PROTOCOL

TITLE: QUANTITATIVE SUBHARMONIC BREAST IMAGING

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STUDY DURATION: 4 year project

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SYNOPSIS

Protocol Title: Quantitative Subharmonic Breast Imaging

Trial Objectives: The primary objective of this trial is:

- To evaluate if quantitative 3D Subharmonic imaging (SHI) or pulse inversion harmonic imaging (HI) can improve the characterization of benign and malignant breast masses (independently or in combination with other imaging modes) compared to x-ray mammography, fundamental grayscale ultrasound (US) or power Doppler imaging (PDI).

The secondary aim of this trial is:

- To compare quantitative (bifurcations & vessel length) and semi-quantitative (blood pool & parametric imaging) measures of the vascular morphology of breast lesions determined by pathology and by SHI.

Trial Design: This is an open-label, non-randomized trial that will be conducted at two clinical sites (the Breast Imaging Centers at Thomas Jefferson University (TJU) Hospital and University of California, San Diego (UCSD) Hospital). All subjects will receive at most two IV bolus injections of Definity™ (Lantheus Medical Imaging, Billerica, MA), will undergo an unenhanced (baseline) and Definity contrast-enhanced US imaging study for evaluation of a breast mass or breast abnormality without mass, and will be scheduled to undergo a clinically indicated biopsy of the breast lesion under investigation.

Trial Population: This trial will consist of up to 450 adult (18 years of age or older) female subjects who are scheduled for a biopsy of a breast mass or abnormality identified on mammography.

Trial Procedures: Subjects eligible for trial enrollment will be identified by the investigators from among TJU and UCSD's patient population who are referred for a breast biopsy. A full demographic profile, known drug allergies or intolerances, and review of the subject's medical/surgical history will be recorded and reviewed to ensure the subject meets inclusion criteria.

A modified Logiq 9 scanner (GE Medical Systems, Milwaukee, WI) with a broad bandwidth, 3D, linear array will be used to acquire conventional images and subharmonic imaging (SHI).

A baseline US grayscale scan will be used to identify the mass or abnormal area seen by mammography (or another concomitant imaging mode, such as US or MRI) and to evaluate the following criteria: diagnosis; size, shape, and orientation of the lesion; echogenicity compared to surrounding tissue. Standard PDI of the lesion or target area will also be performed. The distribution of color signals and the overall color content of the lesion will be evaluated by comparing the pattern and amount of color to the normal surrounding breast. Irregularity of the course of the vessels and anastomoses will be evaluated. Digital clips of the two baseline imaging modes will be acquired (with videotaping as backup).

Patients will then receive a bolus, intravenous (IV) injection of 0.25 ml of Definity in a peripheral vein followed by 3D pulse inversion HI. The pulse inversion HI images will be analyzed similar to the baseline power Doppler images. Patients will then receive a second bolus, IV injection of up to 20 µl/kg of Definity, followed by 3D SHI. The SHI images will be qualitatively analyzed as described above. All subjects will be closely monitored by a physician during and following the study (for 30 minutes as recommended by the FDA).

The entire examination will be recorded as digital loops, as well as on VHS videotape. Baseline and contrast-enhanced US imaging findings will be recorded.

In addition, all subjects will be scheduled to undergo a clinically indicated breast biopsy core / excisional / lumpectomy of the mass or region of abnormality or mastectomy within 30 days after the US study procedure. Apart from diagnosis, the pathologist will provide the following when possible: estrogen progesterone and HER2 receptor presence or absences; presence or absence of hemorrhagic, necrotic, or other component of the lesion (and their location if present); histological margins; lesion size; TNM staging; histological lesion type; presence or absence of vascular invasion; histological and cytological grade; presence or absence of metastases; node staging; and percentage of the lesion that was invasive or *in situ*.

All images will be evaluated by a blinded reader and rated on a quasi-continuous scale from 0 to 100 ranging from "no lesion seen (no findings)" over "probably benign" and "indeterminate" to "malignant." Each case will also be read by the two on-site investigators, blinded to the mammographic and histopathological diagnosis, to allow repeatability to be assessed for each site. Finally, each mammogram will be read by a radiologist as part of the patient's standard clinical assessment and assessed using the BIRADS scale.

Statistical Methodology: Sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) will be calculated for all six imaging modalities (the five different US modes and mammography). The dichotomous parameter needed for these calculations will be derived by ascertaining whether the radiologist would recommend a biopsy or not based on the imaging study in question. Inter- and intra-observer variability will also be calculated.

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 mammography will be analyzed using logistic regression and ROC analyses.

INTRODUCTION

Background

X-ray mammography is efficacious in the detection and differentiation of a high percentage of breast masses. However, in a significant number of patients mammography cannot differentiate between benign and malignant circumscribed masses [Feig 1992]. Solid breast masses are best managed by biopsy or close follow-up [Jackson 1990]. Thus, the development of a technique, which can reliably differentiate benign from malignant solid masses, should result in earlier cancer detection and reduce unnecessary biopsies as well as eliminate patient anxiety from follow-up.

While US imaging complements x-ray mammography in the pre-operative diagnosis of palpable breast tumors, its main use is restricted to differentiating between cystic and solid masses [Burns et al 1982]. To improve the usefulness of US and to aid in the differentiation of benign from malignant lesions both color and pulsed Doppler have been utilized. Pulsed Doppler investigations have found differences in the neovascularity of benign and malignant tumors [Burns et al 1982; Madjar 1991; Bohm-Velez & Mendelson 1989]. However, overlap between the two groups has been reported [Bohm-Velez & Mendelson 1989; Adler et al 1990], indicating that more sensitive techniques, such as color Doppler imaging (CDI) [Adler et al 1990; Madjar 1992; Cosgrove et al 1990; Hamada et al 1992; Cosgrove et al 1993], need to be employed. The results of applying CDI to breast cancer diagnosis are mixed ranging from very positive [Hamada 1992] to quite negative [Adler et al 1992].

Malignant tumors less than 3 mm in diameter will stimulate the growth of new blood vessels by secreting angiogenesis factors [Schor & Schor 1983; Folkman et al 1971; Weidner et al 1991; 1992; Brawer et al 1992; Weind et al 1998; Folkman & Cotran 1976]. Hence examination of tissue perfusion is likely to help in the early detection of malignancies [Brawer et al 1992]. In addition, unlike normal tissue with a relative fixed route between arterial and venous sides, a tumor may have blood flowing directly via arteriovenous shunts and other abnormal connections [Jain 1989], which produce a chaotic (as well as sometimes leaky) angiogenic vasculature [Weidner et al 1992], and have increased inward flow speeds. The morphology of tumor angiogenesis may be an important criterion to evaluate, since the microvessel density (i.e., number and size) detected pathologically is an early and independent prediction of metastatic disease [Weidner et al 1992; Brawer et al 1992; Weind et al 1998].

