



THOMAS JEFFERSON UNIVERSITY
Kimmel Cancer Center

Detection of high grade prostate cancer with subharmonic ultrasound imaging, A pilot study.

Principal Investigators:	<i>Ethan J Halpern, MD</i> Department of Radiology Thomas Jefferson University 132 South 10 th Street Philadelphia, PA 19107-5244 215-955-5345	<i>Flemming Forsberg, PhD</i> Department of Radiology Thomas Jefferson University 132 South 10 th Street Philadelphia, PA 19107-5244 215-955-4870
Co-Investigator(s):	<i>Edouard Trabulsi</i> Department of Urology <i>Leonard Gomella</i> Department of Urology <i>Costas Lallas</i> Department of Urology	
Funding Sponsor:	NIH R21CA202214	
Regulatory Sponsor:	<i>Flemming Forsberg PhD</i>	
IND Number:	112,241	
Study Product:	<i>Definity</i>	
Protocol Number:	<i>15F.607</i>	

Version:	Date:
1.0	11/2015
1.1	11/2016
1.2	12/01/17

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Study Summary

Title	Detection of high grade prostate cancer with subharmonic ultrasound imaging, A pilot study
Short Title	Using Contrast-enhanced Ultrasound (CE-TRUS) to Identify Aggressive forms of Prostate Cancer
Protocol Number	
Phase	<i>Phase II clinical study – classified as Phase II because the goal is to demonstrate that imaging of the prostate with CEUS is improved with the use of subharmonic imaging</i>
Methodology/Study Design	<i>Pilot cohort study of patients scheduled for prostate biopsy</i>
Study Duration	<i>two years</i>
Study Center(s)	<i>Single-center</i>
Objectives	<ol style="list-style-type: none"> 1. To implement SHI technology on a transrectal probe suitable for prostate imaging and biopsy 2. To demonstrate visualization of prostatic vascularity using subharmonic contrast-enhanced imaging. 3. To provide a preliminary estimate of the diagnostic accuracy of contrast-enhanced subharmonic imaging for detection of clinically significant PCa.
Number of Subjects	<i>55 subjects (5 controls and 50 patients)</i>
Diagnosis and Main Inclusion Criteria	<ol style="list-style-type: none"> 1. <i>Patients who are scheduled for prostate biopsy</i> 2. <i>and who are able to undergo CEUS</i>
Study Therapy, Dose, Route, Regimen	<i>The study will involve infusion of the ultrasound microbubble contrast agent Definity immediately prior to a prostate biopsy procedure.</i>
Duration of administration and follow-up	<i>The contrast administration will require 10-15 minutes, during which time a transrectal ultrasound study of the prostate will be performed, and a targeted biopsy procedure of the prostate will be performed.</i>
Reference therapy	<i>Conventional 12-core systematic prostate biopsy</i>
Statistical Methodology	<i>Computation of sensitivity and specificity for detection of clinically significant prostate cancer with conventional 12 core prostate biopsy as the reference standard. No further statistical testing will be performed as this is a preliminary study, designed to demonstrate the feasibility of subharmonic imaging of the prostate and to obtain an estimate of the diagnostic accuracy of CEUS with subharmonic imaging of the prostate.</i>

<p>Schema</p>	<p><i>Subharmonic imaging software will be expanded to work with an endorectal probe on an FDA approved ultrasound imaging system</i></p> <p>↓</p> <p><i>Patient with elevated PSA or an abnormal rectal exam, or a patient requiring a prostate biopsy on active surveillance</i></p> <p>↓</p> <p><i>Scheduled for prostate biopsy</i></p> <p>↓</p> <p><i>Has CEUS with subharmonic imaging</i></p> <p>↓</p> <p><i>Prostate biopsy with up to 6 targeted cores based upon subharmonic imaging as well as prostate biopsy with 12 standard systematic cores.</i></p>

1.0 INTRODUCTION

This document is a protocol for a human research study. This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

1.1 Specific Aims and Hypothesis

1. To implement SHI technology on a transrectal probe suitable for prostate imaging and biopsy
2. To demonstrate visualization of prostatic vascularity during subharmonic contrast enhanced imaging of the prostate.
3. To provide a preliminary estimate of the diagnostic accuracy of contrast-enhanced subharmonic imaging for detection of prostate cancer.

1.2 Background and Rationale

Prostate cancer (PCa) is the most prevalent cancer among American males, with an estimated 3,922,600 living PCa patients.¹ Biochemical screening with serum prostate specific antigen (PSA) results in the detection of many small cancers such that “the risk of being diagnosed with PCa is increasingly greater than the risk of dying of it”.² Thus, although PCa will account for 27% (233,000/855,220) of all new cancer diagnoses among American males in 2014, PCa is estimated to be directly responsible for only 9.5% (29,480/310,010) of cancer related deaths among American males.³ PCa has been likened to the two faces of Janus: “one benevolent face of small, indolent tumors, abundant among middle-aged and elderly men” and a second “grim face of a great killer, being the second most common cancer-related cause of death within the EU and in the US”.⁴ This dichotomy in the progression and prognosis of PCa creates a dilemma for both physician and patient in deciding between definitive therapy of a potentially lethal disease and expectant management of a potentially indolent disease. Simply stated, the PCa treatment dilemma is related to our inability to distinguish indolent from aggressive PCa at the time of diagnosis.

Two recent clinical trials have sparked an intense controversy related to the benefit of screening and treatment of PCa. The Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial failed to show a mortality benefit from screening and treatment of PCa.⁵ The European Randomized Study of Screening for PCa (ERSPC) suggests that with the current standard of care, 1410 men must be screened and 48 additional cases of PCa treated to prevent

one death.⁶ Both studies suggest that overdiagnosis and overtreatment of insignificant cancer is a major drawback of PCa screening, and point to the need for better diagnostic tools to identify clinically significant PCa.⁷ Based upon the data from these trials, the U.S. Preventive Services Task Force (USPSTF) has issued a grade “D” recommendation against PSA-based screening.⁸

Various treatment options are available to the patient with a new diagnosis of PCa. Aggressive therapy with surgery or radiation constitutes the traditional therapy for localized PCa, with the goal of treating the entire prostate to eradicate the cancer. However, both radical prostatectomy and radiation therapy can be associated with substantial morbidity, including sexual impotence, urinary incontinence and injury to adjacent organs.^{9,10} Expectant management without curative therapy has demonstrated favorable 15-20 year follow-up results for localized, low-grade PCa.^{11,12} Prospective cohort studies suggest that aggressive treatment for PCa may be deferred until there is evidence of cancer progression.¹³ For men with “favorable risk” PCa, defined as low volume disease (< 0.5 ml) with a Gleason score below 7, active surveillance (AS) results in similar survival as definitive therapy,¹⁴ and conservative management is associated with a 50% lower incidence of incontinence and impotence as compared to radical prostatectomy.¹⁵ A major barrier to the widespread adoption of AS is the lack of an accurate non-invasive diagnostic test to prospectively identify those patients with aggressive “clinically significant” cancer whose disease will progress without definitive therapy. Patients on AS therefore require a periodic invasive biopsy for tissue diagnosis. A technique that could improve the accuracy of diagnostic ultrasound for the diagnosis of clinically significant PCa would facilitate wider use of AS.

Contrast-Enhanced Imaging of Prostate Cancer

As prostate imaging is necessary for biopsy guidance in the diagnosis of PCa, much research has focused on optimization of imaging techniques for PCa detection. Our group has focused primarily on prostate imaging with microbubble ultrasound contrast agents, as ultrasound is the least expensive and most commonly used modality for guidance of prostate biopsy. When exposed to an ultrasound pulse at some fundamental frequency (f_0), microbubbles act as nonlinear oscillators, generating significant energy components which span the range of frequency emissions from subharmonics through ultraharmonics.¹⁶ While conventional ultrasound processes received echoes at the fundamental frequency (f_0), contrast-enhanced ultrasound imaging is based upon received echo signals in the harmonic ($2f_0$, $3f_0$, etc) or subharmonic ($1/2 f_0$, $1/3 f_0$, etc) range. Since microbubble contrast agents remain intravascular, contrast-enhanced transrectal ultrasound (CE-TRUS) with harmonic imaging (HI) or subharmonic imaging (SHI) selectively images vascularity within the prostate.

Pathologic studies demonstrate a clear association of microvessel density with the presence of PCa,¹⁷ with metastatic disease,¹⁸ with disease stage¹⁹ and with disease-specific survival.^{20,21} Although microvessels are below the resolution of conventional ultrasound Doppler imaging, CE-TRUS provides a practical solution to imaging the microvasculature. Clinical studies using HI have demonstrated that enhancement of the prostate with CE-TRUS is related to increased microvessel density,^{22,23} that CE-TRUS can improve detection of PCa,^{24,25} and that CE-TRUS can increase cancer detection with fewer biopsy cores.^{26,27} We review preliminary results with CE-TRUS HI and SHI below, but note that there are no published studies using SHI of the prostate.

