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CONTRAST ENHANCED ULTRASOUND (CEUS) IN BREAST TUMORS

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“I hear and I forget. I see and I remember. I do and I understand”

Confucius

Chinese philosopher & reformer (551 BC-479 BC)

“Los escritos nunca se terminan, solo se abandonan...”

“Writings are never finished, just abandoned...”

Jorge Luis Borges

Argentine writer (1899-1986)

To Laura

ABSTRACT

Contrast-enhanced ultrasound or CEUS is a combination of the pharmacokinetics of contrast agents (or microbubbles-based ultrasound contrast agents), the signal processing of these agents and the contrast-specific imaging modality.

One of the main objectives of this method is to gather vascular information (from large vessels to capillaries) in different organs or tumors by increasing the signal intensity from blood.

The **aim** of this thesis is to evaluate the kinetic value of real-time harmonic imaging contrast-enhanced ultrasound (CEUS) in breast tumors as a differential diagnostic tool and correlate it with prognostic factors in invasive breast carcinomas.

Successful introduction of a new imaging method always requires studies evaluating the technical parameters for optimal diagnostic performance. Concerning CEUS for imaging breast tumors, the mode of injection and dose of injected agent can be identified among these parameters. Therefore two methodology studies are included in this work (Studies I and III).

In **Study I** we evaluated which method is to prefer between bolus and continuous infusion of equal doses of contrast agent, in order to achieve the best technique to gather vascular information of breast tumors. We observed that the qualitative assessment of the curves after bolus administration of contrast agent provided sharply demarcated tumors with clear visible wash-in and wash-out patterns in all cases. The continuous infusion with the same doses of contrast agent was not able to show a clear wash-in/wash-out in any case.

In **Study II** we investigated whether kinetic parameters of real time harmonic CEUS imaging could be used to differentiate between benign and malignant breast tumors.

The study showed that real time harmonic contrast enhanced ultrasound as a kinetic tool in the breast can detect significant differences between benign and malignant tumors, when evaluating two main parameters: time-to-peak and wash-out ratio. Both parameters have been observed to be earlier/faster in malignancies when compared with benign tumors. We observed the fact that after 21 seconds the malignant tumors tends to eliminate more than 50% of the total amount of contrast, while the benign tumors tends to eliminates less than 50 % (mean values are 69% respective 36% elimination of contrast at 21 seconds).

In **Study III** we compared different doses of injected contrast agent in order to establish an optimal dose for the diagnosis of invasive breast cancer using real-time harmonic CEUS. The results were, in terms of diagnostic features, that the optimal way to evaluate kinetic features of invasive breast tumors using real-time contrast-enhanced ultrasound harmonic imaging was with a bolus injection of contrast agent of either 2.4 mL or 4.8 mL.

In **Study IV** we correlated real time harmonic CEUS kinetic parameters with traditional and molecular prognostic factors in invasive breast cancer. The results were that invasive breast carcinomas exhibiting earlier peak enhancement and fast elimination of microbubbles contrast agent at contrast-enhanced ultrasound are associated with established predictors of poor prognosis.

In **conclusion** this thesis demonstrates that kinetic parameters of real-time harmonic CEUS with a bolus injection of either 2.4 or 4.8 mL contrast agent have potential for diagnosis, differential diagnosis and prognosis of invasive breast cancer tumors.

LIST OF PUBLICATIONS

This thesis is based on the following four original articles:

- I. **Bolus compared with continuous infusion of microbubbles contrast agent using real-time contrast harmonic imaging ultrasound in breast tumors.**
A Saracco MD, P Aspelin MD PhD, K Leifland MD PhD, R Themudo MD, B Wilczek MD PhD, R Axelsson MD PhD
Acta Radiologica, 2009 Oct; 50(8):854-9.

- II. **Differentiation between benign and malignant breast tumors using kinetic features of real-time harmonic contrast-enhanced ultrasound.**
A Saracco MD, B.K. Szabó MD PhD, P. Aspelin MD PhD, K. Leifland MD PhD, B. Wilczek MD PhD, F. Celebioglu MD PhD R. Axelsson MD PhD
Acta Radiologica, 2012 Feb; 53:382-388.

- III. **Contrast-enhanced ultrasound using real-time contrast harmonic imaging in invasive breast cancer: Comparison of enhancement dynamics with three different doses of contrast agent.**
A Saracco MD, B.K. Szabó MD PhD, P. Aspelin MD PhD, K. Leifland MD, E.Tánczos MSC, B. Wilczek MD PhD, Axelsson MD PhD
(Accepted in Acta Radiologica, October 2013).