However, due to the vessel size and tortuosity conventional Doppler is not sufficiently sensitive to detect tumor angiogenesis. US contrast agents can alleviate this problem [Goldberg et al 1994; Needleman & Forsberg 1996; Forsberg et al 1998a; Goldberg et al 2001]. Our preliminary work in animal tumor models as well as in human tumors has shown the capabilities of US contrast to enhance the sensitivity of Doppler signals by increasing the reflectivity of the blood [Forsberg et al 1995; 1996; 1998b; Goldberg et al 1996; 1999; Needleman et al 1998; Halpern et al 2000]. The limitations of non-contrast enhanced Doppler examinations of tumors, as well as the benefits of combining PDI and contrast media, have been confirmed by our research utilizing several gas-filled contrast agents. In this initial work, the emphasis has been just on the enhancement of the Doppler flow signals. Due to the up to 25 dB enhancement of Doppler signals produced by contrast media, a marked increase in the sensitivity of Doppler US for detection of tumor angiogenesis and morphology should be feasible [Goldberg et al 2010]. This has been confirmed by our group showing a statistically significant correlation ($p=0.01$) between contrast enhanced breast US images of tumor vascularity and vessels 20 - 39 μm in diameter seen on pathology [Chaudhari et al 2000; Forsberg et al. 2008].

Given the morphology of tumor angiogenesis, the uptake and washout of contrast over time in a tumor as well as the tumor perfusion may become important diagnostic criteria [Kedar et al 1996; Duda et al 1993; Huber et al 1998], similar to results with breast MRI [Esserman et al 1999]. One study of 34 breast cancers using contrast vascular morphology as well as contrast washout times were statistically significant in discriminating between malignant and benign lesions ($p<0.02$) [Kedar et al 1996]. Furthermore, 4 cases were reclassified after contrast administration, which increased both sensitivity and specificity to 100%. US contrast was used by Wei et al to measure the mean myocardial blood flow i.e., a measure of myocardial perfusion [Wei et al 1998]. The estimated perfusion was compared to radiolabled microsphere derived flow rates (in mL/min/g) and showed a statistically significant linear correlation ($r=0.88$). Tissue perfusion may be better estimated with a new contrast-specific imaging modality harmonic imaging (HI), which transmits at the fundamental transducer frequency (f_o) and receive at the second harmonic ($2f_o$), due to the preferentially enhancement of contrast signals Schrope 1993; Steinbach 1998]. Unfortunately, HI suffers from reduced blood-to-tissue contrast resulting from second harmonic generation and accumulation in tissue [Ward et al 1997].

Consequently, we have proposed using subharmonic imaging (SHI) by transmitting at the fundamental frequency (f_o) and receiving at the subharmonic ($f_o/2$) as an alternative to HI [Shankar et al 1998; 1999; Shi et al 1997; 1999a; 1999b; Forsberg et al 1998c;2000; Bhagavatheeshwaran et al 2004]. Our group

have demonstrated *in vitro* that some contrast agents produce significant subharmonic signal components, which may be greater than the second harmonic echoes [Shankar et al 1998; Shi et al 1997; 1999b]. Aspects of the theory behind SHI have also been documented [Shankar et al 1999]. Furthermore, we are the first ever researchers to produce *in vivo* SHI [Forsberg et al 1998; 2000; 2007; Shi et al 1999b]. Because of no subharmonic generation in tissue and significant subharmonic scattering from some contrast agents, SHI has the potential to detect slow, small volume blood flow associated with tumor neovascularity, making early detection and identification of tumors very likely.

Initial results using contrast enhanced SHI have been promising. As part of a federally funded pilot study using both Definity and Optison (GE Healthcare, Princeton NJ), we have demonstrated, in 14 women with 16 lesions (out of which 4 were cancers) that SHI can detect the slow, small volume blood flow associated with breast tumor angiogenesis [Forsberg et al 2005; 2007]. SHI resulted in an almost complete suppression of tissue signals allowing better visualization of the lesion vascularity relative to baseline power and improved display of small intra-tumoral vessels relative to PDI. The internal morphology of the vascularity associated with the breast masses were also visualized better with SHI than with contrast enhanced PDI. Additionally, we found that the area under the ROC curve for the diagnosis of breast cancer was higher for SHI than for any of the other techniques tested (0.78 versus 0.64, 0.67 and 0.76) [Forsberg et al 2007].

For quantitative analyses, SHI time intensity curves were determined within each lesion using ImagePro Plus software (Media Cybernetics, Silver Spring, MD) [Forsberg et al 2006a]. SHI perfusion estimates were determined using the linear relationship previously established in a canine model [Forsberg et al 2006b]. Perfusion estimates were determined within three regions of interest, (ROIs) encompassing the centre, the periphery or the entire breast lesion. SHI perfusion estimates within the entire lesion were significantly different for malignant and benign tumors ($p = 0.04$) but not for central and peripheral ROIs ($p > 0.32$).

Further quantitative information on the vascular morphology can be extracted post-acquisition by the construction of cumulative maximum intensity (CMI) images from SHI clips. In CMI mode a composite image depicting vascular architecture and blood flow is constructed through maximum intensity projection (MIP) of SHI data over consecutive images [Dave & Forsberg 2009]. Using CMI-SHI the area under the ROC curve was found to be significantly higher than mammography (p=0.031) and the highest level of all methods discussed above with an ROC=0.90 [Dave et al 2010]. Using this technique we have

created parametric images of US contrast kinetic data acquired over a specific time sequence (from contrast injection to washout) showing localized variations in these parameters within a plane and potentially providing additional post-acquisition data for lesion differentiation [Eisenbrey et al 2010].

The proposed agent for the current study, 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 perfluouropropane 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). Definity will be stored in a secure cabinet, with only the study investigators and research personnel having access.

Definity is currently only approved for use in echocardiography. The agent will be used as an off-label indication for this study. We intend to apply for an FDA investigator-instantiated IND for the off-label usage of Definity for breast cancer imaging using SHI.

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. 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 adverse events, 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 adverse events can not 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 affects have been transient, usually lasting only a few minutes.

Table 1.

Selected Adverse Events Reported in ≥ 0.5% of the Subjects who Received Definity in Controlled Clinical Studies

No. of Patients Exposed to Definity	1716	
No. of Patients Reporting an Adverse Event	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 the Definity Package Insert that is included as Appendix B.

1.2 Rationale

US imaging is currently an auxiliary modality in breast imaging. The results from investigations into the possibility of breast cancer diagnosis based on Doppler US flow detection have been mixed. One problem may be the lack of sensitivity of Doppler techniques in detecting vessels and flow associated with tumor neovascularity. This theory is supported by pathological reports of angiogenic vascular morphology being an independent predictor of metastatic disease.

