearing potential, she will have a urine pregnancy test (the results of which will be made available to the subject prior to study initiation). Emergency services (e.g., a crash cart) will be available within the hospital in case of any acute adverse reactions. Adverse events will be monitored during the entire procedure.

6.3.2 Ultrasound Imaging

The ultrasound examinations will be performed by a qualified sonographer. 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. A modified Logiq E9 scanner with a [REDACTED] C10-3v EV transducer

(GE Healthcare) operating in SHI mode will be used to obtain subharmonic radiofrequency (RF) data from the adnexal mass within the ovary and/or fallopian tube. Both the experimental machine and software will be labeled as an investigational device with a GE provided label prior to beginning any clinical experiments (21 CFR 812.5).

A baseline grayscale ultrasound scan of the lesion will be obtained and the following criteria assessed: the size, shape, and orientation of the site; echogenicity compared to surrounding tissue. Standard PDI of the mass will also be performed to select the area with the greatest vascularity. The distribution of color signals and the overall color content of the mass will be evaluated by comparing the pattern and amount of color to normal tissue on the contra-lateral side. If uniform, the color will be categorized as less intense, iso-intense or more intense than normal tissues. If non-uniform, the color will be described by location, intensity and distribution. Anastomoses between adjacent vessels will be noted (as 1-2, 3-5 or >5 vessels connecting). The vascular pattern will also be graded as peripheral, radial, spotty or a combination. Digital clips of the two baseline imaging modes will be acquired.

Two contrast injections will be performed. The first injection will involve pulse inversion grayscale EV SHI (in a dual display format utilizing at a transmit frequency of 5.8 MHz and the subharmonic obtained at 2.9 MHz [Eisenbrey et al. 2011a; Sridharan et al. 2015]) of the area of greatest vascularity within the adnexal mass (as established by baseline PDI) followed by IV administration of 1.3 to 1.5 ml of Definity and acquisition of the contrast enhancement covering baseline, wash-in and peak enhancement to complete wash-out (typically 1-2 minutes). There will be a 15 minute pause between injections to allow for complete contrast clearance. For the second injection, we will infuse up to 1.5 ml of Definity mixed in 25 ml of saline over 5 minutes and then perform subharmonic destruction/reperfusion SHI imaging in multiple imaging planes covering the adnexal mass through the entire period of enhancement (in practice 3-4 minutes of equilibrium contrast concentration); similar to our previous work [Linden et al. 2007; Shi et al. 2001]. The digital clip(s) obtained from the SHI injection will be transferred to a PC for off-line analysis. The SHI images will be qualitatively analyzed as described above. It should be noted, that all subjects will be

closely monitored by a physician during the study [FDA Alert 2007; 2008].

6.4 Efficacy Assessments

The pre- and then post-contrast ultrasound imaging will be evaluated for each patient as follows: grayscale, grayscale and PDI (baseline), and grayscale and SHI. While this may introduced some bias from pre- to post-contrast results, we considered this the more realistic approach to how CEUS may be used in clinical practice. Each case will be read independently by the 2 experienced co-investigators (15+ years of expertise in CEUS imaging) blinded to the surgical results and contrast-enhanced MRI evaluations, to allow repeatability to be assessed. All ultrasound studies will be rated on a quasi-continuous scale from 0 to 100 ranging from “no lesion seen (no findings)” over “definitely benign,” “indeterminate” to “definitely malignant.” Using a quasi-continuous 100-point rating scale has an intuitive probabilistic interpretation and is known to improve the assessment of the capabilities of the imaging modes studied [Wagner et al. 2001]. The SHI clips will be processed to produce CMI-SHI images [Dave & Forsberg 2009; Dave et al 2010], which will also be read by the blinded readers. Finally, sensitivity, specificity and accuracy will be calculated for all imaging modalities (baseline and SHI as well as MRI) and compared to the RMI values used to select patients for surgery [Ferrazzi et al. 1997; 2005; Moolthiya & Yuenyao 2009].

