PROTOCOL

TITLE: Quantitative Subharmonic Breast Imaging: Subharmonic Imaging and Pressure Estimation for Monitoring Neoadjuvant Chemotherapy

VERSION & DATE: Version 1.5- February 20th, 2013

PRINCIPAL INVESTIGATOR: Flemming Forsberg, PhD
Professor, Department of Radiology
Thomas Jefferson University
132 South 10th Street, 7th Floor
Philadelphia, PA 19107
Email address: flemming.Forsberg@jefferson.edu
Tel: 215-955-4870
Fax: 215-955-8549

KEY PERSONNEL:

Barbara M. Cavanaugh, MD
Clinical Associate Professor of Radiology
Department of Radiology
Thomas Jefferson University

Aninna Wilkes, MD
Clinical Assistant Professor of Radiology
Department of Radiology
Thomas Jefferson University

Tiffany Avery, MD
Assistant Professor of Medical Oncology
Department of Medical Oncology
Thomas Jefferson University

LOCATION OF STUDY:
Thomas Jefferson University

925 Chestnut Street, 2th Floor and 132 South 10th Street, 7th Floor
Philadelphia, PA 19107

STUDY DURATION: 2 year project

SYNOPSIS

Protocol Title: Quantitative Subharmonic Breast Imaging; Subharmonic Imaging and Pressure Estimation for Monitoring Neoadjuvant Chemotherapy

Trial Objectives:

The primary objective of this *first-in-humans* trial is:

To evaluate the ability of 3D Subharmonic Imaging (SHI) and Subharmonic Imaging and Pressure Estimation (SHAPE) to track changes in locally advanced breast cancer (LABC) angiogenesis and interstitial fluid pressure (IFP), respectively, by studying women undergoing neoadjuvant chemotherapy before as well as with around 10 % and 60% of the neoadjuvant chemotherapy treatment delivered and after completion of the neoadjuvant chemotherapy treatment. Results will be compared to MRI and pathology.

The secondary aim of this trial is:

- To compare the 3D SHI depiction of breast cancer angiogenesis in humans to CD31, an immunohistochemical predictor of angiogenesis.

Trial Design:

This is an open-label, non-randomized, *first-in-humans* trial that will be conducted at one clinical site Thomas Jefferson University (TJU). All subjects will undergo an unenhanced (baseline) imaging study followed by one infusion of Definity™ (Lantheus Medical Imaging, Billerica,

MA), and a Definity contrast-enhanced US imaging study for evaluation of a breast mass or breast abnormality without mass.

Trial Population: This trial will consist of between 20 and 50 adult (21 years of age or older) female subjects who are undergoing neoadjuvant chemotherapy.

Trial Procedures: Subjects eligible for trial enrollment will be identified by the investigators from among TJU's patient population who are undergoing neoadjuvant chemotherapy. 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 (the 4D10L) will be used to acquire conventional images and subharmonic imaging (SHI). The US software has been modified by GE Medical Systems on the clinical system, the Logiq 9 scanner, to permit acquisition of subharmonic data. All acoustic parameters have been verified and are all within the current standards of the machine, making the modified US system a non-significant risk device. US imaging will be performed at a transmit frequency of 5.8 MHz and the subharmonic obtained at 2.9 MHz. Using this setup, acoustic pressure amplitudes will all be below 1.5 MPa peak negative pressure, 2.5 MPa peak positive pressure (MI < 0.33).

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 Power Doppler (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

The contrast administration will be an intravenous infusion of 2 vials of Definity/50ml saline through the subjects' vascular port (established as part of their standard of care), with infusion rates of 4 to 10 ml/min (titrated to effect). Next, we will run a power optimization algorithm to establish the individualized acoustic parameter settings for the case (on the first imaging study only). We will use those parameters for all subsequent imaging studies to sweep the area of the LABC to acquire 3D, pulse inversion SHI grayscale volume data. The digital clips and radio-frequency (RF) data obtained from each 3D SHI injection will be transferred to a PC for off-line analysis. The SHI images will be qualitatively analyzed as described above. This protocol will be followed for all the contrast studies for each subject. All subjects will be closely monitored by a physician during and following the study.

Moreover, the 3D SHAPE results (i.e., IFP) will be compared to lesion size, tumor response, DFS and overall survival. Results will also be compared to MRI enhancement kinetics and imaging measurements of lesion size obtained before and after completion of the chemotherapy. Once surgery has been performed 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* will be collected as part of the patient's clinical care and correlated with the imaging results.

Statistical Methodology: The ability of the imaging tests to distinguish responders from non-responders will be compared using ROC analysis, while the incremental validity of combining imaging assessments will be analyzed using logistic regression and ROC analyses. Given 40 subjects split evenly into 20 responders and 20 non-responders, the analysis will have 80 % statistical power to detect a difference of 0.174 between a diagnostic test with an area under the ROC curve (A_z) of 0.800 (the null hypothesis) and an actual A_z of 0.974 using a one sample, two-sided z-test with a significance level of 0.05. All analyses will be performed with Stata 12.0 (Stata Corp., College Station, TX).