The introduction of new contrast enhancing agents with the use of a novel imaging technique, three-dimensional (3D) SHI, is expected to detect slow, small volume blood flow associated with tumor neovascularity, making early detection and identification of tumors very likely. SHI may allow tumor perfusion, a measure of angiogenesis, to be estimated via subharmonic uptake and washout curves.

Hence, the current project proposes to increase the ability of breast US to differentiate between benign and malignant lesions and to determine the degree of intratumoral microvascular density (iMVD) by combining injection of an US contrast agent with 3D SHI.

2. TRIAL OBJECTIVES

Trial Objectives: The primary objective of this trial is:

- To evaluate if quantitative 3D SHI or pulse inversion HI can improve the characterization of benign and malignant breast masses (independently or in combination with other imaging modes) compared to x-ray mammography, fundamental grayscale US or PDI.

The secondary aim of this trial is:

- To compare quantitative and semi-quantitative measures of the vascular morphology of breast lesions determined by pathology and by SHI.

3. TRIAL DESIGN

This is an open-label, non-randomized trial that will be conducted at two clinical sites (the Breast Imaging Centers at Thomas Jefferson University (TJU) Hospital and University of California, San Diego (UCSD) Hospital). All subjects will receive at most two IV bolus injections of Definity™ (Lantheus Medical Imaging, Billerica, MA), will undergo an unenhanced (baseline) and Definity contrast-enhanced US imaging study for evaluation of a breast mass or breast abnormality without a mass, and will be scheduled to undergo a clinically indicated biopsy of the breast lesion under investigation.

3.1 Trial Duration

Individual participation in this trial will be limited to US imaging studies (baseline and contrast-enhanced) and a breast biopsy performed up to 30 days after the US study procedures. Baseline and contrast-enhanced images will be acquired on the same day. The entire imaging protocol will require approximately two hours. Subject recruitment is expected to last 3 years (June 2011-2014).

4. TRIAL POPULATION

This trial will consist of up to 450 adult (18 years of age or older) female subjects who are scheduled for excisional biopsy for a breast mass or abnormality identified on mammography.

4.1 Inclusion Criteria

All subjects accepted for this trial must:

- Be a female diagnosed by x-ray mammography (performed within 90 days prior to the study procedure) as having a solid breast mass or abnormal area without a mass.
- Be scheduled for a biopsy (core / excisional / lumpectomy) of the mass or region of abnormality or for mastectomy within 30 days after this study procedure.
- Be at least 18 years of age.
- Be medically stable.
- If a female of child-bearing potential, must have a negative pregnancy test.
- Have signed Informed Consent to participate in the study.

4.2 Exclusion Criteria

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

- Males
- Females who are pregnant or nursing.
- Patients whose breast lesion is unequivocally a cyst by unenhanced US.
- Patients currently on chemotherapy or with other primary cancers requiring systemic treatment.
- Patients who are medically unstable, patients who are seriously or terminally ill, and patients whose clinical course is unpredictable. For example:
- Patients on life support or in a critical care unit.
- Patients with unstable occlusive disease (eg, crescendo angina)
- Patients with clinically unstable cardiac arrhythmias, such as recurrent ventricular tachycardia.
- Patients with uncontrolled congestive heart failure (NYHA Class IV)
- Patients with recent cerebral hemorrhage.
- Patients with clinically significant and unstable renal and/or liver disease (eg, transplant recipients in rejection)
- Patients who have undergone surgery within 24 hours prior to the study sonographic examination.
- Patients with known hypersensitivity to perflutren
- Patients who have received any contrast medium (X-ray, MRI, CT, of US) in the 24 hours prior to the research US exam
- Patients with cardiac shunts.
- Patients with congenital heart defects.
- Patients with severe emphysema, pulmonary vasculitis, or a history of pulmonary emboli.
- Patients with confirmed or suspected liver lesions.
- Patients with respiratory distress syndrome.
- Patients who have had excisional biopsy/lumpectomy of the current area of interest within the past 6 weeks.

Subject identification will be maintained with a study specific alphanumeric code including the study site, patient number for that specific site (001-225) and the patient's initials.

5. MEDICATIONS

Definity will be provided by Lantheus Medical Imaging, Billerica, MA. An FDA Sponsor-Investigator IND will be obtained prior to beginning the trial.

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 perfluouropropane 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). Detailed resuspension instructions are provided in the Definity Product Insert, found in Appendix B.

Definity will be stored in a secure cabinet, with only the study investigators and research personnel having access. Unused drug and empty vials will be properly disposed of after reconciling in the log of study drug.

5.1 Administration

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 the bolus injection at a steady rate not to exceed 1 ml/sec with doses of 0.25 ml up to 20 μl/kg in a thirty minute period with a maximum total dose of 1.5 ml in any one patient. Each bolus injection of Definity will be followed with a very slow flush of 10 ml of normal saline.

5.2 Contraindications

Definity should not be administered to patients with known or suspected hypersensitivity to perflutren. The safety of Definity in patients with 1) right-to-left, bi-directional or transient right-to-left cardiac shunts; 2) severe emphysema, pulmonary vasculitis or a history of pulmonary emboli; 3) confirmed or suspected severe liver lesions; and 4) respirator distress syndrome has not been studied. Therefore, patients with any of these conditions will be excluded from the participation.

5.3 Randomization

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

5.4 Blinding and Unblinding Methods

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

5.5 Storage

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

6. TRIAL PROCEDURES

6.1 Patient Enrollment and Consent

Subjects eligible for trial enrollment will be identified by the investigators from among TJU and UCSD's patient population who are referred for a breast biopsy. An investigator or research coordinator will explain the study to the patient. The patient will be given time to consider the risks and benefits of the study and ask questions about participation. The consent form will be reviewed with the patient and then the patient will be given the form to review. If consent interview is conducted by a coordinator, a study investigator will then discuss the study with the subject and answer any additional questions. The patient, person conducting study interview (if applicable), and a study investigator will all sign the consent form. The patient will be given a copy of the signed consent form for her records.

6.2 Screening Assessments

Screening assessments will be performed within 24 hours prior to the administration of Definity. Trial participants will have the presence of inclusion criteria and absence of exclusion criteria verified by providing a medical history. A full demographic profile, known drug allergies or intolerances, and a review of the subject's medical/surgical history will be recorded. If the subject is a woman of childbearing potential, she will have a urine pregnancy test (the results of which will be made available to the subject prior to study initiation).