Off-line SHI TICs will be created on a pixel by pixel basis. Parametric images will be generated showing time-to-peak, perfusion rate, peak intensity (calculated in dB as the ratio of the peak to the baseline values) and area under the curve (AUC) in the area of greatest vascularity as well as parameters across the mass (based on the data from second infusion) [Eisenbrey et al. 2011b]. The TIC parameters will be compared to the lesion characterization of the other imaging modalities with pathology as the reference standard. It is our hypothesis that these new SHI based biomarkers will improve our capabilities to characterize ovarian cancers. Results will also be compared to MRI enhancement kinetics and imaging assessments obtained as part of the subjects’ standard of care.

Dr. Rosenblum will mark the transaxial plane of the lesion by sutures upon excision of the adnexal mass, so the specimen can be serially sectioned in the transaxial plane and immunohistochemical staining performed to determine the following variables: tumor type (benign or malignant); if malignant is the cancer invasive or a tumor of low metastatic potential; histological lesion type (epithelial, stromal or germ cell), RMI as well as staging and other markers will be collected as part of the patient's clinical care and correlated with the imaging results. Finally, the ability of EV SHI to image angiogenesis (specifically fractional tumor vascularity) will be compared to so called "hot spots" within surgical specimens stained for an immunohistochemical predictor of angiogenesis, specifically a monoclonal antibody against the CD31 endothelial cell marker (Dako Corporation, Carpinteria, CA). The principal investigator of this project has, as part of previously NIH and DOD funded projects, developed the necessary software for quantitative analysis of CEUS images and specimens stained with CD31 using a histomorphometry system based on an Labophot-2 microscope (Nikon, Melville, NY) and ImagePro Plus software [Forsberg et al. 2008].

6.5 Safety Assessments

Emergency services (e.g., a crash cart) will be available within the hospital in case of any acute adverse reactions. AEs will be monitored during the entire procedure. Specifically, the patient will be monitored with non-leading questions to monitor the patient for the occurrence of transient side effects that are described below.

6.5.1 Risks/Benefits Assessment.

The known risks from the administration of Definity are minimal. Definity is well tolerated and has been used extensively in echocardiography applications. A total of 1716 subjects were evaluated in pre-market clinical trials of Definity. Of these patients 144 (8.4%) reported at least one treatment-related adverse reaction. The most common AEs were reported in the Central and peripheral nervous system (3.1%), Body as a Whole (2.4%) and Gastrointestinal system (1.8%). The most common events were headache (2.3%), back and renal pain (1.2%), flushing (1.1%) and nausea (1.0%). All of the non-serious reported side-effects have been transient, usually lasting only a few minutes.

There were 26 serious AEs and 15 (0.9%) subjects discontinued because of an AE. Nineteen (1.1%) suffered serious cardiopulmonary adverse events including eight deaths. The deaths occurred several days after Definity administration and appear to be related to the course of underlying disease. Of the 11 other serious AEs, which appeared within days of the drug administration (2-15 days), all appeared to be a progression of underlying cardiac and non-cardiac disease. However, a role for Definity in the initiation or course of these AEs cannot be ruled out. As a result of post market analysis, the FDA added a black box warning to Definity in 2008 for patients with unstable cardiac or respiratory conditions. In one of the largest retrospective multicenter study evaluating the effect of Definity administration in 66,164 cases, no deaths were reported attributable to the drug use [Wei et al. 2008]. The rate of severe allergic reactions for either cardiac or abdominal ultrasound imaging following Definity was 0.0006% [Wei et al. 2008]. It was concluded that the severe AE rate following the use of ultrasound contrast agents is no greater and may be lower, than the reported event rate for other contrast agents used in other cardiac imaging studies [Wei et al. 2008].

A prospective, open-label, nonrandomized, multicenter phase 4 surveillance registry was conducted at 15 clinical sites in the USA to assess the risk for adverse cardiopulmonary events during or within a 30 minute period after administering Definity to patients between February 2008 and April 2009 [Weiss et al. 2012]. In this study, 1053 patients were administered Definity and no deaths, serious AEs and other significant AEs were reported [Weiss et al. 2012]. The overall drug related adverse event rate was only 3.5% and within these cases, 96.5% of the AEs were of mild or moderate intensity [Weiss et al. 2012]. Based on study by Weiss et al [2012], the FDA concluded that deaths or serious AEs are “unlikely to occur at a rate of more than 0.3% when Definity is used according to recommendations” [FDA 2011].