- IV. **Correlation of contrast-enhanced ultrasound kinetics with prognostic factors in invasive breast cancer.**
B.K. Szabó MD PhD, A. Saracco MD, E. Tánczos MSC, P. Aspelin MD PhD, K. Leifland MD PhD, B. Wilczek MD PhD, R. Axelsson MD PhD
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LIST OF ABBREVIATIONS

ACR	American College of Radiology
ADH	Atypical Ductal Hyperplasia
ASCO-CAP	American Society of Clinical Oncology/College of American Pathologist
AUC	Area Under the Curve
BI-RADS	Breast Imaging-Reporting and Data System
BRCA 1-2	Breast Cancer Susceptibility Gene 1-2
CBE	Clinical Breast Examination
CC	Craneo-Caudal
c-erbB-2	Human Epidermal Grow Factor Receptor 2
CEUS	Contrast-Enhanced Ultrasound
CHI	Contrast Harmonic Imaging
CTR	Contrast-to-Tissue Ratio
DCE	Dynamic Contrast Enhanced
DCIS	Ductal Carcinoma In-Situ
ER	Estrogen Receptor
FISH	Fluorescent In-Situ Hybridization
FNAB	Fine Needle Aspiration Biopsy
Her2/neu	Human Epidermal Grow Factor Receptor 2
HRT	Hormone Replacement Therapy
IBC	Invasive Breast Cancer
IHC	Immunohistochemistry
Ki-67	Human Protein-Antigen Marker for Cell Proliferation
LM	Latero Medial
MI	Mechanical Index
MLO	Medio Lateral Oblique
MRI	Magnetic Resonance Imaging
MTT	Mean Transit Time
MVD	Microvessel Density
NHG	Nottingham Histology Grade System
NPV	Negative Predictive Value
PR	Progesterone Receptor
ROI	Region Of Interest
TGC	Time Gain Control
TNM	Tumor, Nodes, Metastases
US	Ultrasound/Ultrasonography
VAB	Vacuum-Assisted Biopsy
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

1 GENERAL INTRODUCTION

1.1 BACKGROUND

[...] It was in the time of Hippocrates, around 400 B.C. that the word for cancer first appeared in the medical literature: *karkinos*, from the Greek word for ‘crab’. The tumor, with its clutch of swollen blood vessels around it, reminded Hippocrates of a crab dug in the sand with its legs spread in a circle. The image was peculiar [...] but also vivid.

“The Emperor of all Maladies, a biography of cancer” by Siddhartha Mukherjee.

Breast Cancer is a heterogeneous and progressive disease with a potentiality of spreading locally and to other organs.

It is the leading cancer amongst women in developed industrialized countries, both in incidence and mortality (1). Almost 1.4 million new cases worldwide were reported in 2008 representing around 23% of all cancer in women and around 458,000 estimated deaths (2)(Fig. 1).

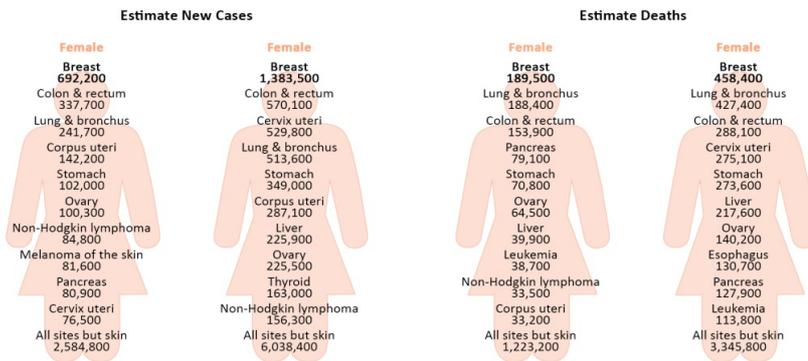


Fig.1. Women’s estimated new cancer cases and deaths for leading cancer sites, by level of economic development and worldwide respectively (Adapted from Global Cancer, Facts and Figures, 2nd Edition, Globocan 2008).

In Sweden this disease represents around 30% of all the cancer cases in women in a year, affecting them in 1 to 2 out of 10 during their life time and with an increase rate in incidence in the last 20 years of 1.4 % per year (3). According to the Swedish National Breast Cancer Registrar there were 8,363 new breast cancer cases reported in the whole country during 2011(compared with 7,659 in 2008). Since survival from this disease depends mainly on removal of the neoplasm before it spreads, early diagnosis and an adequate treatment are likely to be the key for success (4, 5).

The last report from The Swedish Two-County Trial published by Tabár et al. in 2011 (6), showed a 30 % reduction in breast cancer mortality from screening mammography alone, among women 40 to 74 year-old, during 3 decades control time. Bock et al. in 2013 (7) referred in a correspondence article that for women with breast cancer, early detection also results in improved quality of life from less extensive surgical treatment. Women with screen-detected breast cancer in the UK have half the mastectomy rate of women with symptomatic cancers-i.e., 27% versus 53%.

In spite of some controversies, there are many studies showing that the survival rates of this disease has been continuously increasing in the last 15 years (8-11). The main reasons are: the early detection done in well organized screening programs, public education and the improvement of the therapies (both neo and adjuvant therapy).

1.2 BREAST CANCER ETIOLOGY AND RISK FACTORS

The identification of the cause of breast cancer and a clear strategies to prevent this disease remains unsolved. Although breast cancer can be seen also in men (Sweden reported 33 male cases against almost 8000 cases in the female population during 2010), it is rare among that population, therefore female gender is still the strongest risk factor for breast cancer.