6.3 Ultrasound Imaging

The US examinations will be performed by a qualified sonographer. Procedures and equipment for this trial will be used in accordance with typical clinical procedures. In particular, the mechanical index will be set to less than 0.8 on the US scanner. All trial procedures will be conducted in accordance with Good

Clinical Practice. For the ultrasound examination, the patient will be asked to lie in the supine position and a catheter will be placed in a superficial vein (preferably an antecubital vein). Acoustic coupling gel will be applied to the breast area of interest. A baseline US grayscale scan will be used to identify the mass or abnormal area seen by mammography (or another concomitant imaging mode such as US or MRI). Standard PDI of the lesion or target area will also be performed.

Following baseline imaging, patients will receive a bolus, IV injection of 0.25 ml of Definity and the mass or area of abnormality will be imaged with the broad bandwidth linear array (selected in the development phase described above) and a 30 to 40 second digital clip of the 3D pulse inversion HI volume data, covering baseline to beyond peak enhancement, will be acquired. The point of maximum enhancement in the pulse inversion harmonic images will be assessed visually (by the on-site investigators in consensus) and at least 10 seconds of data will be acquired past this time point. Subsequently more sweeps of the abnormality will be stored on the scanner hard drive every 1 to 2 minutes through the period of enhancement (up to 5 minutes).

After a 30 minutes waiting period patients will receive a second bolus, IV injection of up to 20 μl/kg of Definity, sweeps of the mass or abnormal area will be made to acquire 3D SHI grayscale volume data through the period of enhancement (up to 2 minutes in our pilot study). It should be noted, that all subjects will be closely monitored by a physician during and following the study (for 30 minutes as recommended by the FDA) [FDA Alert 2007/2008]. Additionally, resuscitation equipment and trained personnel will be in immediate proximity to the patient during the study.

All images (baseline and contrast-enhanced) will be obtained using a broad bandwidth, 3D, linear array, and will include images of the entire area of interest. The entire examination will be recorded as digital loops, as well as on VHS videotape.

6.4 Safety Monitoring

Patients will be monitored for AEs during and 30 mins after contrast administration. All other procedures will be performed according to TJU/UCSD standard of care.

6.5 Surgical Biopsy

All subjects will be scheduled to undergo a breast biopsy (core / excisional / lumpectomy) of the mass or region of abnormality or mastectomy within 30 days after the US study procedure. The date of the

biopsy and the pathology results will be recorded. An experienced pathologist will complete a worksheet for each biopsy specimen. If the patient undergoes a surgical biopsy at TJU, the surgeon (Dr. Gordon Schwartz) will mark the transaxial plane of the lesion by sutures upon excision of the breast mass to allow additional data collection from this subgroup of patients in order to partially fulfill the secondary aim of this trial. At UCSD surgical biopsy specimens will be treated according to clinical standard i.e., the imaging plane and the pathology sections will not be matched.

6.6 Efficacy Assessments

The primary blinded read will be performed on all 450 human cases by a physician with more than 15 years of expertise in general breast imaging and with specific expertise in breast SHI (Dr. Piccoli). All imaging studies will be rated on a quasi-continuous scale from 0 to 100 ranging from "no lesion seen (no findings)" over "probably benign" and "indeterminate" to "malignant." Using a quasi-continuous 100-point rating scale has an intuitive probabilistic interpretation and is know to improve the assessment of the characterization capabilities of the imaging modes studied [Wagner et al 2001].

The SHI images will be qualitatively analyzed as described above for the pulse inversion HI injection.

The pre- and then post-contrast diagnostic US criteria (as detailed below) will be evaluated for each patient in two readings as follows: grayscale; grayscale and PDI (baseline); grayscale and contrast enhanced pulse inversion HI, and then in the second reading grayscale/baseline and SHI and, finally grayscale and SHI with dynamic CMI-SHI added. While this may introduced some bias from pre- to post-contrast results, we considered this the more realistic approach to how US contrast may be used in clinical practice. Moreover, such a "sequential" reading scheme may well produce the same total reader variance as a more independent reading scheme (where the evaluation of each imaging mode is separated in time by 1 month or more), while having the advantage of being logistically much less demanding [Beiden et al 2002]. Each case will also be read by the two on-site investigators, blinded to the mammographic and histopathological diagnosis, to allow repeatability to be assessed for each site. Finally, each mammogram will be read by a radiologist as part of the patient's standard clinical assessment and assessed using the BIRADS scale [ACR 1998].

Diagnostic US criteria (Evaluated for each imaging mode):

- Diagnosis; size, shape, and orientation of the lesion; echogenicity compared to surrounding tissue.

- Distribution of color signals and the overall color content of the lesion evaluated by comparing the pattern and amount of color to the normal surrounding breast.
    - If uniform, the color will be categorized as less intense, iso-intense or more intense than normal breast.
    - If non-uniform, the color will be described by location, intensity and distribution.
- Irregularity of the course of the vessels will be scored on a scale from 1.0 to 5.0 (smooth to severe irregularity)
- Anastomoses between adjacent vessels will be noted (as 1-2, 3-5 or >5 vessels connecting).
- Enhancement pattern will also be graded as peripheral, radial from one or multiple sites, spotty or a combination.

Sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) will be calculated for all six imaging modalities (the five different US modes and mammography). The dichotomous parameter needed for these calculations will be derived by ascertaining whether the radiologist would recommend a biopsy or not based on the imaging study in question. Inter- and intra-observer variability will also be calculated

6.7 Pathology

All subjects will be scheduled to undergo a breast biopsy (core / excisional / lumpectomy) of the mass or region of abnormality or mastectomy within 30 days after the US study procedure. The date of the biopsy and the pathology results will be recorded. An experienced pathologist will complete a worksheet for each biopsy specimen. If the patient undergoes a surgical biopsy at TJU, the surgeon will mark the transaxial plane of the lesion by sutures upon excision of the breast mass. At UCSD surgical biopsy specimens will be treated according to clinical standard i.e., the imaging plane and the pathology sections will not be matched. Careful attention will be paid to the labeling of each section to ensure the correct orientation. Whole mount paraffin blocks will be prepared from which central 5 mm sections will be cut and mounted on 2 x 3 inch glass slides and stained with hematoxylin and eosin (H&E) according to standard methods and examined microscopically by the pathologist as part of the patient's clinical care. Apart from diagnosis, the pathologist will provide the following variables: estrogen progesterone and HER2 receptor presence or absences; presence or absence of hemorrhagic, necrotic, or other component of the lesion (and their location if present); histological margins; lesion size; TNM staging; histological lesion type; presence or absence of vascular invasion; histological and cytological grade; presence or absence of metastases; node staging; and percentage of the lesion that was invasive or *in situ*. If the

patient undergoes a core biopsy, it will not be possible to assess lesion size. All other histopathological parameters will be the same as for the excisional biopsy described above and the specimens will be treated identically.