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 Definity are unlikely. The use of contrast with the new ultrasound imaging techniques is expected to provide significantly more information than conventional ultrasound techniques. This may lead to a non-invasive method for characterization of adnexal masses.

To minimize and/or eliminate risks a nurse will be present during the entire procedure. Emergency services (e.g., a crash cart) will be available within the hospital in case of any acute adverse reactions. Adverse events will be monitored during the entire procedure.

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

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 Definity. 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 observed or volunteered problems, complaints, signs or symptoms, and diagnoses, occurring from the initiation of

Definity dosing until the completion of the Definity administration will be recorded on a serious or non-serious AE data form, whether or not associated with the use of the trial medication. GE Healthcare will be informed of all AEs. In addition all unexpected and serious AEs are reported to the TJU IRB and to the FDA. The investigator is required to submit all unexpected and serious AEs to the TJU IRB within 48 hours, and to GE Healthcare. Fatal AEs related to treatment which are unexpected must be reported within 24 hours to the TJU IRB and to GE Healthcare. Fatalities not related to the study drug/device must be reported within 5 days. The PI of the study has previous experience running ultrasound clinical trials and will serve as the study sponsor. He will be responsible for ensuring all FDA requirements are met and all AE are properly reported.

The AE forms will include: subject identification number and initials; subject's date of birth, gender, and ethnicity; name of the institution, date of Definity 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(s) will evaluate and note the duration, intensity, and relationship to (association with) the Definity 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 drug therapy or other

treatment.

When the intensity of the AE changes over time, the maximum intensity will be recorded.

RELATIONSHIP TO DEFINITY ADMINISTRATION

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

An "unlikely" relationship indicates that there is little or no chance that Definity 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 Definity 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 Definity 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 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 serious AE.]
- 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.

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 Definity administration. The medical monitor will also indicate whether he concurs with the details of the report provided by the principal investigator.

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

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

6.7.1 Discontinuation of Subjects

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 all of the following trial requirements:

- Has received one bolus injection and one infusion of Definity
- Has undergone the complete ultrasound imaging study as described in this protocol

If a subject's participation in the trial is interrupted for any reason (e.g., because of an AE or if the subject is lost to follow-up) and the subject has met the criteria described above for completing the trial, the subject's trial participation will be considered completed. If a subject's trial participation is interrupted for any reason

by the subject's or investigator's choice and the subject has not met all of the criteria listed above, then the subject will be considered a discontinued subject.

7. DATA MANAGEMENT AND STATISTICAL ANALYSES

7.1 Data Management

Data forms will be completed for all subjects enrolled in the trial. The patient study files will be stored in a secure file cabinet and maintained by the research study coordinators at TJU. Patient study files will be kept for 7 years after the completion of the study. The PI of the study has previous experience running ultrasound clinical trials and will serve as the study sponsor. He will be responsible for ensuring all FDA requirements are met and all AE are properly reported.

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.

7.2 Statistical Analyses

7.2.1 Hypotheses

H₁: EV SHI will significantly differentiate malignant from benign adnexal masses using surgical pathology as the reference standard. As an initial end-point we speculate that an area under the ROC curve of 0.75 or better will be achieved by EV SHI.

H₂: EV SHI biomarkers will improve the characterization of benign and malignant adnexal masses compared to standard ultrasound or contrast enhanced MRI or other clinical parameters. As an initial end-point we speculate that an area under the ROC curve of 0.75 or better will be achieved by EV SHI, which will be better than low-end estimates of the accuracy of standard ultrasound, contrast enhanced MRI and other clinical parameters (which is on the order of 0.70 to 0.72).

H₃: A correlation coefficient of 0.70 will be achieved between *in vivo* SHI ovarian neovascularity measurements and iMVD.