1.3 Study Therapy

Since the subjects in this study are scheduled to have prostate biopsy as part of their standard of care, the only study related test that will be performed is a contrast-enhanced ultrasound with subharmonic imaging. The proposed ultrasound contrast agent for the current study, Definity™, (Perflutren Lipid Microsphere, Lantheus Medical Imaging, Inc; N. Billerica, MA), is a microbubble contrast agent that demonstrates enhancement of the prostate on both grayscale and Doppler imaging. Definity is a sterile, non-pyrogenic suspension of liposome-encapsulated perfluoropropane microbubbles. This contrast agent is composed of a blend of three phospholipids contained in a matrix of sodium chloride, propylene glycol and glycerin in water. The contrast agent is supplied in a vial that contains the phospholipids and perfluoropropane gas. The microbubble agent is prepared by shaking the standard size vial with the aid of a shaking device (Vialmix; ESPE, Seefeld, Germany). Based upon our previous experience with Definity, two vials of Definity will be mixed and diluted in 50 ml of normal saline, yielding a concentration of 49.4 µl/ml. For the purpose of contrast-enhanced imaging, Definity will be infused over approximately 10-12 minutes, during which time pulse inversion harmonic and flash replenishment imaging will be performed. Definity remains stable within the circulation for 3 to 6 minutes after infusion; all contrast-enhanced procedures will be completed during the infusion time.

1.3 Clinical Data – Breast and Liver

Feasibility studies of this new SHI mode have been conducted *in vitro* and *in vivo* by our group^{28,29} as well as others^{30;31,32,33,34,35;36;37;38}. Researchers at Jefferson produced the first ever *in vivo* grayscale SHI images (of canine kidneys) using a modified US scanner. We also produced the first ever *in vivo* human grayscale SHI images, demonstrating (in 14 women with 16 breast lesions) that SHI can detect the slow, small volume blood flow associated with breast tumor angiogenesis in humans.³⁹

Fig. 1 presents an example of a fibroadenoma imaged after administration of Optison with power Doppler imaging (PDI) and in SHI mode (transmitting at 4.4 MHz and receiving at 2.2 MHz; optimized for SHI) using a modified Logiq 9 scanner (GE Healthcare, Milwaukee, WI). Notice, the almost complete lack of signal from non-vascular tissue, and that the internal morphology of the tumor vascularity was visualized better with SHI than with contrast enhanced PDI.



Figure 1. Fibroadenoma (arrows) imaged in PDI mode (a) and in SHI mode (b) after injection of contrast.

Fig. 2 shows a breast lesion with benign ductal microcalcifications and fibrocystic changes. Branching vessels within and around the lesion are depicted in finer detail with SHI (Fig 3b) and without the color blooming of contrast enhanced PDI (Fig 3a). Finally, SHI depicts neovessels ($\sim 20\text{-}40\mu\text{m}$ diameter⁴⁰) not observed with conventional imaging.

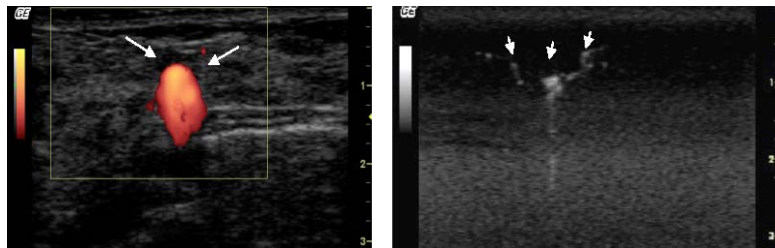


Figure 2. Benign ductal microcalcifications and fibrocystic changes (arrows) depicted with contrast PDI (a) and with SHI (b).

More recently we have focused on translating grayscale SHI into human clinical trials in the breast and liver^{41,42} and on improved processing of SHI images.⁴³ We have introduced the concept of CMI-SHI, where the inflow of contrast bubbles in tumor neovessels is reconstructed by temporal maximum intensity projection of pixels traced from successive image frames.^{44;45} A dynamic cumulative maximum intensity display for subharmonic imaging (CMI-SHI) was developed using a new automated kernel tracking algorithm by identifying motion induced displacements. As demonstrated by the receiver operating characteristic analysis in Fig 3, the area under the curve for diagnosis of breast cancer was greatest for dynamic CMI-SHI (0.90) and was

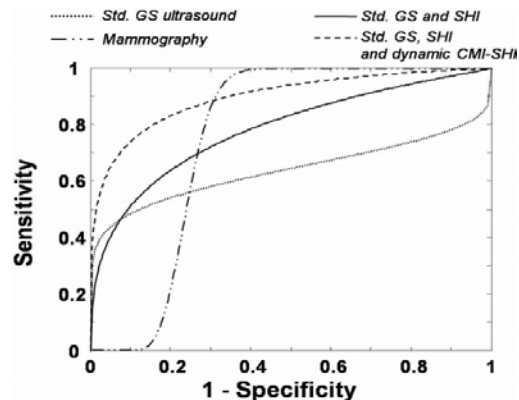


Figure 3. ROC curves for four of the imaging modalities; (GS: grayscale).

significantly better than for mammography ($p = 0.031$). With regard to tissue perfusion, we have developed a novel method for deriving quantitative estimates of tissue perfusion, based on *in vivo* grayscale SHI⁴⁶ as an alternative to the well-known destruction/reperfusion technique.⁴⁷ A modified Logiq 9 scanner operating in SHI mode was used to measure renal perfusion. Fractional blood volumes were estimated from subharmonic signal intensities from blood in tissue.^{48,49} Perfusion was estimated from the initial slope of the SHI time intensity curve and correlated with the reference standard neutron activation assay technique ($r = 0.62$; $p < 0.001$).⁵⁰ Finally, we created parametric images of SHI contrast kinetics, demonstrating increased perfusion in breast cancer relative to benign lesions (0.103 ± 0.030 vs. 0.054 ± 0.017 ; $p = 0.0014$).⁵¹

1.5 Clinical Data - Prostate

Two basic ultrasound technologies have been used to image microbubble contrast agents in the prostate: Doppler and grayscale HI. These technologies are summarized in a textbook by one of the PIs.⁵² Color and power Doppler imaging techniques, available on most modern ultrasound systems, utilize relatively high energy levels that destroy a large proportion of the microbubbles as they are imaged. Pulse inversion and grayscale HI are technological advances that together permit high resolution, low energy grayscale imaging of contrast agents.^{53,54,55,56} Various pulsing sequences can further improve grayscale HI of the prostatic microcirculation.^{57,58} Flash replenishment imaging was first described as a grayscale harmonic technique that can demonstrate tissue perfusion and may be used for blood flow estimation.^{59,60,61} Contrast-Enhanced Flash Replenishment may be combined with Maximum Intensity Projection (CE-FR-MIP) of each pixel in the image over 2-4 seconds of low power image frames to demonstrate microvascular patterns associated with neovascularity⁶² and PCa.⁶³ Whereas assessment for neovascularity with contrast-enhanced Doppler imaging is based upon the magnitude of contrast enhancement, CE-FR-MIP provides additional information based upon changes to the normal intraprostatic microvascular architecture (Fig. 4).

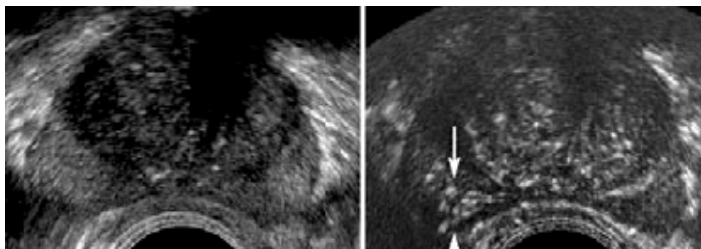


Figure 4. Gleason 7 PCa in the right mid-gland is invisible on grayscale (left image), but tumor vascularity is clearly defined with CE-FR-MIP imaging (right image; arrows).