In a subset of cases at TJU (approximately 85) staining will also be performed for an immunohistochemical predictor of tumor angiogenesis, specifically a monoclonal antibody against the PECAM endothelial cell marker (anti-CD31; Dako Corporation, Carpinteria, CA) in addition to the standard H&E staining described above. The PI, as part of previously NIH and DOD funded projects, has developed the necessary software for quantitative analysis of contrast enhanced US images and specimens stained with CD31 using a histomorphometry system based on an SMZ-10A microscope (magnification 100x; Nikon, Melville, NY) and ImagePro Plus software [Chaudhari et al. 2000; Forsberg et al. 2008]. Vessels will be identified in the digitized image by the presence of CD31 staining. ROIs around visible vessels will be defined for the ImagePro software. This labor-intensive task will be performed by a graduate student under the direct supervision of the PI. A pathologist will be available for consultation (Dr. Juan Palazzo).

6.8 Safety Assessments

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

6.8.1 Risks/Benefits Assessment.

Serious cardiopulmonary and allergic reactions including fatalities have occurred during or following administration of Definity, causing the FDA to place a black box warning on the agent. However these occurrences have been rare (less than 1 in 5,000 patients). As a result, patients with cardiac shunts or unstable cardiopulmonary conditions will be excluded. Patients will also be monitored for 30 minutes after contrast administration for any adverse reactions. The majority of adverse events from Defnity were mild to moderate in severity. Transient side effects that have been described as possibly related to Definity administration include headache (2.3%), back and renal pain (2.1%), flushing (1.1%) and nausea (1.0%). Hypersensitivity reactions to perflutren may occur, although rare.

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 US imaging techniques is expected to provide significantly more information than from conventional US techniques. This may lead to additional information about the characteristics of breast tumors which may be clinically relevant.

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

The risk benefit ratio is low. Based on the available non-clinical 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. In a relatively healthy outpatient population referred for surgical breast biopsy we do not expect any severe reactions. However, in order to ensure that the 20 μl/kg dose used for SHI is not altering the risk benefit ratio, data from the first ten (10) patients and then the next ten patients (i.e., the first 20 subjects) will be scrutinized by the TJU DSMB immediately upon completion. If AEs are encountered to a higher degree than what is expected given the Definity label, the dosing will be adjusted downward.

6.8.2 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 both 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. All adverse events are reported to the Clinical Research Management Office (CRMO) via the password protected Kimmel Cancer Center Adverse Event Reporting System. In addition all unexpected and serious adverse events (SAEs) are reported to the TJU IRB and to the Food and Drug Administration (FDA) if applicable. The investigator is required to submit all unexpected and serious adverse events to the TJU IRB and the DSMB within 48 hours. Fatal adverse events related to treatment which are unexpected must be reported

within 24 hours to the TJU IRB and the DSMB through CRMO. Fatalities not related to the study drug/device must be reported within 5 days.

The AE forms will include: subject identification number and initials; subject's date of birth, gender, and ethnicity; date of 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 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 Grade 1 -5 according to the NCI CTCAE 4.03:

Grade 1: Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.

Grade 2: Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living (ADL).

Grade 3: Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL.

Grade 4: Life-threatening consequences; urgent intervention indicated.

Grade 5: Death related to AE.

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 Package Insert 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 (SAE; Grades 3-5) is defined as a significant clinical hazard, contraindication, or precaution that:

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

All unexpected and serious adverse events are reported to the TJU/UCSD IRB and to FDA if applicable. The investigator is required to submit all unexpected and serious adverse events to the TJU/UCSD IRB

and the data safety monitoring board within 48 hours. Fatal adverse events related to treatment which are unexpected must be reported within 24 hours to the TJU/UCSD IRB and the DSMB through CRMO.

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

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.

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

6.9 End-of-Treatment and End-of-Trial Evaluations

6.9.1 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 two injections of Definity
- Has undergone the complete US imaging study (baseline and contrast-enhanced) as described in this protocol
- Undergoes a breast biopsy (core / excitional / lumpectomy) or mastecomy within 30 days of the contrast-enhanced US imaging study

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 coordinator. Patient study files will be kept for 7 years after the completion of the study.

The final data will be entered into a database. The investigator will be responsible for management of the database. The database will be maintained within an organized and secure directory system.

7.2 Statistical Analyses

7.2.1 Hypotheses:

H_1: SHI or pulse inversion HI will improve the diagnostic accuracy of mammography, grayscale US or power Doppler imaging to distinguish benign from malignant breast disease. The fundamental hypothesis is that the area under the ROC curve will improve by 0.10 to 0.15 with SHI compared to the other imaging modalities.

H_2: Some findings of SHI (including perfusion estimates and CMI-SHI) or pulse inversion HI correlate with the pathological diagnosis of benign/malignant or with other significant physiological/pathological parameters. The fundamental hypothesis is that higher correlation coefficients can be achieved with SHI than with pulse inversion HI.

H_3: 3D SHI estimates of perfusion correlate with absolute perfusion measured in the kidneys of dogs. The fundamental hypothesis is that correlation coefficients above 0.75 can be achieved for *in vivo* SHI perfusion estimates.

H_4: I*n vivo* fractional tumor vascularity measurements obtained with power Doppler imaging, pulse inversion HI or SHI correlate with iMVD or any other pathological measure of breast tumor vascularity obtained in the surgical biopsy specimens. The fundamental hypothesis is that higher correlation coefficients can be achieved with SHI than with the other two US flow modes.

7.2.2 Analysis of Results

The findings of SHI, pulse inversion HI and PDI will be correlated to vascular morphology (i.e., tumor angiogenesis) and other pathological findings; including a) size b) vascularity, and c) type of mass. For cancers, pathological criteria, which will be evaluated, are the stage of the cancer, degree of proliferation and presence or absence of receptors. 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 which measures significance of changes in related samples (pairwise comparisons). For variables in which accurate numerical measurement is possible (e.g., perfusion), the techniques will be compared using an ANOVA.

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 mammography will be analyzed using logistic regression and ROC analyses [DeLong et al. 1988; Metz 1986; Wagner et al. 2001]. The tests are: x-ray mammography, fundamental grayscale US imaging, PDI, pulse inversion HI and SHI (as well as SHI with CMI-SHI added), while pathology (based on excisional or core biopsies) will provide the reference standard. All studies will be rated on a quasi-continuous scale from 0 to 100, since this has an intuitive probabilistic interpretation and improves the assessment of the characterization capabilities of the imaging modalities compared to using a coarser 6-point scale [Wagner 2001]. Logistical regression techniques will be used to combine the 4 US imaging modes and mammography as well as to incorporate the quantitative parameters into the SHI diagnosis before repeating the ROC analysis. This will allow all possible combinations to be compared to one another (e.g., mammography versus all the US modes combined or mammography and grayscale US versus mammography and grayscale US and SHI, etc.). Differences between ROC curves will be tested by computing Mann-Whitney statistics.