1. Introduction

Systemic preoperative therapy (neoadjuvant chemotherapy alone or in combination with endocrine therapy) is widely used in the treatment of locally advanced, primary breast cancer (LABC) and is considered the standard of care for these patients.[1,2,3,4] Some 20 % of all primary breast cancers are diagnosed as LABC or are carcinomas with an unfavorable tumor-to-breast size ratio. In the largest study to date (>1500 patients), there was no statistical differences

between survival among patients that underwent neoadjuvant therapy (69%) and patients receiving adjuvant therapy (70%; p=0.80) after 9 years.[5] Likewise, for disease free survival (DFS), which was 53 and 55 %, respectively, in the adjuvant and the neoadjuvant groups (p=0.5). These results were supported by a recent meta-analysis involving almost 4000 women, which found no statistical or clinical differences between neoadjuvant and adjuvant therapy arms associated with survival or disease progression[5] and this result was recently confirmed even after 10 years of follow-up.[6] Moreover, neoadjuvant therapy is increasingly being used to treat operable, palpable breast cancers as well, in an attempt to avoid mastectomies and allow for breast conserving surgery instead.[4,7,8] Neoadjuvant therapies have the potential to improve survival and reduce morbidity by downstaging tumors, since a reduction in the primary tumor volume can be used to predict a reduction in micrometastatic tumor volume and clinical benefit.[4,9] However, monitoring the *in vivo* response to therapy is essential to distinguish between patient responders and non-responders as early as possible to allow for better, individualization of treatment options. A noninvasive technique that reliably determines breast cancer response to therapy may improve treatment outcomes.

Scintimammography utilizing Tc-99m sestamibi[10,11] , positron emission tomography (PET), computed tomography (CT) and optical imaging .[12,13,14] as well as Magnetic resonance imaging (MRI) has been used to monitor the response of LABC to neoadjuvant therapy.[15,16,17,18,19] While current guidelines recommend that patients diagnosed with breast cancer are followed post-surgery with x-ray mammography every 6 to 12 months,[20] a study comparing MRI, mammography and ultrasound (US) imaging for evaluation of residual disease following neoadjuvant therapy concluded that MRI and US were equivalent and better than mammography

for this application.[21] Also, mammography does not provide functional information and contrast-enhanced MRI is expensive, not readily available in many communities and associated with nephro-toxicity.[20]

Measuring a functional parameter such as breast tumor neovascularity with color and power Doppler imaging (CDI and PDI, respectively) have meet with initial success (κ=0.87) in small studies.[22,23] However, the sensitivity of CDI and PDI can be markedly improved with intravenous (IV) injection of gas microbubbles as US contrast agents, as we and others have demonstrated in patients with LABC.[24,25,26] Following our initial and somewhat disappointing experiences with two-dimensional (2D) contrast enhanced PDI (no statistically improvements in sensitivity and specificity were found with contrast,[26] we embarked on a study of 55 patients (with 16 cancers and 38 benign lesions) utilizing contrast enhanced three-dimensional (3D) PDI (supported by DAMD17-97-1-7116).[27] The sensitivity of contrast enhanced 3D PDI was significantly greater than that of standard 2D and 3D US imaging (grayscale as well as PDI; $p<0.04$). [27] Recent studies of breast tumors by our group have indicated significant correlation of direct pathologic vascularity assessments, such as the intra-tumoral micovascular density (iMVD), with ultrasonic vascularity measurements post contrast particularly for vessels 20 to 39 μm in diameter ($p<0.03$).[28] This indicates the potential of contrast-enhanced US to provide a noninvasive measure of breast tumor neovascularity. It is important, since angiogenesis may be an independent prognostic marker for breast cancer and, thus, an important parameter to monitor when assessing LABC therapy response.[29,30]

Moreover, solid tumors are known to have higher interstitial fluid pressures (IFPs) than surrounding normal tissues (10–25mmHg depending on tumor type and size),[31] due to the leaky nature of angiogenic tumor vessels that makes the microvascular pressure (MVP) equivalent to the IFP.[31,32,33,34] The development of tumor angiogenesis is closely related to IFP,[35] as confirmed by results in human breast cancer patients where the IFP was significantly higher in cancers than in surrounding normal tissues (29 vs. -0.3mmHg; p=0.001).[36] The high IFP and elevated vascular permeability in tumors may prevent the delivery of therapeutic drugs to large regions of the tumors.[37] Thus, it could be hypothesized that breast cancers with high IFP would respond poorly to chemotherapy because of poor drug delivery. This concept is supported by a recent study in 54 patients with LABC, which measured IFP before and after treatment with one of two chemotherapy agents, and found that one therapy (paclitaxel) significantly decreased the mean IFP (by 36%; p=0.02) whereas the other agent did not.[38] While clinical outcomes are not available for this study, another study demonstrated that tumor IFP can predict survival in patients with cervix cancer, independently of other prognostic factors.[39] These results suggest that tumor IFP may be a useful quantitative endpoint for assessing response to neoadjuvant therapy of LABC.