Numerous trials of CE-TRUS using HI to detect PCa have been conducted at the Jefferson Prostate Diagnostic Center.^{64,65} An early study ($n=100$ subjects) demonstrated improved sensitivity for PCa detection from 38% at baseline to

65% after infusion of the microbubble agent Definity (Lantheus Medical Imaging, N Billerica, MA); $p \leq 0.004$.⁶⁶ Furthermore, lesions detected by contrast-enhancement were generally larger and with a higher Gleason score – that is, they were the more clinically significant cancers. The last 40 subjects in this study were evaluated with targeted biopsy. A suspicious site identified by CE-TRUS was 3.5 times more likely to have a positive biopsy than an adjacent site without contrast enhancement ($p < 0.025$).⁶⁷

A Department of Defense funded clinical trial of 301 men demonstrated that targeted cores based upon CE-TRUS were twice as likely to return a positive biopsy as systematic cores.⁶⁸ Using CE-FR-MIP, our group demonstrated positive biopsies in 29/225 targeted cores as compared with 50/600 systematic cores, yielding an odds ratio of 2.0 for cancer detection with CE-TRUS ($p = 0.034$).⁶⁹ A recently completed National Cancer Institute (NCI) funded clinical trial ($n = 272$) confirmed increased PCa detection with CE-TRUS, and specifically with CE-FR-MIP (OR=2.1, $p < 0.001$). More significantly, CE-TRUS preferentially detected lesions with greater tumor volume and Gleason score ≥ 7 ($p < 0.001$). ROC analysis for detection of high volume / high grade PCa with CE-TRUS yielded an area under the curve of 0.90 ($p = 0.001$).⁷⁰ Comparison of CE-TRUS HI with whole mount prostatectomy specimens in our DOD and NCI study subjects demonstrated microbubble enhancement in 93% of malignant foci. In summary, PCa's visualized with CE-TRUS tend to be larger and of higher grade.

In summary, clinical studies demonstrate selective detection of higher grade PCa with HI, based upon the increased neovascularity of these tumors. Nonetheless, although CE-TRUS with HI selectively detects clinically significant PCa, overall by-patient detection of high grade PCa with targeted biopsy based upon HI alone remains inferior to a 12 core systematic biopsy. This limitation is related, in part, to difficulty discerning enhancing foci of PCa adjacent to the hypervascular transition zone (see abstract in appendix). A major limitation of HI is related to reduced blood-to-tissue contrast resulting from second harmonic generation and accumulation in tissue.⁷¹ Hence, subharmonic imaging (SHI), transmitting at the fundamental frequency (f_0) and receiving at the subharmonic ($f_0/2$), becomes an attractive alternative contrast-specific imaging mode, because of the weaker subharmonic generation in tissue and the significant subharmonic scattering produced by some new contrast agents.⁷² As demonstrated in the previous section, preliminary data with SHI suggest that this technique provides a superior signal to background ratio for evaluation of blood flow, that SHI can improve depiction of neovessels, and that SHI may be useful for quantitative assessment of tissue perfusion in cancer. In order to optimize the detection of clinically significant PCa, we propose the use of SHI with CE-TRUS for targeted biopsy of the prostate.

1.6 Dose Rationale and Risk/Benefits

Based upon our previous experience with Definity and according to the package insert, two vials of Definity will be mixed and diluted in 50 ml of normal saline, yielding a concentration of 49.4 $\mu\text{l/ml}$. For the purpose of contrast-enhanced imaging, Definity will be infused over approximately 10-12 minutes, during which time pulse inversion HI and SHI will be performed. Definity remains stable within the circulation for 3 to 6 minutes after infusion; all contrast-enhanced procedures will be completed during the infusion time. The risk of this procedure is basically the risk of an adverse reaction to Definity. The largest safety studies published to date on the use of ultrasound contrast agents in humans (involving up to 4,300,966 subjects) concluded that these agents have a good safety profile in both cardiac and non-cardiac applications.^{73,74,75,76} In our experience, adverse reactions to Definity include only minor sensations of discomfort which resolve as soon as the infusion is stopped.

2.0 STUDY OBJECTIVES

1. To implement SHI technology on a transrectal probe suitable for prostate imaging and biopsy
2. To demonstrate visualization of prostatic vascularity during subharmonic contrast enhanced imaging of the prostate.
3. To provide a preliminary estimate of the diagnostic accuracy of contrast-enhanced subharmonic imaging for detection of prostate cancer.

3.0 STUDY DESIGN

3.1 General Design

This study is a pilot phase II clinical trial of 55 patients (5 controls and 50 patients) over the course of 2 years. Experimental software used for subharmonic imaging of other body parts will be adopted to work with the transrectal prostate probe of an FDA approved ultrasound system, specifically the IC5-9D transducer on the Logiq E9 system (GE Healthcare; Milwaukee, WI). In vitro testing will be performed on 5 patients to ensure that the power output of the transducer with the new subharmonic software is within the FDA approved limits for the system power output. After in vitro optimization of the subharmonic mode for imaging, we will conduct a pilot clinical trial of 50 patients over 2 years. All participants will be evaluated with contrast-enhanced transrectal ultrasound (CE-TRUS) prior to prostate biopsy. Subharmonic imaging of the CE-TRUS study will be used to direct targeted biopsy of the prostate with up to 6 biopsy cores. This will be followed by a conventional systematic biopsy of the prostate with 12 cores.

3.2 Primary Study Endpoints

- a. Demonstration of prostate vascularity with subharmonic contrast-enhanced imaging. Specifically, demonstration of increased prostate vascularity in patients with high risk, aggressive prostate cancer.
- b. Preliminary measures of efficacy (sensitivity and specificity) will be obtained for subharmonic CE-TRUS in the detection of high risk, aggressive prostate cancer after biopsy procedure on 50 patients.

3.3 Secondary Study Endpoints

n/a

3.4 Primary Safety Endpoints

No safety issues are expected from the use of subharmonic imaging, as the power output of the ultrasound system will be similar to its operation in conventional mode. No safety issues are expected from the administration of Definity for CEUS, as this agent has been used safely in numerous clinical trials and is FDA approved for intravenous administration during cardiac imaging.

4.0 SUBJECT SELECTION AND WITHDRAWAL

4.1 Inclusion Criteria

1. Subject must be scheduled for a clinically indicated needle biopsy of the prostate based upon an elevated PSA, abnormal digital rectal examination, or based upon active surveillance of prostate cancer.
2. Subject must be able and willing to give written informed consent for a contrast enhanced ultrasound study of the prostate.
3. Subject must be a male at least 18 years of age when informed consent is obtained.
4. Subject must have a life expectancy that exceeds the duration of the clinical trial.

4.2 Exclusion Criteria

1. Participant in a clinical trial involving an investigational drug within the past 30 days.
2. Patients with known or suspected hypersensitivity to perflutren
3. Previous treatment for PCa.
4. Clinically unstable, severely ill, or moribund.

4.3 Gender/Minority/Pediatric Inclusion for Research

This study is only applicable to adult males, as PCa is only present in the adult male population. The study will include men of all races; there will be no exclusion based upon race or ethnicity.

4.4 Subject Recruitment and Screening

Fifty adult male patients will be recruited to this study by our urology co-investigators from the population scheduled to undergo prostate biopsy at Thomas Jefferson University Hospital. Subjects will be screened for eligibility by the attending urologist. The urologist will explain the procedure and may obtain written informed consent from each study participant at that time or in radiology on the day of study and prior to any study procedures being performed.

4.5 Early Withdrawal of Subjects

4.5.1 When and How to Withdraw Subjects

Any subject may withdraw from the study up until the time the CEUS study is performed. However, once that study is performed, the data will be included in our analysis.

5.0 STUDY DRUG/THERAPY

5.1 Description

This is not a therapeutic trial. The only drug that will be administered is Definity, which is a microbubble ultrasound contrast agent. The risk of the procedure is the risk of an adverse reaction to Definity, which is considered minimal. Although the study participants may not benefit from the findings, future patients may benefit from development of a non-invasive imaging technique that can detect and characterize aggressive PCa. If successful, this technique may reduce the number of biopsy procedures performed for prostate cancer, and it will likely be used to guide a limited number of targeted biopsy cores into those areas that demonstrate imaging characteristics of aggressive prostate cancer.

5.2 Treatment Regimen

Based upon our previous experience with Definity and according to the manufacturer's instruction in the package insert, two vials of Definity will be mixed and diluted in 50 ml of normal saline, yielding a concentration of 49.4 µl/ml. For the purpose of contrast-enhanced imaging, Definity will be infused over

approximately 10-12 minutes, during which time pulse inversion HI and SHI will be performed.

5.3 Risks

The risk of this procedure is basically the risk of an adverse reaction to Definity, and the risks of conventional prostate biopsy. The largest safety studies published to date on the use of ultrasound contrast agents in humans (involving up to 4,300,966 subjects) concluded that these agents have a good safety profile in both cardiac and non-cardiac applications.^{77,78,79,80} In our experience, adverse reactions to Definity include only minor sensations of discomfort which resolve as soon as the infusion is stopped.