All histopathological variables will be compared to imaging judgments on diagnoses and characteristics of vascularity for the different modalities. When both sets of variables are nominal, chi-square tests will be conducted. When both types of variables are ordinal or continuous, correlations will be calculated. When one type of variable is nominal and one continuous non-parametric rank order tests such as Mann-Whitney U-tests or Kruskal-Wallis tests will be performed [Rosner 1990]. Inter- and intra-observer variability will also be determined by calculating the intraclass correlation coefficient and the kappa statistic [Shrout 1979; McGraw 1996].

Comparisons of sensitivities, specificities and accuracies for diagnosing cancer with the different imaging modalities will be conducted with McNemar's test for correlated proportions (against the histopathological gold standard). All of the statistical analyses proposed for the 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 3D SHI to diagnose breast cancer.

Finally, the vascularity measures obtained from the subgroup of up to 85 CD31 stained breast tumor specimens will be compared to the fractional tumor neovascularity data to determine if any correlation exists (in baseline PDI or one of the two contrast enhanced modes). The existence of a linear relationship between ultrasonic and pathologic data will be assessed using single variable linear regression techniques and reverse stepwise multiple linear regression analysis.

All analyses and computations will be performed using NCSS/PASS 2005 and Stata 9.0 (Stata Corporation, College Station, TX), while the study database will be designed and implemented in Filemaker Pro 10.0 (Filemaker Inc, Santa Clara, CA). This database will contain all patient information (except names and other identifiers), including the answers to the questionnaire, and the results of the various US imaging modes as well as the pathology results.

7.2.3 Efficacy Measures

Qualitative evaluation of the baseline and Definity contrast-enhanced US images will be conducted at the trial site by two (2) blinded reviewers. Interpretation of the biopsy results will also occur at the trial site by an independent pathologist, blinded to the imaging studies. Subsequently one (1) overall blinded reader (Dr Piccoli) will read all cases from both sites.

The blinded reviewers will complete a worksheet for each imaging test (x-ray mammography, conventional gray scale imaging, power Doppler (with and without contrast enhancement), and SHI). All studies will be rated on a scale from 1 to 5 (1=definitely benign; 2=probably benign; 3=possible; 4=probably malignant; 5=definitely malignant). For the US modalities, the distribution of color signals and the overall color content of the lesion will be evaluated by comparing the pattern and amount of color to the normal surrounding breast. If uniform, the color will be less intense, isointense or more intense than normal breast. If non-uniform, the color will be described by location, intensity and distribution. Irregularity of the course of the vessels and anastomoses between adjacent vessels will be noted. The enhancement pattern will also be graded as peripheral, radial from one or multiple sites,

spotty or a combination. The degree of tumor neovascularity will be estimated. These findings will be compared to histological findings to determine overall efficacy of the imaging methods.

7.2.4 Sample Size Calculation

A power analysis was performed using NCSS/PASS 2005 (NCSS, East Kaysville, UT) to estimate the number of cases required in the positive and the negative group (i.e., the malignant and benign cases, respectively) to achieve significance at the 0.05 level (on a two-sided test) when comparing binormal ROC curves (i.e., for specific aim 3) based on the primary reader (Dr. Piccoli) reading all cases.

For this study to demonstrate that 3D SHI produces a 10 % increase in diagnostic accuracy with 90 % statistical power requires between 400 and 600 patients (depending on the positive biopsy rate). If the real increase in accuracy is 15 %, then between 167 and 300 cases are needed to achieve 90 % power. Consequently, we selected a sample size of 450 patients for this clinical trial, which will allow us to detect a 15 % increase in the diagnostic accuracy of diagnosing breast cancer with over 90 % statistical power (assuming a positive biopsy rate of around 20 %). If the actual positive biopsy rate is approximately 30 %, then we will have more 90 % power to detect a 10 % increase in accuracy.

For Hypothesis # 3, we assumed that the correlation coefficient achievable with 3D SHI perfusion estimates will be 0.75 whereas our preliminary work with 2D SHI achieved a coefficient of 0.57 [Forsberg et al. 2006a]. This difference will be detectable with more than 80 % power at the 0.05 significance level (on a two-sided test) when 152 data points are analyzed. Hence, the decision to study 5 dogs for a total of 160 perfusion estimates. Finally, for Hypothesis # 4, we assumed that the correlation coefficient between power Doppler imaging and pathology will be approximately 0.40 (since our preliminary work [Caudhari et al. 2000; Forsberg et al. 2008] achieved coefficients between 0.25 and 0.49). Then we will have more than 80 % power to detect an improvement in the correlation by 0.3 or more due to SHI at the 0.05 significance level (using a two-sided test) when 84 patients are analyzed.

REFERENCES

ACR (American College of Radiology). Breast imaging reporting and data system 3rd ed. Reston, Va: American College of Radiology, 1998.

Adler DD, Carson PL, Rubin JM, Quinn-Reid D. Doppler ultrasound color flow imaging in the study of breast cancer: preliminary findings. *Ultrasound Med. Biol.*, 16:553-559, 1990.

Beiden SV, Wagner RF, Doi K, Nishikawa RM, Freedman M, Lo SC, Xu XW. Independent versus sequential reading in ROC studies of computer-assist modalities: analysis of components of variance. *Acad Radiol*.,9:1036-1043, 2002.

Bhagavatheeshwaran G, Shi WT, Forsberg F, Shankar PM. Subharmonic generation from contrast agents in simulated neovessels. *Ultrasound Med Biol*, 30:199–203, 2004.

Bohm-Velez M, Mendelson EB: Computed tomography, duplex Doppler ultrasound and magnetic resonance imaging in evaluating the breast. *Semin Ultrasound CT MR*, 10:171-176, 1989.

Brawer MK, Bigler SA, Deering RE: Quantitative morphometric analysis of the microcirculation in prostate carcinoma. *J Cell Bioch* 16H:62-64, 1992.

Burns PN, Halliwell M, Wells PNT. Ultrasonic Doppler Studies of the Breast. *Ultrasound Med Biol.*, 8:127-143, 1982.

Chaudhari MH, Forsberg F, Voodarla A, et al. Breast tumor vascularity identified by contrast enhanced ultrasound and pathology: initial results. *Ultrasonics,* 38:105-109, 2000.

Cosgrove DO, Bamber JC, Davey JB, McKinna JA, Dudley Sinnet H: Color Doppler signals from breast tumors. *Radiology*, 176:175-180, 1990.