Consequently, a proposal to establish a new technique for monitoring the response to neoadjuvant therapy through the novel and innovative use of contrast-enhanced US based pressure estimation to measure IFP in breast tumors was funded by the DOD (BCRP award W81XWH-08-1-0503). In that project we measured IFP using subharmonic pressure estimation, which requires the use of subharmonic signals from US contrast agents. These 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, which span the range of possible frequency emissions from subharmonics through ultraharmonics.[25,40] Nonlinear bubble echoes can be separated from tissue echoes and used to create contrast specific imaging modalities, which allows blood flow in small vessels and capillaries (e.g., tumor angiogenesis) to be detected. One such modality is harmonic imaging (HI), which has been extensively studied.[25,41] HI preferentially enhances and displays contrast signals rather than surrounding tissue echoes by transmitting at the fundamental transducer frequency (f_0) and receiving at the second harmonic ($2f_0$). HI is commercially available on most state-of-the-art US scanners. However, in practice HI suffers from reduced blood-to-tissue contrast due to second harmonic generation in tissue.[39]

Alternatively, our group has been developing a novel contrast-specific imaging modality called 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., a much better signal-to-noise ratio) relative to other US contrast modes.[42] Most importantly, as part of a DOD funded study (DAMD17-00-1-0464) conducted at Thomas Jefferson University (TJU), we have demonstrated, in a first-in-humans study of 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 demonstrating that subharmonic signals can be obtained in humans with clinical dosages of US contrast agents.[43] This concept is now being explored further in a NCI funded clinical trial of 3D SHI in 450 women with breast lesions (under R01 CA140338).

Microbubbles can also act as pressure sensors due to the difference in compressibility between the bubble and the surrounding medium.[44] Our group has proposed, patented and investigated a novel and innovative technique called subharmonic-aided pressure estimation (SHAPE).[45,46,47,48,49,50,51,52,53] We have demonstrated that the subharmonic signal component from contrast microbubbles is an excellent indicator of the hydrostatic pressure variations. Changes in the first, second, and subharmonic amplitudes of five different ultrasound contrast agents were measured *in vitro* at static hydrostatic pressures from 0 to 186 mmHg (i.e., the range of human blood pressures). Over the pressure range studied the first and second harmonic amplitudes reduced approximately 2 dB for all contrast agents. Over the same pressure range, the subharmonic amplitudes decreased by 9-14 dB and excellent linear regressions were achieved between the subharmonic amplitude and the hydrostatic pressure variations ($r^2 \geq 0.98$, $p<0.001$).[45,46,48]

We implemented an initial, off-line version of the SHAPE algorithm on a commercial US scanner.[49,50] SHAPE was realized on a phased array transducer (PA4-2) with a frequency range from 1.5-4.5 MHz and *in vivo* experiments were carried out in 4 canines with simultaneous measurements of pressure in the heart chambers performed with SHAPE and with a solid state pressure catheter (SPC-350; the reference standard).

Using SHAPE for IFP estimation in LABC requires a high frequency linear. As an *in vivo* proof-of-concept, 5 Sinclair swine with naturally occurring melanomas,[54] were studied. Subharmonic signals were acquired (in triplicate) in tumors and surrounding tissues with the US scanner using an L14-5 probe. Definity was infused at a rate of 6.25 ml/min. RF data was

acquired in pulse inversion grayscale mode at different transmit frequencies and acoustic power levels (6.7 and 10.0 MHz as well as -4, -8 and -12 dB).[51] SHAPE results were compared to IFP measurements obtained with an invasive pressure monitor (Stryker, Berkshire, UK) as the reference standard. The difference between the subharmonics and the IFP in tumors and their surrounding tissues was most linear for 10 MHz transmission ($r^2 \geq 0.67$; $p<0.05$; Fig 6).[51] The slope from 3 out of 4 animals was similar to each other (-0.19±0.07) and to the *in* vitro slope (-0.22), while the last swine (i.e., swine 1) showed a large spread within the normal tissue IFP. These results provide proof-of-concept for the feasibility of using SHAPE as a noninvasive monitor of IFP.

2. Hypothesis

The main hypothesis is that subharmonic signals from US contrast microbubbles can be employed to image breast cancer neovascularity as well as noninvasively measure the IFP of breast cancer (i.e., using SHAPE) and that this information will improve the monitoring of tumor response to neoadjuvant therapies ultimately allowing for better, individualized treatment options to be selected. This in turn should result in improved clinical outcomes and greater survivability for women afflicted with breast cancer.

3. Specific Aims for the Human Clinical Trial.

1) To evaluate the ability of 3D SHI and SHAPE to track changes in LABC angiogenesis and IFP, respectively, by studying women undergoing neoadjuvant chemotherapy before as well as with around 10% and 60% of the neoadjuvant chemotherapy treatment delivered and after

completion of the neoadjuvant chemotherapy treatment and comparing results to MRI and pathology.

2) Finally, as a secondary aim, to compare the 3D SHI depiction of breast cancer angiogenesis in humans to CD31, an immunohistochemical predictor of angiogenesis.

4. Research Strategy

Ultrasound Contrast Agent: We have selected the contrast agent Definity (which will be provided free of charge by Lantheus) 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 employed by our group under an FDA issued IND (73,313).[55] However, the FDA has yet to approve Definity for use in breast 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 breast cancer undergoing neoadjuvant chemoptherapy.

Definity is a sterile, non-pyrogenic suspension of liposome-encapsulated perfluoropropane microbubbles.[25] The agent will be stored in a secure cabinet, with only the study investigators and research personnel having access. *In vivo:* The administration of the contrast will be done as an intravenous infusion of 2 vials of Definity/50ml saline through the subjects' vascular port (established as part of their standard of care), with infusion rates of 4 to 10 ml/min (titrated to effect); as in our prior human trial.[55] Next, we will run a power optimization algorithm to establish the individualized acoustic parameter settings for the case (on the first imaging study only).[56] We will use those parameters for all subsequent imaging studies to sweep the area of the LABC to acquire 3D, pulse inversion SHI grayscale volume data. The digital clips and RF data obtained from each 3D SHI injection

will be transferred to a PC for off-line analysis. The SHI images will be qualitatively analyzed as described above. This protocol will be followed for all the contrast studies for each subject (except for the power optimization algorithm, which will only be run once for each patient). All subjects will be closely monitored by a physician during and following the study.