5.4 Method for Assigning Subjects to Treatment Groups

n/a, as all subjects will be studied with HI and SHI.

6.0 STUDY PROCEDURES

6.1 Study Visit Schedule

The CEUS study procedure will be performed during a single episode of care at which time the patient will undergo a clinically indicated biopsy of the prostate. No additional visits will be required to participate in the study.

All ultrasound imaging will be performed with a commercially available Logiq E9 ultrasound system that provides conventional color and power Doppler imaging, and which will be modified to provide contrast-enhanced HI and SHI. Because of the very low SHI signal obtained from tissue, SHI is performed with a dual imaging technique that displays a conventional grayscale image alongside the SHI image. The conventional grayscale image is necessary to define the tissue anatomy and determine location within the prostate while the SHI is used to demonstrate vascularity and neovessels.

The proposed microbubble contrast agent for the current study, Definity®, (Perflutren Lipid Microsphere, Lantheus), is a microbubble contrast agent that demonstrates enhancement of the prostate on both grayscale and Doppler imaging. Definity remains stable within the circulation for 3 to 6 minutes after infusion and all contrast-enhanced procedures will need to be completed during the infusion time. This time constraint has not been a problem in prior studies. Based on our previous experience with Definity (and in accordance with the manufacturer's instructions), two vials will be mixed and diluted in 50 ml of

normal saline, yielding a concentration of 49.4 $\mu\text{l/ml}$. A 20 gauge intravenous catheter will be inserted into an arm vein for administration of contrast material. Definity will be delivered as an infusion at an initial rate of 4 ml/min. The infusion rate will be titrated to achieve adequate enhancement of the prostatic microvasculature. Post contrast imaging will begin as soon as contrast is visible. The infusion of Definity will continue for approximately 10-12 minutes during which time the contrast-enhanced diagnostic ultrasound, the assignment of rating scores, and the SHI guided biopsy procedure will be performed.

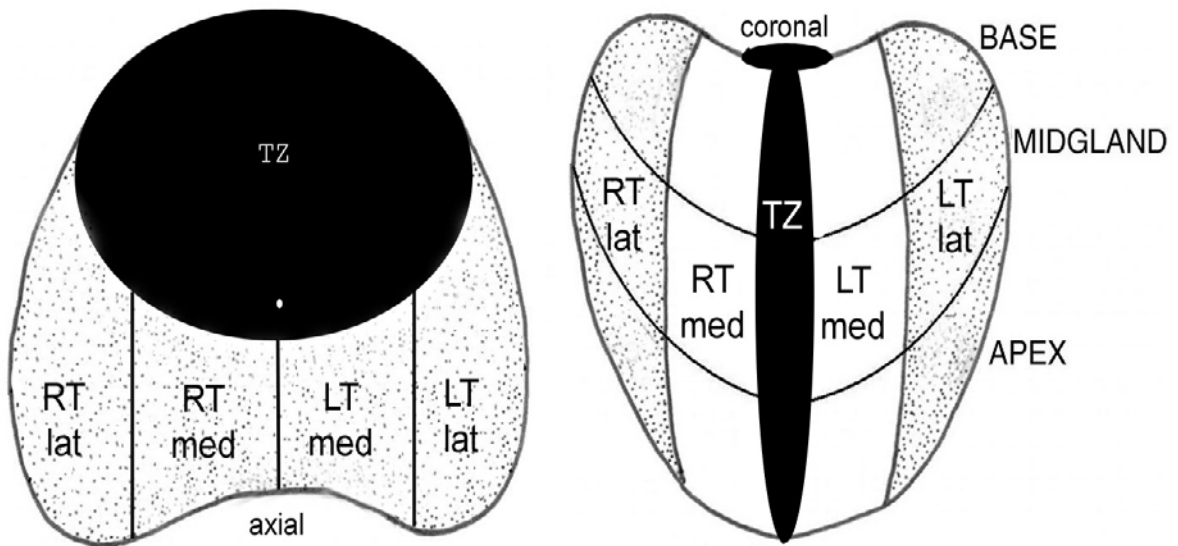
Study participants will be prepared for biopsy according to standard clinical operating procedure for subjects undergoing TRUS-guided biopsy at the Jefferson Prostate Diagnostic Center. Study subjects will be imaged in the lithotomy position with grayscale ultrasound performed to measure gland size.⁸¹ Next, the prostate will be evaluated with both baseline and contrast-enhanced imaging modes. The dynamic range and imaging frequencies for grayscale, and color Doppler imaging will be standardized for all subjects (scanning frequency of 7.0 MHz with dynamic range of 65 dB). The Doppler window for color imaging will include the entire gland. The baseline examination (~5 minutes) will consist of conventional grayscale imaging and color Doppler imaging. Contrast-enhanced imaging (~5-7 minutes) will consist of color Doppler imaging, HI and SHI. In order to allow comparison of the various imaging modes, a standard imaging sweep through the prostate will be performed in the axial plane from the base of the gland to the apex for each of the 2 baseline imaging modes and 3 contrast-enhanced imaging modes.

Study participants will be monitored for AEs during and 30 minutes after contrast administration.

6.2 Study Interpretation

The peripheral zone of each prostate will be rated for suspicion of PCa at 12 sites, including medial and lateral component of each sextant. A 5-point rating score will be assessed in real-time by the examining physician for each ultrasound technique. These scores have been previously described and validated,⁸² with proven utility for predicting the risk of malignancy in prior studies.[Error! Bookmark not defined.](#)[Error! Bookmark not defined.](#) A maximum of 6 sites will be identified as the most suspicious areas on SHI; targeted biopsy specimens will be obtained from these sites under ultrasound guidance during contrast-enhanced imaging. If fewer sites are identified, fewer targeted biopsy cores may be obtained.

The interpretation of CEUS studies will follow a 12 part extended sextant grid as demonstrated in the illustration below:



7.0 STATISTICAL PLAN

7.1 Sample Size Determination

The study is a pilot study, for the purpose of generating preliminary data for a future grant submission. The study is not powered to demonstrate any specific accuracy of CEUS, but rather to accumulate several demonstration cases of SHI in high grade prostate cancer.

Based upon our past experience we expect approximately 30% of our patients to be positive for prostate cancer and half of these (15%) to have a diagnosis of high grade disease. Thus, in a population of 50 patients, the mean expected number of patients with high grade disease would be 7.5. Using an exact binomial probability distribution, the probability of finding at least 5 patients in this sample with high grade disease is 88%. The probability of finding 3 or fewer patients with high grade disease is 4.6%. The probability of not finding a single patient with high grade disease is 0.03%. Thus, the sample size of $n=50$ provides a greater than 95% probability of demonstrating the performance of SHI in at least 3 patients with high grade prostate cancer.

7.2 Statistical Methods

Rough estimates of the sensitivity and specificity of SHI for high grade disease may be obtained if there are sufficient patients with high grade disease. No additional statistical analysis will be performed

7.3 Subject Population(s) for Analysis

All study participants will be included in the analysis.

8.0 SAFETY AND ADVERSE EVENTS

8.1 Definitions

Adverse Event

An ***adverse event*** (AE) is any symptom, sign, illness or experience that develops or worsens in severity during the course of the study. Intercurrent illnesses or injuries should be regarded as adverse events. Abnormal results of diagnostic procedures are considered to be adverse events if the abnormality:

- results in study withdrawal
- is associated with a serious adverse event
- is associated with clinical signs or symptoms
- leads to additional treatment or to further diagnostic tests
- is considered by the investigator to be of clinical significance

Serious Adverse Event

Adverse events are classified as serious or non-serious.

A ***serious adverse event*** is any AE that is:

- fatal
- life-threatening
- requires or prolongs hospital stay
- results in persistent or significant disability or incapacity
- a congenital anomaly or birth defect
- an important medical event

Important medical events are those that may not be immediately life threatening, but are clearly of major clinical significance. They may jeopardize the subject, and may require intervention to prevent one of the other serious outcomes noted above. For example, drug overdose or abuse, a seizure that did not result in in-patient hospitalization, or intensive treatment of bronchospasm in an emergency department would typically be considered serious.

All adverse events that do not meet any of the criteria for serious should be regarded as ***non-serious adverse events***.

Adverse Event Reporting Period

The study period during which adverse events will be reported will extend from the time of administration of the Definity contrast agent, up to 24 hours after the administration.

Preexisting Condition

A preexisting condition is one that is present at the start of the study. A preexisting condition should be recorded as an adverse event if the frequency, intensity, or the character of the condition worsens during the study period.

General Physical Examination Findings

At screening, any clinically significant abnormality should be recorded as a preexisting condition. At the end of the study, any new clinically significant findings/abnormalities that meet the definition of an adverse event must also be recorded and documented as an adverse event.