Cosgrove DO, Kedar RP, Bamber JC, al-Murrani B, Davey JB, Fisher C, McKinna JA, Sevensson WE, Tohno E, Vagios E, et al: Breast disease: color Doppler US in differential diagnosis. *Radiology* 189:99-104, 1993.

Dave JK, Forsberg F. Novel automated motion compensation technique for producing cumulative maximum intensity subharmonic images. *Ultrasound Med Biol;* 35, 155-1563, 2009.

Dave JK, Forsberg F, Fernandes S, Piccoli CW, Fox TB, Merton DA, Leodore LM, Hall AL. Static and dynamic cumulative maximum intensity display mode for subharmonic breast imaging - a comparative study with mammography and conventional ultrasound techniques. *J Ultrasound Med,* 2010; 29, 1177-1185.

DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 1988; 44:837-845.

Duda VF, Rode G, Schlief R: Echocontrast agent enchanced color flow imaging of the breast. *Ultrsound Obstet Gynecol* 3: 191-194, 1993.

Eisenbrey JR, Dave JK, Merton DA, Palazzo JP, Hall AL, Forsberg F. Parametric imaging using subharmonic signals from ultrasound contrast agents in patients with breast lesions. Submitted to *J Ultrasound Med.* 2010.

Esserman L, Hylton N, George R, Weidner N: Contrast-enhanced magnetic resonance imaging to assess tumor histopathology and angiogenesis in breast carcinoma. *Breast journal* 5: 13-21, 1999.

FDA Alert. Information for Healthcare Professionals. Micro-bubble Contrast Agents (marketed as Definity (Perflutren Lipid Microsphere) Injectable Suspension and Optison (Perflutren Protein-Type A Microspheres for Injection). 10/12/2007, updated 7/17/2008 (accessed May 23, 2010).

FDA Alert. Public Health Advisory. Information for Healthcare Professionals Gadolinium-Based Contrast Agents for Magnetic Resonance Imaging (marketed as Magnevist, MultiHance, Omniscan, OptiMARK, ProHance). 12/22/2006, updated 5/23/2007.

Feig SA: Breast masses: Mammographic and sonographic evaluation. *Radiologic Clin North Am* 30:67-92, 1992.

Folkman J, Meerler E, Abernathy C and Williams G. Isolation of a tumour factor responsible for angiogenesis. *J Exp Med.* 33:275-278, 1971.

Folkman J, Cotran R: Relation of vascular proliferation to tumor growth. *Int Rev Exp Pathol* 16:207-248, 1976.

Forsberg F, Merton DA, Liu JB, Needleman L, Goldberg BB. Clinical applications of ultrasound contrast agents. *Ultrasonics* 36:695-701, 1998a.

Forsberg F, Liu JB, Merton DA, Rawool NM, Goldberg BB. Parenchymal enhancement and tumor visualization using a new sonographic contrast agent. *J Ultrasound Med,* 14:949-957, 1995.

Forsberg F, Goldberg BB, Liu JB, Merton DA, Rawool NM. On the feasibility of real-time, in vivo harmonic imaging with proteinaceous microspheres. *J. Ultrasound Med.,* 15:853 - 860, 1996.

Forsberg F, Needleman L, Goldberg BB, Schultz SM, Merton DA, Rawool NM. Contrast enhancement of breast masses (abstract). *Ultrasonic Imaging*, 20:52-53, 1998b.

Forsberg F, Shi WT, Hall A, Thomenius KE, Goldberg BB: Comparison of nonlinear US imaging modes using contrast agents. *Radiology* 209(P):190 (Abstr.), 1998c.

Forsberg F, Shi WT, Goldberg BB: Subharmonic imaging of contrast agents. *Ultrasonics* 38:93–98, 2000.

Forsberg F, Piccoli CW, Ro R, et al. Contrast enhanced subharmonic breast imaging: work in progress. *Proc. IEEE Ultrason Symp*, 625-628, 2005.

Forsberg F, Piccoli CW, Merton DA, Palazzo JJ, Hall AL. Breast lesions: imaging with contrast-enhanced subharmonic US - initial experience. *Radiology*, 244:718-726, 2007.

Forsberg F, Soparawala R, Merton DA, Piccoli CW, Palazzo JJ, Hall AL. Quantitative analysis of contrast enhanced subharmonic breast images. *Proc IEEE Ultrason Symp*, 1584 – 1587, 2006a.

Forsberg F, Liu JB, Shi WT, Ro R, Lipcan KJ, Deng X, Hall AL. In vivo perfusion estimation using subharmonic contrast microbubble signals. *J Ultrasound Med,* 25:15-21, 2006b.

Forsberg F, Kuruvilla B, Pascua M, Chaudhari MH, Merton DA, Palazzo JP, Goldberg BB. Comparing contrast-enhanced color flow imaging and pathological measures of breast lesion vascularity. Ultrasound Med Biol, 34:1365-1372, 2008.

Goldberg BB, Raichlen JS, Forsberg F. Ultrasound Contrast Agents: Basic Principles and Clinical Applications, 2nd Ed, Martin Dunitz Ltd., London, 2001.

Goldberg BB, Liu JB, Forsberg F: Ultrasound contrast agents: a review. *Ultrasound Med. Biol.*, 20:319-333, 1994.

Goldberg BB, Merton DA, Forsberg F, Liu JB, Rawool NM. Color amplitude imaging: preliminary results using vascular sonographic contrast. *J Ultrasound Med*, 15: 127-134, 1996.

Goldberg BB, Leen E, Needleman L, et al. Contrast enhanced ultrasound imaging of liver lesions: a phase II study with NC100100 (abstract). *J Ultrasound Med,* 18:S43, 1999.

Halpern EJ, Verkh L, Forsberg F, Mattrey R, Goldberg BB: Initial clinical experience with contrast-enhanced imaging of the prostate using. *AJR* 2000; 174:1575-1580.

Hamada M, Konishi Y, Shimada K, et al: Color Doppler echography of breast diseases: detection rate of color flow signals and significance of color Doppler echography in breast diseases. *Jpn J Med Ultrasound*, 19:329-336, 1992.

Huber S, Helbich T, Kettenbach J, Dock W, Zuna I, Delorme S. Effects of microbubble contrast agents on breast tumors: computer-assisted quantitative assessment with color Doppler US - early experience. *Radiology*, 208:485-489, 1998.

Jackson VP: The Role of US in Breast Imaging. *Radiology*, 177:305-311, 1990.

Jain RK: Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. *J. National Cancer Institute* 81: 570-576, 1989.

Kedar RP, Cosgrove DO, McCready VR, et al. Microbubble contrast agent for color Doppler US: Effect on breast masses. *Radiology* 198:679-686, 1996.