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.[57,58,59] 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 (as recommended by the FDA).[60,61]

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 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 ≥ 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.

Patient Enrollment

At TJU, Dr Avery (the medical oncologist on this project) and her colleagues treat more than 500 women with breast cancer each year out of which1-2 women/week will be treated with neoadjuvant chemotherapy. We expect to enroll between 20 and 50 subjects undergoing neoadjuvant chemotherapy (i.e., 10 – 25 women/year) in this *first-in-humans* project who will be studied with contrast US (SHI and SHAPE) before as well as with around 10 % and 60% of the neoadjuvant chemotherapy treatment delivered and after completion of the last chemotherapy treatment. Thus, a total of some 144 contrast studies will be performed in this project (range: 80 – 200). The patients studied in this project will be women over the age of 21, who will receive an MRI as part of their standard of care (before and after completion of the chemotherapy). The MRI results together with pathology findings will be compared to the 3D SHI and SHAPE studies as a means for treatment monitoring.

Given that these women have been diagnosed with breast cancer, they are usually very motivated to participate in research studies that include additional monitoring and, therefore, we do not expect recruitment to be an issue. 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. Breast cancer in males accounts for only about 1% of cases at TJU and, thus, will not be included in the patient population.

The inclusion criteria for this trial are as follows:

- Females
- Be diagnosed with T1 or greater LABC, any N and M0.

- Be scheduled for neoadjuvant chemotherapy
- Be at least 21 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.

The exclusion criteria are:

- Males
- Females who are pregnant or nursing.
- Patients with other primary cancers requiring systemic treatment.
- Patients with any metastatic disease.
- Patients undergoing neoadjuvant endocrine therapy.
- Patients with known hypersensitivity or allergy to any component of Definity.
- Patients with cardiac shunts or congenital heart defects.
- Patients with unstable cardiopulmonary conditions or respiratory distress syndrome.
- Patients with uncontrollable emphysema, pulmonary vasculitis, pulmonary hypertension or a history of pulmonary emboli.
- Patients who have received any contrast medium (X-ray, MRI, CT or US) in the 24 hours prior to the research US exam.

US Examination Protocol.

A baseline ultrasound grayscale (US) scan of the treated 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 LABC will also be performed. The distribution of color

signals and the overall color content of mass will be 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) and 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 from one or multiple sites, spotty or a combination. Digital clips of the two baseline imaging modes will be acquired.

The contrast administration will be an intravenous infusion of 2 vials of Definity/50ml saline through the subjects' vascular port (established as part of their standard of care), with infusion rates of 4 to 10 ml/min (titrated to effect).[55] Next, we will run a power optimization algorithm to establish the individualized acoustic parameter settings for the case (on the first imaging study only).[56] We will use those parameters for all subsequent imaging studies to sweep the area of the LABC to acquire 3D, pulse inversion SHI grayscale volume data. The digital clips and RF data obtained from each 3D SHI injection will be transferred to a PC for off-line analysis. The SHI images will be qualitatively analyzed as described above. This protocol will be followed for all the contrast studies for each subject (except for the power optimization algorithm, which will only be run once for each patient). All subjects will be closely monitored by a physician during and following the study.

Data Assessments.

The pre- and then post-contrast US imaging will be evaluated for each patient at all 4 time points in one reading as follows: grayscale, grayscale and PDI (baseline), and grayscale and 3D SHI. 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. Each case will be read independently by the 2 experienced co-investigators (more than 15 years of expertise in breast imaging) blinded to the contrast-enhanced MRI evaluations, to allow repeatability to be assessed. All US studies will be rated on a quasi-continuous scale from 0 to 100 ranging from "no change relative to pre-therapy" over "indeterminate" to "definite changes seen." 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.[62]

Moreover, the 3D SHAPE results (i.e., IFP estimates) will be compared to lesion size, tumor response, DFS and overall survival. Results will also be compared to MRI enhancement kinetics and imaging measurements of lesion size obtained before and after completion of the chemotherapy. Once surgery has been performed 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* will be collected as part of the patient's clinical care and correlated with the imaging results. Finally, the ability of 3D SHI to image angiogenesis (specifically fractional tumor vascularity) will be compared to specimens stained for an immunohistochemical predictor of angiogenesis, specifically a monoclonal antibody against the PECAM endothelial cell marker (anti-CD31; Dako Corporation, Carpinteria, CA). The PI 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 (Nikon, Melville, NY) and ImagePro Plus software.[28]

Statistical Analysis. Our statistical analysis will address the primary objective of this study by answering two major questions:

1. Can *in vivo* 3D SHAPE monitor the effect of neoadjuvant chemotherapy on breast cancer IFP?
2. Can *in vivo* 3D SHI monitor the effect of neoadjuvant chemotherapy (i.e., responders/non-responders) on breast cancer?