Post-study Adverse Event

All unresolved adverse events should be followed by the investigator until the events are resolved, the subject is lost to follow-up, or the adverse event is otherwise explained. The investigator will instruct each subject to report any subsequent event(s) that the subject, or the subject's personal physician, believes might reasonably be related to participation in this study.

Hospitalization, Prolonged Hospitalization or Surgery

Any adverse event that results in hospitalization or prolonged hospitalization should be documented and reported as a serious adverse event unless specifically instructed otherwise in this protocol. Any condition responsible for surgery should be documented as an adverse event if the condition meets the criteria for and adverse event.

Neither the condition, hospitalization, prolonged hospitalization, nor surgery are reported as an adverse event in the following circumstances:

- Hospitalization or prolonged hospitalization for diagnostic or elective surgical procedures for a preexisting condition. Surgery should ***not*** be reported as an outcome of an adverse event if the purpose of the surgery was elective or diagnostic and the outcome was uneventful.
- Hospitalization or prolonged hospitalization required to allow efficacy measurement for the study.
- Hospitalization or prolonged hospitalization for therapy of the target disease of the study, unless it is a worsening or increase in frequency of hospital admissions as judged by the clinical investigator.

8.2 Recording of Adverse Events

The following subsections detail what information must be documented for each adverse event occurring during the time period specified in Section 8.3.

8.2.1 Relationship to Study Intervention

The relationship to study intervention or study participation must be assessed and documented for all adverse events. Evaluation of relatedness must consider etiologies such as natural history of the underlying disease, concurrent illness, concomitant therapy, study-related procedures, accidents, and other external factors.

The following guidelines are used to assess relationship of an event to study intervention:

1. Related (Possible, Probable, Definite)
 - a. The event is known to occur with the study intervention.
 - b. There is a temporal relationship between the intervention and event onset.
 - c. The event abates when the intervention is discontinued.
 - d. The event reappears upon a re-challenge with the intervention.
2. Not Related (Unlikely, Not Related)
 - a. There is no temporal relationship between the intervention and event onset.
 - b. An alternate etiology has been established.

8.2.2 Expectedness

The PI is responsible for determining whether an AE is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the intervention. Risk information to assess expectedness can be obtained from preclinical studies, the investigator's brochure, published medical literature, the protocol, or the informed consent document.

8.2.3 Severity of Event

Adverse events will be graded for severity according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0.

8.2.4 Intervention

Any intervention implemented to treat the adverse event must be documented for all adverse events.

8.3 Safety Assessment and Follow Up

The PI will follow adverse events with start dates occurring any time after informed consent is obtained until 7 (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation. At each study visit, the investigator (or

designee) will inquire about the occurrence of AE/SAEs since the last visit. Events will be followed for outcome information until resolution or stabilization.

8.4 Reporting to IRB

8.4.1 Unanticipated Problems

All incidents or events that meet criteria for unanticipated problems (UAPs) as defined in Section 8.1.1 Unanticipated Problems require the creation and completion of an unanticipated problem report form (OHR-20).

UAPs that pose risk to participants or others, and that are not AEs, will be submitted to the IRB on an OHR-20 form via the eazUP system within 10 working days of the investigator becoming aware of the event.

UAPs that do not pose risk to participants or others will be submitted to the IRB at the next continuing review.

8.4.1.1 Adverse Events

Grade 1 AEs will be reported to the IRB at continuing review.

Grade 2 AEs will be reported to the IRB at the time of continuing review.

8.4.1.2 Serious Adverse Events

SAEs will be reported to the IRB on OHR-10 forms via the electronic reporting system (eSAEy) according to the required time frames described below.

Grade 3-4 AEs that are unexpected and deemed to be at least possibly related to the study will be reported to the IRB within 2 working days of knowledge of the event.

Grade 3-4 AEs that are deemed unrelated to the study will be reported to the IRB within 5 working days.

Grade 5 AEs will be reported to the IRB within one working day of knowledge of the event.

All SAEs will be submitted to the IRB at continuing review, including those that were reported previously.

8.5 Reporting to SKCC DSMC

All AEs and SAEs, safety and toxicity data, and any corrective actions will be submitted to the DSMC per the frequency described in the SKCC DSMP. The

report to the SKCC DSMC will also include any unanticipated problems that in the opinion of the PI should be reported to the DSMC.

For expedited reporting requirements, see table below:
DSMC AE/SAE Reporting Requirements

	Grade 1	Grade 2		Grade 3				Grades 4 and 5
	Unexpected and Expected	Unexpected	Expected	Unexpected		Expected		Unexpected and Expected
				With Hospitalization	Without Hospitalization	With Hospitalization	Without Hospitalization	
Unrelated Unlikely	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	5 Working Days	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	5 Working Days	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Phase I - 48 Hours (Death: 24 Hours) Phase II - 5 working days
Possible Probably Definite	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	48 Hours (Death: 24 Hours)	Phase I - 48 Hours Phase II - 5 working days	48 Hours (Death: 24 Hours)	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Phase I and Phase II - 48 Hours (Death: 24 Hours)

8.6 Report to FDA

In accordance with 21 CFR 212.32, sponsor-investigators of studies conducted under an IND must comply with following safety reporting requirements:

Expedited IND Safety Reports:

7 Calendar-Day Telephone or Fax Report:

The Sponsor-Investigator is required to notify the FDA of any fatal or life-threatening adverse event that is unexpected and assessed by the investigator to be possibly related to the use of Definity. An unexpected adverse event is one that is not already described in the Investigator Brochure.

Such reports are to be telephoned or faxed to the FDA within 7 calendar days of first learning of the event. Each telephone call or fax transmission (see fax number below) should be directed to the FDA new drug review division in the Center for Drug Evaluation and Research or in the product review division for the Center for Biologics Evaluation and Research, whichever is responsible for the review of the IND.

15 Calendar-Day Written Report:

The Sponsor-Investigator is also required to notify the FDA and all participating investigators, in a written IND Safety Report, of any serious, unexpected AE that is considered possibly related to the use of Definity. An unexpected adverse event is one that is not already described in the Investigator Brochure.

Written IND Safety Reports should include an Analysis of Similar Events in accordance with regulation 21 CFR § 312.32. All safety reports previously filed with the IND concerning similar events should be analyzed. The new report should contain comments on the significance of the new event in light of the previous, similar reports.

Written IND safety reports with Analysis of Similar Events are to be submitted to the FDA and all participating investigators within 15 calendar days of first learning of the event. The FDA prefers these reports on a MedWatch 3500A Form but alternative formats are acceptable (e.g. summary letter).

FDA fax number for IND Safety Reports:

1 (800) FDA - 0178

All written IND Safety Reports submitted to the FDA by the Sponsor-Investigator must also be faxed to:

Thomas Jefferson University IRB

IND Annual Reports

In accordance with the regulation 21 CFR § 312.32, the Sponsor-Investigator shall within 60 days of the anniversary date that the IND went into effect submit a brief report of the progress of the investigation. Please refer to Code of Federal Regulations, 21 CFR § 312.32 for a list of the elements required for the annual report. All IND annual reports submitted to the FDA by the Sponsor-Investigator should be copied to the National Institutes of Health as the sponsor of this study.

8.5.2 Data and Safety Monitoring Committee

Data and Safety Monitoring Committee (DSMC) is the Data and Safety Monitoring Board (DSMB) for the KCC. The DSMC is a multidisciplinary committee charged with overseeing the monitoring of safety of participants in clinical trials, and the conduct, progress, validity, and integrity of the data for all clinical trials at the Thomas Jefferson University KCC. The committee meets quarterly to review the progress and safety of all active research protocols that are not monitored by another safety and data monitoring committee or board.

- The DSMC meets quarterly. Additional DSMC meetings are scheduled based on the nature and number of trials being monitored over a specified time period. The DSMC meets (by conference call) within 24 hours following the notification of an unexpected adverse event felt to be related to the study drug.
- Prior to each DSMC meeting, each board member, is provided a printout of all reported AEs and SAEs occurring during the reporting period for this clinical trial. The principal investigator provides a detailed and comprehensive narrative assessment of current adverse events to date, indicating their possible significance and whether these toxicities have affected the conduct of the trial. DSMC members are provided with the principal investigator's assessment, a written report summarizing adverse events, safety data, and activity data observed during the specified time period described in each protocol, as well as recommendations from the Medical Monitor. A review of outcome results (response, toxicity and adverse events) and factors external to the study (such as scientific or therapeutic developments) is discussed, and the Committee votes on the status of each study.
- A summary of the board's action is sent to each investigator, the CCRRC and TJU IRBs. The DSMC actions may include recommendations/requirements that will lead to improved patient safety and/or efficacy, significant benefits or risks that have developed, or other changes determined to be necessary. The DSMC may also take note of slow accrual or lack of scientific progress, and refer such issues to the CCRRC. The DSMC provides the investigator with the rationale for any decision made.