Madjar H, Sauerbrei W, Munch S et al: Continuous wave and pulsed Doppler studies of the breast: Clinical results and effect of transducer frequency. *Ultrasound Med Biol*, 17:31-39, 1991.

Madjar H: Breast examination with continuous wave and color Doppler ultrasound. *Obstet. & Gynecol.*, 2:215-220, 1992.

McGraw KO, Wong SP. Forming inferences about some intraclass correlation coefficients. *Psychol Methods*, 1:30-46, 1996.

Metz 1986; Metz CE. ROC Methodology in radiologic imaging. *Invest. Radiol.*, 21:720-733, 1986.

Miller AP, Nanda NC. Contrast echocardiography: new agents. *Ultrasound Med Biol*., 30:425-434, 2004.

Needleman L, Forsberg F. Contrast agents in ultrasound. *Ultrasound Quarterly* 13:121-138, 1996.

Needleman L, Leen E, Kyriakopoulou K, et al. NC100100: A new US contrast agent for fundamental an harmonic imaging of Hepatic lesions (abstract). *Radiology* 209(P):189, 1998.

Rosner B. Fundamentals of Biostatistics (3rd Ed.). Boston, MA: PWS-Kent Publishing Co., 1990.

Schor AM and Schor SL. Tumour angiogenesis. *J Pathol.* 141:385-413, 1983.

Schrope BA, Newhouse VL: Second harmonic ultrasound blood perfusion measurement. *Ultrasound Med Biol* 19: 567-579, 1993.

Shankar PM, Krishna PD, Newhouse VL: Advantages of subharmonic over second harmonic backscatter for contrast-to-tissue echo enhancement. *Ultrasound Med Biol* 24:395-399, 1998.

Shankar PM, Krishna PD, Newhouse VL: Subharmonic scattering properties of ultrasound contrast agents. *Phys Med Biol* 44:681, 1999.

Shi WT, Forsberg F, Gupta M, Alessandro J, Wheatley MA, Goldberg BB: Subharmonic response of a new US contrast agent. *Radiology* 205(P): 353 (Abstr), 1997.

Shi WT, Forsberg F, Raichlen JS, Needleman L, Goldberg BB: Pressure dependence of subharmonic signals from contrast microbubbles. *Ultrasound Med Biol* 25, 275-283, 1999a.

Shi WT, Forsberg F, Hall AL, Chiao RY, Liu JB, Miller S, Thomenius KE, Wheatley MA, Goldberg BB: Subharmonic imaging with contrast agents: initial results. *Ultrasonic Imaging* 21:79-94, 1999b.

Shrout PE, Fleiss JL. Intraclass correlations: uses in assessing rater reliability. *Psychological Bulletin*, 86:420-428, 1979.

Steinbach GC, Lee YZ, Lazenby JC, Peterson TM, Mattrey RF: Vascular Echogenicity on B-Mode High Resolution Harmonic Imaging as a Function of Velocity and Contrast Concentration. *J Ultrasound Med* 17: S55 (Abstr), 1998.

Wagner RF, Beiden SV, Metz CE. Continuous versus categorical data for ROC analysis: some quantitative considerations. *Acad Radiol*, 8:328-334, 2001.

Ward B, Baker AC, Humphrey VF: Nonlinear propagation applied to the improvement of resolution in diagnostic medical ultrasound. *J. Acoust. Soc. Am*. 101: 143-154, 1997.

Wei K, Jayaweera AR, Firoozan S, Linka A, Skyba DM, Kaul S. Quantification of myocardial blood flow with ultrasound-induced destruction of microbubbles administered as a constant venous infusion. *Circulation* 97:473-483, 1998.

Weidner N, Folkman J, Pozza F, et al. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast cancer. *J Natl. Cancer Inst.,* 84: 1875-1887, 1992.

Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis- Correlation in invasive breast carcinoma. *N Engl J Med* 324:1-8, 1991.

Weind KL, Maier CF, Rutt BK, Moussa M. Invasive carcinoma and fibroadenomas of the breast: comparison of microvessel distributions - implications ofr imaging modalities. *Radiology*, 208:477-483, 1998.

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, including advertisements used to recruit participants, 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 reapproval 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. A witness must also sign, date, and initial the consent form. Two copies of the consent form should be completed so that the subject can get an original copy and a copy can be kept for the investigator’s study records.

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 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 photographs, 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.

Roles and Responsibilities of Study Personnel

Flemming Forsberg, Ph.D., Professor of Radiology and Director of Ultrasound Physics, TJU, will serve as Principal Investigator on this grant. 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.

Barbara M. Cavanaugh, MD, Clinical Associate Professor of Radiology, and Director of the Division of Breast Imaging, TJU, is an authority in diagnostic breast ultrasound imaging with extensive experience in ultrasound research. As a co-investigator, she will assist in patient recruitment, interpret breast ultrasound studies and provide general clinical guidance.

Robert F. Mattrey, MD, Professor of Radiology, UCSD, has extensive experience in contrast-enhanced breast imaging. As a co-investigator, will oversee patient recruitment at UCSD, interpret breast ultrasound studies and provide general clinical guidance.

Yuko Kono, MD, PhD, Assistant Clinical Professor of Medicine, UCSD, will interpret ultrasound studies, assist in patient recruitment and imaging, and provide clinical guidance.

Aninna Wilkes, MD Clinical Assistant Professor of Radiology, TJU, will interpret ultrasound studies, assist in patient recruitment and provide clinical guidance on flow measurement related issues.

Catherine Piccoli, M.D., will interpret mammograms and ultrasound images and advise on clinical issues.

Laurence Parker, Ph.D., TJU, Research Assistant Professor of Radiology will serve as the project statistician and he will perform the ROC analysis along with other statistical analyses required.

John Eisenbrey, Ph.D., Research Fellow, Department of Radiology, TJU, will aid patient recruitment, data collection, and data analysis.

To Be Named, is a research sonographer. She/he will be responsible for performing the ultrasound examination under the supervision of the radiologists listed as co-investigators and the PI of the study.

Colleen Dascenzo, CCRC, is an IV certified research coordinator. She will be responsible for screening, recruiting, and scheduling patients and will explain the study to them. In addition, she will perform data entry.

To Be Named – Research Nurse - He/she will prepare and administer the contrast agent, record medications, and monitor the patients appropriately during and after the procedure. His/her effort is calculated to be <5%

To Be Named, TJU, will act as the medical monitor for this project.

Additionally, Drs. Gordon Schwartz and Juan Palazzo, TJU, have agreed to assist the project, if necessary, in their standard clinical roles i.e., as a breast surgeon and as a pathologist.

Signature of PI: _____

Flemming Forsberg, PhD

APPENDIX B – DEFINITY PACKAGE INSERT