The findings of SHI/SHAPE will be correlated to vascular morphology (i.e., tumor angiogenesis ascertained with CD31) and other pathological findings; including size, vascularity, presence or absence of receptors. For the pre and post-contrast enhanced comparisons, dichotomous parameters (e.g., responder/non-responder) 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 each time point. For variables in which accurate numerical measurement is possible (e.g., IFP and vascularity), the techniques will be compared using repeated measures logistical regression and ANOVA.

To answer statistical question 2, the ability of the imaging tests to distinguish responders from non-responders will be compared using ROC analysis, while the incremental validity of combining imaging assessments will be analyzed using logistic regression and ROC analyses.[62,63,64] The tests are fundamental grayscale US imaging, PDI, 3D SHI and contrast

enhanced MRI, while patient outcomes and pathology will provide the reference standards. A power analysis was performed using PASS 12 (NCSS, East Kaysville, UT) to estimate the number of cases required to produce clinically acceptable results. A sample size of 20 responders and 20 non-responders (i.e., approximately halfway between 20 and 50 subjects) achieve 80% power to detect a difference of 0.174 between a diagnostic test with an area under the ROC curve (A_z) of 0.800 (the null hypothesis) and an actual A_z of 0.974 using a one sample, two-sided z-test with a significance level of 0.05. The ratio of the standard deviation of the responses in the two groups was assumed to be 1.00. All analyses will be performed with Stata 12.0 (Stata Corp., College Station, TX).

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.

5.1 Administration

The contrast administration will be an intravenous infusion of 2 vials of Definity/50ml saline through the subjects' vascular port (established as part of their standard of care), with infusion rates of 4 to 10 ml/min (titrated to effect).[55] Next, we will run a power optimization algorithm to establish the individualized acoustic parameter settings for the case (on the first imaging study only).[56] We will use those parameters for all subsequent imaging studies to sweep the area of the LABC to acquire 3D, pulse inversion SHI grayscale volume data. The digital clips and RF data obtained from each 3D SHI injection will be transferred to a PC for off-line analysis. The SHI images will be qualitatively analyzed as described above. This protocol will be followed for all the contrast studies for each subject (except for the power optimization algorithm, which will only be run once for each patient). All subjects will be closely monitored by a physician during and following the study.

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) respiratory 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 coordinator 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 patient population who are who are undergoing neoadjuvant chemotherapy. 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. 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. Acoustic coupling gel will be applied to the breast area of interest. A baseline US grayscale scan will be used to identify the breast cancer seen by mammography (or another concomitant imaging mode such as US or MRI). Standard PDI of the tumor will also be performed. If the mass is too large to be completely imaged by the 4D10 L probe, the most vascular region seen on 2D PDI will be selected for the 3D SHI studies.

To perform the 3D SHI imaging, US software has been modified by GE Medical Systems on the clinical system, the Logiq 9 scanner, to permit acquisition of subharmonic data. All acoustic parameters have been verified and are all within the current standards of the machine, making the modified ultrasound system a non-significant risk device. US imaging will be performed at a transmit frequency of 5.8 MHz and the subharmonic obtained at 2.9 MHz. Using this setup, acoustic pressure amplitudes will all be below 1.5 MPa peak negative pressure, 2.5 MPa peak positive pressure (MI < 0.33).

The contrast administration will be an intravenous infusion of 2 vials of Definity/50ml saline through the subjects' vascular port (established as part of their standard of care), with infusion rates of 4 to 10 ml/min (titrated to effect).[55] Next, we will run a power optimization algorithm to establish the individualized acoustic parameter settings for the case (on the first imaging study only).[56] We will use those parameters for all subsequent imaging studies to sweep the area of the LABC to acquire 3D, pulse inversion SHI grayscale volume data. The digital clips and radio frequency data obtained from each 3D SHI injection will be transferred to a PC for off-line analysis. The SHI images will be qualitatively analyzed as described above. This protocol will be followed for all the contrast studies for each subject (except for the power optimization algorithm, which will only be run once for each patient). All subjects will be closely monitored by a physician during and following the study.

6.4 Safety Monitoring

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

6.5 Safety Assessments

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

6.6.1 Risks/Benefits Assessment.

Serious cardiopulmonary and allergic reactions including fatalities have occurred during or following administration of 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. 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.

Clinically significant adverse effects from the administration of Definity are unlikely based on our previous experiences.[55] 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.

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. All adverse events are reported to the Clinical Trial Support Office (CTSO) via the password protected Kimmel Cancer Center Adverse Event Reporting System. In addition all unexpected and serious adverse events (SAEs) are reported to the TJU IRB and to the Food and Drug Administration (FDA) if applicable. The investigator is required to submit all unexpected and serious adverse events to the TJU IRB and the DSMB within 48 hours. Fatal adverse events related to treatment which are unexpected must be reported within 24 hours to the TJU IRB and the DSMB through CTSO. Fatalities not related to the study drug/device must be reported within 5 days.

The AE forms will include: subject identification number and initials; subject's date of birth, gender, and ethnicity; date of 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 mild, moderate, or severe.

Mild AEs are usually transient, require no special treatment, and do not interfere with the subject's daily activities.

Moderate AEs traditionally introduce a low level of inconvenience or concern to the subject and may interfere with daily activities but are usually ameliorated by simple therapeutic measures.

Severe AEs interrupt a subject's usual daily activity and typically require systemic drag therapy or other treatment.

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

RELATIONSHIP TO 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) is defined as a significant clinical hazard, contraindication, or precaution that:

Results in death

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

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

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

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 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.