9.0 DATA HANDLING AND RECORD KEEPING

9.1 Confidentiality

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Those regulations require a signed subject authorization informing the subject of the following:

- What protected health information (PHI) will be collected from subjects in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research subject to revoke their authorization for use of their PHI.

In the event that a subject revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization. For subjects that have revoked authorization to collect or use PHI, attempts should be made to obtain permission to collect at least vital status (i.e. that the subject is alive) at the end of their scheduled study period.

9.2 Source Documents

Source data is all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents. Examples of these original documents, and data records include: hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories, and at medico-technical departments involved in the clinical trial.

9.3 Case Report Forms

The study case report form (CRF) is the primary data collection instrument for the study. CRFs for the CEUS studies will be completed for each study subject, and are included in section 14 of this protocol. All data requested on the CRF must be recorded. All missing data must be explained. If a space on the CRF is left blank because the procedure was not done or the question was not asked, write "N/D". If the item is not applicable to the individual case, write "N/A". All entries should be printed legibly in black ink. If any entry error has been made, to correct such an error, draw a single straight line through the incorrect entry and enter the correct data above it. All such changes must be initialed and dated.

DO NOT ERASE OR WHITE OUT ERRORS. For clarification of illegible or uncertain entries, print the clarification above the item, then initial and date it.

9.4 Records Retention

It is the investigator's responsibility to retain study essential documents for at least 2 years after the last approval of a marketing application in their country and until there are no pending or contemplated marketing applications in their country or at least 2 years have elapsed since the formal discontinuation of clinical development of the investigational product. These documents should be retained for a longer period if required by an agreement with the sponsor. In such an instance, it is the responsibility of the sponsor to inform the investigator/institution as to when these documents no longer need to be retained.

10.0 STUDY MONITORING, AUDITING, AND INSPECTING

Clinical site monitoring and auditing is conducted to ensure that the rights of human participants are protected, that the study is implemented in accordance with the protocol and/or other operating procedures, and that the quality and integrity of study data and data collection methods are maintained. Monitoring and auditing for this study will be performed in accordance with the SKCC's Data and Safety Monitoring Plan (DSMP) developed by the SKCC Data and Safety Monitoring Committee (DSMC). The DSMP specifies the frequency of monitoring, monitoring procedures, the level of clinical site monitoring activities (e.g., the percentage of participant data to be reviewed), and the distribution of monitoring reports. Some monitoring activities may be performed remotely, while others will take place at the study site(s). Appropriate staff will conduct monitoring activities and provide reports of the findings and associated action items in accordance with the details described in the SKCC DSMP.

11.0 ETHICAL CONSIDERATIONS

This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to a properly constituted independent Institutional Review Board (IRB), in agreement with local legal

prescriptions, for formal approval of the study conduct. The decision of the IRB concerning the conduct of the study will be made in writing to the investigator before commencement of this study.

All subjects for this study will be provided a consent form that is compliant with local and federal regulations, describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. See Attachment for a copy of the Subject Informed Consent Form. This consent form will be submitted with the protocol for review and approval by the IRB for the study. The formal consent of a subject, using the IRB-approved consent form, must be obtained before that subject is submitted to any study procedure. This consent form must be signed by the subject or legally acceptable surrogate, and the investigator-designated research professional obtaining the consent.

12.0 STUDY FINANCES

12.1 Funding Source

Funding for this study will be provided by an R21 grant from the NIH (R21 CA202214).

12.2 Conflict of Interest

Any investigator who has a conflict of interest with this study (patent ownership, royalties, or financial gain greater than the minimum allowable by their institution, etc.) must have the conflict reviewed by a properly constituted Conflict of Interest Committee with a Committee-sanctioned conflict management plan that has been reviewed and approved by the study sponsor prior to participation in this study. All Jefferson University Investigators will follow the TJU Conflicts of Interest Policy for Employees (107.03).

12.3 Subject Stipends or Payments

n/a

13.0 PUBLICATION PLAN

The investigators involved in this study will be solely responsible for analysis of the data, and publication of any results. It is expected that the data from this study will be published in a peer reviewed urology journal.

14.0 REFERENCES

¹ Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, Cooper D, Gansler T, Lerro C, Fedewa S, Lin C, Leach C, Cannady RS, Cho H, Scoppa S, Hachey M, Kirch R, Jemal A, Ward E. Cancer treatment and survivorship statistics, 2012. *CA Cancer J Clin* 2012; 62:220-241. [PMID: 22700443]

² Penson DF, Rossignol M, Sartor AO, Scardino PT, Abenham LL. PCa: epidemiology and health-related quality of life. *Urology*. 2008; 72(6 Suppl):S3-11. [PMID: 19095126]

³ Siegel R, Ma J, Zou Z, Jemal A. Cancer Statistics, 2014. *CA A Cancer Journal for Clinicians*. 2014; 64:9-29. [PMID: 23335087]

⁴ Bratt O. Watching the face of Janus--active surveillance as a strategy to reduce overtreatment for localised PCa. *Eur Urol*. 2006; 50(3):410-2. [PMID: 16650924]

⁵ Andriole GL, Crawford ED, Grubb RL, 3rd, Buys SS, Chia D, Church TR, Fouad MN, Gelmann EP, Kvale PA, Reding DJ, Weissfeld JL, Yokochi LA, O'Brien B, Clapp JD, Rathmell JM, Riley TL, Hayes RB, Kramer BS, Izmirlian G, Miller AB, Pinsky PF, Prorok PC, Gohagan JK, Berg CD; PLCO Project Team. Mortality results from a randomized prostate-cancer screening trial [published erratum in *N Engl J Med*. 2009;360:1797] *N Engl J Med*. 2009;360:1310–1319. PMID: [19297565]

⁶ Schröder FH, Hugosson J, Roobol MJ, Tammela TL, Ciatto S, Nelen V, Kwiatkowski M, Lujan M, Lilja H, Zappa M, Denis LJ, Recker F, Berenguer A, Määttänen L, Bangma CH, Aus G, Villers A, Rebillard X, van der Kwast T, Blijenberg BG, Moss SM, de Koning HJ, Auvinen A; ERSPC Investigators. Screening and prostate-cancer mortality in a randomized European study. *N Engl J Med*. 2009; 360(13):1320-8. [PMID: 19207566]

⁷ Gomella LG, Liu XS, Trabulsi EJ, Kelly WK, Myers R, Showalter T, Dicker A, Wender R. Screening for PCa: the current evidence and guidelines controversy. *Can J Urol*. 2011 Oct;18(5):5875-83. PMID: [22018148]

⁸ Moyer VA, on behalf of the U.S. Preventive Services Task Force*. Screening for PCa: U.S. Preventive Services Task Force Recommendation Statement. *Ann Intern Med*. 2012;157:120-134.

⁹ Penson DF, McLerran D, Feng Z, Li L, Albertsen PC, Gilliland FD, Hamilton A, Hoffman RM, Stephenson RA, Potosky AL, Stanford JL. 5-year urinary and sexual outcomes after radical prostatectomy: results from the PCa outcomes study. *J Urol*. 2005 May;173(5):1701-5. [PMID: 15821561]

¹⁰ Zelefsky, MJ; Fuks, Z; Happersett, L; Lee, HJ; Ling, CC; Burman, CM; Hunt, M; Wolfe, T; Venkatraman, ES; Jackson, A; Skwarchuk, M; Leibel, SA. Clinical experience

with intensity modulated radiation therapy (IMRT) in PCa. *Radiother Oncol.* 2000;55:241–249. [PMID: 10869739]

11 Albertsen PC, Hanley JA, Fine J. 20-year outcomes following conservative management of clinically localized PCa. *JAMA.* 2005 293(17):2095-101. [PMID: 15870412]

12 Johansson JE, Holmberg L, Johansson S, Bergström R, Adami HO. Fifteen-year survival in PCa. A prospective, population-based study in Sweden. *JAMA.* 1997 277(6):467-71. [PMID: 9020270]

13 Patel MI, DeConcini DT, Lopez-Corona E, Ohori M, Wheeler T, Scardino PT. An analysis of men with clinically localized PCa who deferred definitive therapy. *J Urol.* 2004; 171(4):1520-4. [PMID: 15017211]