7.2.2 Analysis of Results

The findings of SHI will be correlated to vascular morphology (i.e., tumor angiogenesis ascertained with CD31) and other imaging and pathological findings; including size, vascularity, presence or absence of receptors and RMI values. The techniques will be compared using an analysis of variance (ANOVA) with method (i.e., baseline ultrasound imaging, SHI, or MRI) as the dependent variable and outcome as the independent variables. For the pre and post-contrast enhanced comparisons, dichotomous parameters (e.g., benign/malignant) and ranked data (less, iso, or more intense) will be analyzed with the McNemar test. The ability of the imaging tests to distinguish benign from malignant masses will be compared using ROC analysis, while the incremental validity of imaging diagnosis and morphological characteristics as well as serum biomarkers (i.e., RMI) will be analyzed using logistic regression and ROC analyses [DeLong et al. 1988; Metz 1986; Wagner et al. 2001]. Differences between ROC curves will be tested by computing Mann-Whitney statistics. All of the statistical analyses proposed for this human clinical trial will be repeated split by racial and ethnic groups to determine if clinically important race/ethnicity differences exist in the ability of SHI to characterize adnexal masses (although the limited sample size of this pilot study makes it unlikely that such differences – if any exist - will be detectable).

All analyses and computations will be performed using NCSS/PASS 2008 and Stata 12.0 (Stata Corporation, College Station, TX), while the study database will be designed and implemented in REDCap (Vanderbilt University, Nashville, TN). This database will contain all patient information (except names and other identifiers), including pathology, MRI and SHI measurements as well as other clinical variables.

7.2.3 Sample Size Considerations

As this project involves a pilot study of a new ultrasound imaging mode (i.e., SHI) in a completely new application, no power analysis can be performed *a priori*. As a pilot study this project is not powered to

provide definitive answers to the hypotheses presented above (cf., section 7.2.1), but rather is a first step towards establishing feasibility and to provide data for a future more exhaustive study. The planned sample size of 45 subjects will provide important information on the precision of the anticipated correlation coefficient of 0.70 between in vivo SHI ovarian neovascularity measurements and iMVD such that the 95% confidence interval would be (0.51, 0.82).

The Gynecological Oncology Division at TJU evaluate more than 500 women with adnexal masses each year out of which around 1 woman/week will require surgical intervention based on their morphological characteristics (on standard imaging), CA125 levels and menopausal status [Ferrazzi et al. 1997; 2005; Moolthiya & Yuenyao 2009]. The patient population of this project will reflect the population demographics found at major American urban academic health centers. The overall hospital demographics for TJU include 60 % Caucasian, 16 % African American, 13 % Hispanic, 5 % Asian, 1 % Other, and 5 % unknown patients. We anticipate enrolling approximately 45 subjects scheduled for surgery in this project (with some 15-20 % expected to be cancers), which means we are aiming for a recruitment rate of approximately 58% (45/78).

7.2.4 Disposition of Software

Investigational software containing the modified SHI mode will be removed from the EPIQ scanner upon completion of the study to avoid the investigational device being used for any unapproved investigations. At the conclusion of the study, the developed software will be saved on CD for future experimentation. These investigations may ultimately further the development of SHI technology as a cancer imaging system. However, under no circumstances will the software be used in a clinical environment (i.e., for human studies) without GE consent. This will be ensured by the use of a GE controlled password for installation of experimental software which is currently in use and will expire at the conclusion of the study.

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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

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

Ji-Bin Liu, MD, Research Professor of Radiology will assist with the animal studies and interpret ultrasound images from animals as well as humans.

Andrej Lyshchik, MD, PhD, Assistant Professor of Radiology will interpret ultrasound images from animals as well as humans and advise on clinical issues.

Norman Rosenblum, MD, PhD, Professor of Obstetrics and Gynecology will assist with the patient recruitment, interpret surgical findings and advise on clinical issues.

John Eisenbrey, PhD, Research Assistant Professor of Radiology will assist with the animal studies, aid in patient recruitment, informed consent, ultrasound studies, and the data entry and statistical analyses.

Signature of PI: _____
Flemming Forsberg, PhD

APPENDIX B – DEFINITY PACKAGE INSERT