6.7 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 trial requirements that is to participate in 4 US contrast studies during the durantion of their clinical cancer treatment.

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: Our hypothesis is that subharmonic signals from US contrast microbubbles can be employed to image breast cancer neovascularity as well as noninvasively measure the IFP of breast cancer (i.e., using SHAPE) and that this information will improve the monitoring of tumor response to neoadjuvant therapies ultimately allowing for better, individualized treatment options to be selected. This in turn will result in improved clinical outcomes and greater survivability for women afflicted with breast cancer.

7.2.2 Analysis of Results

For the pre and post-contrast enhanced comparisons, dichotomous parameters (e.g., responder/non-responder) 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., IFP and vascularity), the techniques will be compared using logistical regression and an ANOVA.

The ability of the imaging tests to distinguish responders from non-responders will be compared using ROC analysis, while the incremental validity of imaging assessments will be analyzed using logistic regression and ROC analyses.[62,63,64] The tests are fundamental grayscale US imaging, PDI, 3D SHI and contrast enhanced MRI , while patient outcomes and pathology will provide the reference standards. 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.[62] Logistical regression techniques will be used to combine the 3 US imaging modes and MRI as well as to incorporate the quantitative parameters (i.e., SHAPE IFP estimates) into the US evaluation before repeating the ROC analysis. This will allow all possible combinations to be compared to one another (e.g., MRI versus all the US modes combined or MRI and grayscale US versus MRI 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 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.[65] Inter- observer variability will also be determined by calculating the intraclass correlation coefficient.[66,67]

Comparisons of subject outcomes (i.e., responders vs. non-responders) established by monitoring the treatment with the different imaging modalities will be conducted with Cox Survival analysis (against

the pathological reference standard). For the US techniques where data from multiple time points are available, this analysis will be repeated for each time point. 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/SHAPE to monitor breast cancer treatment.

Finally, the vascularity measures obtained from the CD31 stained breast tumor specimens will be compared to the fractional tumor neovascularity data to determine if any correlation exists (in baseline PDI or contrast enhanced mode). The existence of a linear relationship between US 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 Stata 12.0 (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) and the results of the various US imaging modes as well as the pathology results.

References Cited

[1] Green MC, Esteva FJ, Hortobagyi GN. Neoadjuvant chemotherapy. *Breast Dis*, 21:23-31, 2004.

[2] Dixon JM. Role of endocrine therapy in the neoadjuvant surgical setting. *Ann Surg Oncol* 11(Suppl):18S-23S, 2004.

[3] Greenberg S, Stopeck A, Rugo HS. Systemic treatment of early breast cancer--a biological perspective. *J Surg Oncol*. 103:619-626, 2011.

[4] Kaufmann M, von Minckwitz G, Smith R, et al. International expert panel on the use of primary (preoperative) systemic treatment of operable breast cancer: review and recommendations. *J Clin Oncol*, 21:2600 – 2608, 2003.

[5] Mauri D, Pavlidis N, Ioannidis JP. Neoadjuvant versus adjuvant systemic treatment in breast cancer: a meta-analysis. *J Natl Cancer Inst*, 97:188-194, 2005.

[6] van Nes JG, Putter H, Julien JP, Tubiana-Hulin M, van de Vijver M, Bogaerts J, de Vos M, van de Velde CJ. Cooperating Investigators of the EORTC. Preoperative chemotherapy is safe in early breast cancer, even after 10 years of follow-up; clinical and translational results from the EORTC trial 10902. *Breast Cancer Res Treat*.; 115:101-113, 2009.

[7] Wolmark N, Wang J, Mamounas E, Bryant J, Fisher B. Preoperative chemotherapy in patients with operable breast cancer: nine-year results from national surgical adjuvant breast and bowel project B-18. *J Natl Cancer Inst Monogr*; 30:96–102, 2001.

[8] Ezzat AA, Ibrahim EM, Ajarim DS, et al. Phase II study of neoadjuvant paclitaxel and cisplatin for operable and locally advanced breast cancer: analysis of 126 patients. *Br J Cancer*, 90:968-974, 2004.

[9] Mamounas EP. Neoadjuvant chemotherapy for operable breast cancer: is this the future? *Clin Breast Cancer,* 4(Suppl.):S10-S19, 2003.

[10] Tiling R, Kessler M, Untch M, et al. Breast cancer: monitoring response to neoadjuvant chemotherapy using Tc-99m sestamibi scintimammography. *Onkologie,* 26:27-31, 2004.

[11] Dunnwald LK, Gralow JR, Ellis GK, et al. Residual tumor uptake of [99mTc]-sestamibi after neoadjuvant chemotherapy for locally advanced breast carcinoma predicts survival. *Cancer*, 103:680-688, 2005.

[12] Baum RP, Przetak C. Evaluation of therapy response in breast and ovarian cancer patients by positron emission tomography (PET). *Q J Nucl Med,* 45:257-268, 2001.

[13] Moyses B, Haegele P, Rodier JF, et al. Assessment of response by breast helical computed tomography to neoadjuvant chemotherapy in large inflammatory breast cancer. *Clin Breast Cancer,* 2:304-310, 2002.

[14] Jakubowski DB, Cerussi AE, Bevilacqua F, Shah N, Hsiang D, Butler J, Tromberg BJ. Monitoring neoadjuvant chemotherapy in breast cancer using quantitative diffuse optical spectroscopy: a case study. *J Biomed Opt*, 9:230-238, 2004.