14 Dall'Era MA, Cooperberg MR, Chan JM, Davies BJ, Albertsen PC, Klotz LH, Warlick CA, Holmberg L, Bailey DE Jr, Wallace ME, Kantoff PW, Carroll PR. Active surveillance for early-stage PCa: review of the current literature. *Cancer.* 2008; 112(8):1650-9. [PMID: 18306379]

15 Steineck G, Helgesen F, Adolfsson J, Dickman PW, Johansson JE, Norlén BJ, Holmberg L; Scandinavian Prostatic Cancer Group Study Number 4. Quality of life after radical prostatectomy or watchful waiting. *N Engl J Med.* 2002; 347(11):790-6. [PMID: 12226149]

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

17 Bigler SA, Deering RE, Brawer MK. Comparison of microscopic vasculature in benign and malignant prostate tissue. *Human Pathology* 24:220-226, 1993. [PMID: 8432518]

18 Weidner N, Carroll PR, Flax J, Blumenfeld W, Foldman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *American Journal of Pathology* 143:401-409, 1993. [PMID: 7688183]

19 Bostwick DG, Wheeler TM, Blute M, Barrett DM, MacLennan GT, Sebo TJ, Scardino PT, Humphrey PA, Hudson MA, Fradet Y, Miller GJ, Crawford ED, Blumenstein BA, Mahran HE, Miles BJ. Optimized microvessel density analysis improves prediction of cancer stage from prostate needle biopsies. *Urology* 48:47-57, 1996. [PMID: 8693651]

20 Lissbrant IF, Stattin P, Damber JE, Bergh A. Vascular density is a predictor of cancer-specific survival in prostatic carcinoma. *Prostate* 33:38-45, 1997. [PMID: 9294625]

21 Borre M, Offersen BV, Nerstrom B, Overgaard J. Microvessel density predicts survival in PCa patients subjected to watchful waiting. *British Journal of Cancer* 78:940-944, 1998. [PMID: 9764587]

22 Sedelaar JP, van Leenders GJ, Hulsbergen-van de Kaa CA, van der Poel HG, van der Laak JA, Debruyne FM, Wijkstra H, de la Rosette JJ. Microvessel density: correlation between contrast ultrasonography and histology of PCa. *European Urology*. 40(3):285-93, 2001. [PMID: 11684844]

23 Strohmeyer D, Frauscher F, Klauser A, Recheis W, Eibl G, Horninger W, Steiner H, Volgger H, Bartsch G. Contrast-enhanced transrectal color Doppler ultrasonography (TRCDUS) for assessment of angiogenesis in PCa. *Anticancer Research*. 21(4B):2907-13, 2001. [PMID: 11712785]

24 Bogers HA, Sedelaar JP, Beerlage HP, de la Rosette JJ, Debruyne FM, Wijkstra H, Aarnink RG. Contrast-enhanced three-dimensional power Doppler angiography of the human prostate: correlation with biopsy outcome. *Urology*. 54(1):97-104, 1999. [PMID: 10414734]

25 Roy C, Buy X, Lang H, Saussine, Jacquemin D. Contrast enhanced color Doppler endorectal sonography of the prostate: efficiency for detecting peripheral zone tumors and role for biopsy procedure. *The Journal of Urology* 170:69-72, 2003. [PMID: 12796647]

26 Frauscher F, Klauser A, Halpern EJ, Horninger W, Bartsch G. Detection of PCa with a Microbubble Ultrasound Contrast Agent. *The Lancet* 357:1849-1850, 2001.

27 Frauscher F, Klauser A, Volgger H, Halpern EJ, Pallwein L, Steiner H, Schuster A, Horninger W, Rogatsch H, Bartsch G. Comparison of Contrast-enhanced Color Doppler Targeted Biopsy to Conventional Systematic Biopsy: Impact on PCa Detection. *The Journal of Urology* 167:1648-1652, 2002. [PMID: 11912381]

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

29 Eisenbrey JR, Sridharan A, Machado P, Zhao H, Halldorsdottir VG, Dave JK, Liu JB, Park S, Dianis S, Wallace K, Thomenius KE, Forsberg F. Three-dimensional subharmonic ultrasound imaging in vitro and in vivo. *Acad Radiol*, 2012; 19: 732-739. [PMID: 22464198].

30 Chomas J, Dayton P, May D, Ferrara K. Nondestructive subharmonic imaging. *IEEE Trans. Ultrason. Ferroelec. Freq. Contr.*, 2002; 49:883-892.

31 Faez T, Renaud G, Defontaine M, Calle S, de Jong N. Dynamic manipulation of the subharmonic scattering of phospholipid-coated microbubbles. *Phys Med Biol* 2011b; 56:6459-73.

32 Faez T, Emmer M, Docter M, Sijl J, Versluis M, de Jong N. Characterizing the subharmonic response of phospholipid-coated microbubbles for carotid imaging. *Ultrasound Med Biol*. 2011a; 37:958-970. [PMID: 21531498].

-
- ³³ Goertz DE, Cherin E, Needles A, Burns PN, Foster FS. High frequency nonlinear B-scan imaging of microbubble contrast agents. *IEEE Trans Ultrason Ferroelectr Freq Control*. 52:65-79, 2005.
- ³⁴ Goertz DE, Frijlink ME, de jong N, van der Steen AF. Nonlinear intravascular ultrasound contrast imaging. *Ultrasound Med Biol*, 32:491-502, 2006.
- ³⁵ Goertz DE, Frijlink ME, Tempel D, Bhagwandas V, Gisolf A, Krams R, de Jong N, van der Steen AF. Subharmonic contrast intravascular ultrasound for vasa vasorum imaging. *Ultrasound Med Biol*, 33:1859-1872, 2007.
- ³⁶ Helfield BL, Cherin E, Foster FS, Goertz DE. Investigating the subharmonic response of individual phospholipid encapsulated microbubbles at high frequencies: a comparative study of five agents. *Ultrasound Med Biol*. 2012; 38:846-863. [PMID: 22402024].
- ³⁷ Needles A, Goertz DE, Karshafian R, Cherin E, Brown AS, Burns PN, Foster FS. High-frequency subharmonic pulsed-wave Doppler and color flow imaging of microbubble contrast agents. *Ultrasound Med Biol*, 34:1139-1151, 2008.
- ³⁸ Shankar PM, Krishna PD, Newhouse VL. Advantage of subharmonic over second harmonic backscatter for contrast-to-tissue echo enhancement. *Ultrasound Med. Biol*, 1998; 24:395-399.
- ³⁹ Forsberg F, Piccoli CW, Merton DA, Palazzo JJ, Hall AL. Breast lesions: imaging with contrast-enhanced subharmonic US - initial experience. *Radiology* 2007, 244:718-726.
- ⁴⁰ 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*, 2008; 34:1365-1372.
- ⁴¹ Forsberg F, Liu JB, Chiou HJ, Rawool NM, Parker L, Goldberg BB. Comparison of fundamental and wideband harmonic contrast imaging of liver tumors. *Ultrasonics* 2000a, 38:110-113.
- ⁴² Eisenbrey JR, Dave JK, Halldorsdottir VG, Merton DA, Miller C, Gonzalez JM, Machado P, Park S, Dianis S, Chalek CL, Kim CE, Baliff JP, Thomenius KE, Brown DB, Navarro VJ, Forsberg F. Noninvasive subharmonic aided pressure estimation of portal vein pressures in patients with chronic liver disease. *Radiology*, 2013; 266. In press.
- ⁴³ Eisenbrey JR, Joshi N, Dave JK, Forsberg F. Assessing algorithms for defining vascular architecture in subharmonic images of breast lesions. *Phys Med Biol*, 2011c; 56:1 – 12.
- ⁴⁴ Dave JK, Forsberg F. Novel automated motion compensation technique for producing cumulative maximum intensity subharmonic images. *Ultrasound Med Biol*, 2009; 35:155-1563.