[15] Pavic D, Koomen MA, Kuzmiak CM, Lee YH, Pisano ED. The role of magnetic resonance imaging in diagnosis and management of breast cancer. *Technol Cancer Res Treat*, 3:527-541, 2004.

[16] Denis F, Desbiez-Bourcier AV, Chapiron C, Arbion F, Body G, Brunereau L. Contrast enhanced magnetic resonance imaging underestimates residual disease following neoadjuvant docetaxel based chemotherapy for breast cancer. *Eur J Surg Oncol,* 30:1069-1076, 2004.

[17] Murata Y, Ogawa Y, Yoshida S, et al. Utility of initial MRI for predicting extent of residual disease after neoadjuvant chemotherapy: analysis of 70 breast cancer patients. *Oncol Rep*, 12:1257-1262, 2004.
[18] Martincich L, Montemurro F, De Rosa G, et al. Locally advanced breast cancer: comparison of mammography, sonography and MR imaging in evaluation of residual disease in women receiving neoadjuvant chemotherapy. *Eur Radiol,* 14:1371-1379, 2004.
[19] Arlinghaus LR, Li X, Levy M, Smith D, Welch EB, Gore JC, Yankeelov TE. Current and future trends in magnetic resonance imaging assessments of the response of breast tumors to neoadjuvant chemotherapy. *J Oncol*. 2010:1-17, 2010.
[20] Pestalozzi B, Castiglione M; ESMO Guidelines Working Group. Primary breast cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann Oncol*. 19 Suppl 2:ii7-10, 2008.
[21] Londero V, Bazzocchi M, Del Frate C, Puglisi F, Di Loreto C, Francescutti G, Zuiani C. Locally advanced breast cancer: comparison of mammography, sonography and MR imaging in evaluation of residual disease in women receiving neoadjuvant chemotherapy. *Eur Radiol*, 14:1371-1379, 2004.
[22] Lagalla R, Caruso G, Finazzo M. Monitoring treatment response with color and power Doppler. *Eur J Radiol*, 27(Suppl 2):S149-S156, 1998.
[23] Huber S, Medl M, Helbich T, et al. Locally advanced breast carcinoma: computer assisted semiquantitative analysis of color Doppler ultrasonography in the evaluation of tumor response to neoadjuvant chemotherapy (work in progress). *J Ultrasound Med*, 19:601-607, 2000.
[24] Vallone P, D'Angelo R, Filice S, et al. Color-Doppler using contrast medium in evaluating the response to neoadjuvant treatment in patients with locally advanced breast carcinoma. *Anticancer Res,* 25(:595-599, 2005.
[25] Goldberg BB, Raichlen JS, Forsberg F. *Ultrasound Contrast Agents: Basic Principles and Clinical Applications*, 2nd Ed, Martin Dunitz Ltd., London, 2001.
[26] Goldberg BB, Merritt CRB, Needleman L, et al. Contrast enhanced ultrasound and mammography for breast cancer detection (abstract). *J Ultrasound Me*d, 20:S95, 2001.
[27] Forsberg F**,** Goldberg BB, Merritt CRB, et al. Diagnosing breast lesions with contrast-enhanced 3-dimensional power Doppler imaging. *J Ultrasound Med*, 23:173-182, 2004.
[28] 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.
[29] 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.
[30] 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.
[31] Stohrer M, Boucher Y, StangassingerM, Jain RK. Oncotic pressure in solid tumors is elevated. *Cancer Res*, 60:4251-4255, 2000.
[32] Hashizume H, Baluk P, Morikawa S, et al. Openings between defective endothelial cells explain tumor vessel leakiness. *Am J Pathol*, 156:1363-1380, 2000.
[33] Li WW. Tumor angiogenesis: Molecular pathology, therapeutic targeting, and imaging. *Acad Radiol*, 7:800-811, 2000.

[34] Milosevic MF, Fyles AW, Hill RP. The relationship between elevated interstitial fluid pressure and blood flow in tumors: a bioengineering analysis. *Int J Radiat Oncol Biol Phys*, 43:1111-1123, 1999.
[35] Boucher Y, Leuning M, Jain RK. Tumor angiogenesis and interstitial hypertension. *Cancer Res*, 56:4264-4266, 1996.
[36] Nathanson SD, Nelson L. Interstitial fluid pressure in breast cancer, benign breast conditions and breast parenchyma. *Ann Surg Oncol*, 1:333-338, 1994.
[37] Jain RK. The next frontier of molecular medicine: delivery of therapeutics. *Nat Med*, 4:655-657, 1998.
[38] Taghian AG, Abi-Raad R, Assaad SI, et al. Paclitaxel decreases the interstitial fluid pressure and improves oxygenation in breast cancers in patients treated with neoadjuvant chemotherapy: clinical implications. *J Clin Oncol* 23:1951-1961, 2005.
[39] Milosevic M, Fyles A, Hedley D, et al. Interstitial fluid pressure predicts survival in patients with cervix cancer independent of clinical prognostic factors and tumor oxygen measurements. *Cancer Res*, 61:6400-6405, 2001.
[40] Lauterborne W. Numerical investigation of nonlinear oscillations of gas bubble in liquids. *J Acoust Soc Am*, 59:283-293, 1976.
[41] Hamilton MF, Blackstock DT. *Nonlinear Acoustics*, Academic Press, San Diego, CA, 1998.
[42] Forsberg F, Shi WT, Goldberg BB. Subharmonic imaging of contrast agents. *Ultrasonics*, 38:93-98, 2000.
[43] 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.
[44] Leighton TG. *The Acoustic Bubble*. London: Academic Press; 1994.
[45] Shi WT, Forsberg F, Raichlen JS, Needleman L, Goldberg BB. Pressure dependence of subharmonic signals from contrast microbubbles. *Ultrasound Med Biol*, 25:275-283, 1999.
[46] Shi WT, Forsberg F, Goldberg BB, Raichlen S. *Method and system for pressure estimation using subharmonic signals from microbubble-based ultrasound contrast agents*. U.S. Patent No. 6,302,845, 2001.
[47] Forsberg F, Liu JB, Shi WT, Furuse J, Shimizu M, Goldberg BB. In vivo pressure estimation using subharmonic contrast microbubble signals: proof of concept. *IEEE Trans Ultrason Ferroelec Freq Contr*, 52:581-583, 2005.
[48] Halldorsdottir VG, Dave JK, Leodore LM, Eisenbrey JR, Park S, Hall AL, Thomenius K, Forsberg F. Subharmonic contrast microbubble signals for noninvasive pressure estimation under static and dynamic flow conditions. *Ultrason Imaging*; 33:153-164, 2011.
[49] Dave JK, Halldorsdottir VG, Eisenbrey JR, Liu JB, McDonald ME, Dickie K, Leung C, Forsberg F. Noninvasive estimation of dynamic pressures in vitro and in vivo using the subharmonic response from microbubbles. *IEEE Trans Ultrason, Ferroelec and Freq Control*, 58:2056-2066, 2011.
[50] Dave JK, Halldorsdottir VG, Eisenbrey JR, Raichlen JS, Liu JB, McDonald ME, Dickie K, Wang S, Leung C, Forsberg F. Noninvasive left ventricular pressure estimation using subharmonic emissions from microbubbles - an in vivo pilot study. *JACC Cardiovasc Imaging*, 5:87–92, 2012.
[51] Halldorsdottir VG, Dave JK, Eisenbrey J, Machado P, Liu JB, Merton DA, Cavanaugh BC, Forsberg F. Subharmonic aided pressure estimation for monitoring interstitial fluid pressure in tumors: in vitro and in vivo proof of concept (abstract). *J Ultrasound Med*, 30:S28, 2011.

[52] Paul S, .Katiyar A, Sarkar K, Chatterjee D, Shi WT, Forsberg F. Material characterization of the encapsulation of an ultrasound contrast microbubble and its subharmonic response: strain-softening interfacial elasticity model. *J Acoust Soc Am*., 127:3846-3857, 2010.
[53] Katiyar A, Sarkar K, Forsberg F. Modeling subharmonic response from contrast microbubbles as a function of ambient static pressure. *J Acoust Soc Am*, 129:2325–2335, 2011.
[54] Hook RR, Jr, Berkelhammer J, Oxenhandler RW. Animal model of human disease: Sinclair swine melanoma. *Am J Pathol*, 108:130-133, 1982.
[55] Linden RA, Trabulsi EJ, Forsberg F, Gittens PR, Gomella LG, Halpern EJ. Contrast enhanced ultrasound flash replenishment method for directed prostate biopsies. *J Urology*, 178:2354-2358, 2007.
[56] Dave JK, Halldorsdottir VG, Eisenbrey JR, et al. On the implementation of an automated acoustic output optimization algorithm for subharmonic aided pressure estimation. *Ultrasonics*, 53, 2013. In press.
[57] Main ML, Goldman JH, Grayburn PA. Ultrasound contrast agents: balancing safety versus efficacy. *Expert Opin Drug Saf*; 8:49-56, 2009.
[58] Dolan MS, Gala SS, Dodla S, et al. Safety and efficacy of commercially available ultrasound contrast agents for rest and stress echocardiography a multicenter experience. *J Am Coll Cardiol*; 53:32-38, 2009.
[59] Wei K, Mulvagh SL, Carson L, et al. The safety of Definity and Optison for ultrasound image enhancement: a retrospective analysis of 78,383 administered contrast doses. *J Am Soc Echocardiogr*; 21:1202-1206, 2008.
[60] 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. http://www.fda.gov/Safety/MedWatch/SafetyInformation/SafetyAlertsforHumanMedicalProducts/ucm092270.htm (accessed August 23, 2011).
[61] 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. http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm142884.htm (accessed August 23, 2011).
[62] Wagner RF, Beiden SV, Metz CE. Continuous versus categorical data for ROC analysis: some quantitative considerations. *Acad Radiol*, 8:328-334, 2001.
[63] 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.
[64] Metz CE. ROC methodology in radiologic imaging. Invest Radiol 1986; 21:720-733.
[65] Rosner B. Fundamentals of Biostatistics (3rd Ed.). Boston, MA: PWS-Kent Publishing Co., 1990.
[66] Shrout PE, Fleiss JL. Intraclass correlations: uses in assessing rater reliability. Psychological Bulletin, 86:420-428, 1979.
[67] McGraw KO, Wong SP. Forming inferences about some intraclass correlation coefficients. Psychol Methods, 1:30-46, 1996.