-
- ⁴⁵ 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.
- ⁴⁶ 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* 2006, 25:15-21.
- ⁴⁷ 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* 1998; 97:473-483.
- ⁴⁸ Rubin JM, Adler RS, Fowlkes JB, Spratt S, Pallister JE, Chen JF, Carson PL. Fractional moving blood volume: estimation with power Doppler US. *Radiology* 1995; 197:183-190.
- ⁴⁹ Rubin JM, Bude RO, Fowlkes JB, Spratt RS, Carson PL, Adler RS. Normalizing fractional moving blood volume estimates with power Doppler US: defining a stable intravascular point with the cumulative power distribution function. *Radiology* 1997; 205:757-765.
- ⁵⁰ Sridharan A, Eisenbrey JR, Liu JB, et al. Perfusion estimation using contrast enhanced three-dimensional subharmonic ultrasound imaging: an in vivo study. *Invest Radiol*, 2013; 48 : 654-660.
- ⁵¹ 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. *J Ultrasound Med* 2011b; 30:85–92.
- ⁵² Halpern EJ, Cochlin LI, Goldberg BB. *Imaging of the Prostate*. London: Martin Dunitz Ltd. 2002. [isbn: 1841841986]
- ⁵³ de Jong N, Cornet R, Lancee CT. Higher harmonics of vibrating gas-filled microspheres. Part one: simulations. *Ultrasonics* 32:447-453. 1994. [doi: 10.1016/0041-624X(94)90064-7]
- ⁵⁴ de Jong N, Cornet R, Lancee CT. Higher harmonics of vibrating gas-filled microspheres. Part two: measurements. *Ultrasonics* 32:455-459, 1994. [doi: 10.1016/0041-624X(94)90065-5]
- ⁵⁵ Schrope BA, Newhouse VL, Uhlendorf V. Simulated capillary blood flow measurement using a nonlinear ultrasonic contrast agent. *Ultrasonic Imaging* 14:134-158, 1992. [PMID: 1604755]
- ⁵⁶ Schrope BA, Newhouse VL. Second harmonic ultrasound blood perfusion measurement. *Ultrasound in Medicine and Biology* 19:567-579, 1993. [PMID: 8310553]

-
- 57 Aigner F, Pallwein L, Mitterberger M, Pinggera GM, Mikuz G, Horninger W, Frauscher F. Contrast-enhanced ultrasonography using cadence-contrast pulse sequencing technology for targeted biopsy of the prostate. *BJU Int.* 2009; 103(4):458-63. [PMID: 19021610]
- 58 Yang JC, Tang J, Li J, Luo Y, Li Y, Shi H. Contrast-enhanced gray-scale transrectal ultrasound-guided prostate biopsy in men with elevated serum prostate-specific antigen levels. *Acad Radiol.* 2008; 15(10):1291-7. [PMID: 18790401]
- 59 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.* 1998 Feb 10;97(5):473-83. [PMID: 9490243]
- 60 Kamiyama N, Moriyasu F, Mine Y, Goto Y. Analysis of flash echo from contrast agent for designing optimal ultrasound diagnostic systems. *Ultrasound Med Biol.* 1999 Mar;25(3):411-20. [PMID: 10374984]
- 61 Shi WT, Forsberg F, Liu JB, Rawool NM, Goldberg BB. Blood flow estimation with harmonic Flash Echo Imaging. *Ultrason Imaging.* 2001 Jul;23(3):161-70. [PMID: 11958587]
- 62 Sugimoto K, Moriyasu F, Kamiyama N, Metoki R, Yamada M, Imai Y, Iijima H. Analysis of morphological vascular changes of hepatocellular carcinoma by microflow imaging using contrast-enhanced sonography. *Hepatol Res.* 2008; 38(8):790-9. [PMID: 18507694]
- 63 Linden RA, Trabulsi EJ, Forsberg F, Gittens PR, Gomella LG, Halpern EJ. Contrast enhanced ultrasound flash replenishment method for directed prostate biopsies. *J Urol.* 2007; 178(6):2354-8. [PMID: 17936814]
- 64 Halpern E.J., Verkh L., Forsberg F., Gomella L.G., Mattrey R.F., Goldberg BB. Initial experience with contrast-enhanced sonography of the prostate. *American Journal of Roentgenology* 174:1757-1580, 2000. [PMID: 10845484]
- 65 Halpern EJ, McCue PA, Aksnes AK, Hagen EK, Frauscher F, Gomella LG: Contrast enhanced sonography of the prostate with Sonazoid: Comparison with whole mount prostatectomy specimens in twelve patients. *Radiology* 2002; 222:361-366. [PMID: 11818600]
- 66 Halpern EJ, Rosenberg M, Gomella LG. PCa: contrast-enhanced US for detection. *Radiology* 219:219-225, 2001. [PMID: 11274560]
- 67 Halpern EJ, Frauscher F, Rosenberg M, Gomella LG. Directed Biopsy During Contrast Enhanced Sonography of the Prostate. *American Journal of Roentgenology* 178:915-919, 2002. [PMID: 11906872]

68 Halpern EJ, Ramey JR, Strup SE, Frauscher F, McCue P, Gomella LG. Detection of Prostate Cancer with Contrast Enhanced Sonography Using Intermittent Harmonic Imaging. *Cancer* 2005; 104(11):2372-2383. [PMID: 16240450]

69 Linden RA, Trabulsi EJ, Forsberg F, Gomella LG, Halpern EJ. Contrast enhanced ultrasound flash replenishment method for directed prostate biopsies. *J Urol* 178(6):2354-8, 2007.

70 Halpern EJ, Gomella LG, Forsberg F, McCue PA, Trabulsi EJ. Contrast-Enhanced Transrectal Ultrasound for the Detection of PCa: A Randomized, Double-Blinded Trial of Dutasteride Pretreatment. *J Urol* 2012; 188(5):1739-45. [PMID: 22998915]

⁷¹ Hamilton MF, Blackstock DT. *Nonlinear acoustics*, Academic Press, San Diego, CA, 1998.

⁷² Forsberg F, Shi WT, Goldberg BB. Subharmonic imaging of contrast agents. *Ultrasonics* 2000b, 38:93-98.

73 Dolan MS, Gala SS, Dodla S, Abdelmoneim SS, Xie F, Cloutier D, Bierig M, Mulvagh SL, Porter TR, Labovitz AJ. Safety and efficacy of commercially available ultrasound contrast agents for rest and stress echocardiography a multicenter experience. *J Am Coll Cardiol* 2009; 53:32-38. [PMID: 19118722]

74 Main ML, Ryan AC, Davis TE, Albano MP, Kusnetzky LL, Hibberd M. Acute mortality in hospitalized patients undergoing echocardiography with and without an ultrasound contrast agent (multicenter registry results in 4,300,966 consecutive patients). *Am J Cardiol*. 102:1742-1746, 2008. [PMID: 19064035]

75 Main ML, Goldman JH, Grayburn PA. Ultrasound contrast agents: balancing safety versus efficacy. *Expert Opin Drug Saf* 2009; 8:49-56. [PMID: 19236217]

76 Wei K, Mulvagh SL, Carson L, Davidoff R, Gabriel R, Grimm RA, Wilson S, Fane L, Herzog CA, Zoghbi WA, Taylor R, Farrar M, Chaudhry FA, Porter TR, Irani W, Lang RM. The safety of Definity and Optison for ultrasound image enhancement: a retrospective analysis of 78,383 administered contrast doses. *J Am Soc Echocardiogr* 2008; 21:1202-1206. [PMID: 18848430]

77 Dolan MS, Gala SS, Dodla S, Abdelmoneim SS, Xie F, Cloutier D, Bierig M, Mulvagh SL, Porter TR, Labovitz AJ. Safety and efficacy of commercially available ultrasound contrast agents for rest and stress echocardiography a multicenter experience. *J Am Coll Cardiol* 2009; 53:32-38. [PMID: 19118722]

78 Main ML, Ryan AC, Davis TE, Albano MP, Kusnetzky LL, Hibberd M. Acute mortality in hospitalized patients undergoing echocardiography with and without an ultrasound contrast agent (multicenter registry results in 4,300,966 consecutive patients). *Am J Cardiol*. 102:1742-1746, 2008. [PMID: 19064035]

79 Main ML, Goldman JH, Grayburn PA. Ultrasound contrast agents: balancing safety versus efficacy. *Expert Opin Drug Saf* 2009; 8:49-56. [PMID: 19236217]

80 Wei K, Mulvagh SL, Carson L, Davidoff R, Gabriel R, Grimm RA, Wilson S, Fane L, Herzog CA, Zoghbi WA, Taylor R, Farrar M, Chaudhry FA, Porter TR, Irani W, Lang RM. The safety of Definity and Optison for ultrasound image enhancement: a retrospective analysis of 78,383 administered contrast doses. *J Am Soc Echocardiogr* 2008; 21:1202-1206. [PMID: 18848430]

⁸¹ Halpern EJ, Frauscher F, Forsberg F, Strup SE, Nazarian LN, O'Kane P, Gomella LG. High Frequency Doppler Imaging of the Prostate: Effect of Patient Position. *Radiology* 222:634-639, 2002. [PMID: 11867778]

⁸² Mitterberger M, Aigner F, Pinggera GM, Steiner E, Rehder P, Ulmer H, Halpern EJ, Horninger W, Frauscher F. Contrast-enhanced colour Doppler-targeted prostate biopsy: correlation of a subjective blood-flow rating scale with the histopathological outcome of the biopsy. *BJU Int.* 2010 Mar 25. [Epub ahead of print] [PMID: 20346032]