Epidemiological observations done by Gail et al. in 1989 (12), Claus et al. in 1993(13), Ford et al. in 1994 (14), Tyrer et al. in 2004 (15), Tice et al. and Veronesi et al. in 2005 (16, 17) and Barlow et al. in 2006 (18) concluded in diverse established and suspected risk prediction factor/models for breast cancer from a hereditary and lifestyle point of view with different strengths of association:

- Early menarche, late menopause, null parity, high age at first partum (*the longer in time a woman has menstrual cycles, the higher her risk for breast cancer*)
- No breast feeding
- Age (*elderly individual; in Sweden with a rapidly increase from the age of 50 and the peak age between 70-74 years-old*)
- Geographical location (*developed countries*)
- Extensive dense breast tissue post-menopausal (*visualized on mammography*)
- Family history of cancer (*breast cancer in first-degree relative, ovarian cancer*)
- BRCA 1-2 (*presence of genes mutations*)
- Previous disorders of the breast (*ADH, LCIS*)
- Previous breast cancer
- Expose to ionizing radiation on the chest (*before age of 20*)
- HRT (*long-term use*)
- Oral contraceptives (*depot ones*)
- Diethylstilbestrol (*use during pregnancy*)
- Alcohol consumption (*intake*), obesity at mature/post-menopausal age

1.3 BREAST CANCER CLASSIFICATION AND PROGNOSTIC FACTORS

Breast cancer arises almost always from the epithelial component of the breast, and is therefore considered as adenocarcinoma.

1.3.1 Histopathological classification

From the histopathological point of view, breast cancer can be classified as: Invasive ductal 71%, Invasive lobular 10%, DCIS 7%, Invasive tubular 4%, Mucinous 3%, Medullary 3%, Papillary and others 2% (Fig. 2).

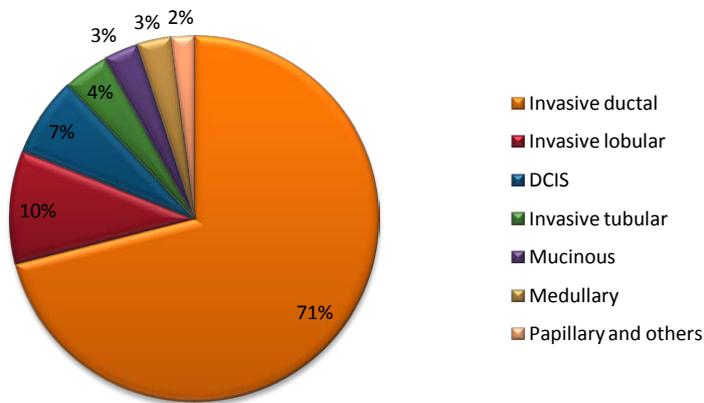


Fig.2. Histopathological classification of breast cancer (Adapted from: *Practical Breast Pathology* by Tot, Tabár and Dean.(Thieme 2002).

The difference between an in-situ and invasive carcinoma is whether the cancer is confined or not by normal basement membrane of the duct or lobuli. The transition from an “in-situ” to an “invasive” carcinoma is a key event in breast cancer progression that is still not well understood (19). The presence of tumor emboli in the vessels or vascular invasion (lymphatics or blood) highly correlates with involvement of regional lymph nodes which provides prognostic information (20).

1.3.2 Staging (TNM classification)

Breast cancer staging is based in three characteristics: size of the tumor (*T, done based on the single invasive largest foci or whether the cancer is non-invasive*)(21), regional lymph node status (*N, whether cancer is in the lymph nodes or not*) and whether the cancer has spread to other parts of the body, beyond the breast (*M, distant metastases*). It is general accepted that the most powerful predictor of the outcome in breast cancer is the lymph nodes status (22-25).

1.3.3 Histology grading

The histological grading of breast cancer has also become widely accepted as a powerful indicator of prognosis. The Nottingham Histology Grade System (*NHG*) introduced in the early '90s by Elston and Ellis is the most used classification for breast cancer histology. It is a scoring system from I to III (less to more aggressive) given according to the tubule formations, mitotic activity and cells size/uniformity (26).

1.3.4 Hormone receptors

Another way to classify an invasive breast carcinoma (primary tumor or recurrences) is based on two hormonal receptor status obtained by immunohistochemistry (*IHC*) which predicts whether the disease will respond to endocrine therapy: *Estrogen (ER)* and *Progesterone (PR)*. Assessment of ER status is primarily performed to predict clinical outcome and in particular responsiveness to endocrine therapy (27) while PR expression is indicative of ER function and the absence of PR is associated with tamoxifen resistance and overexpression of Her2/neu (28). It is now recommended that ER and PR assays be considered positive if there is at least 1% positive tumor nuclei in the sample tested, and tumors with at least those rates may also benefit from endocrine therapy (29).

1.3.5 Her2/neu (c-erbB-2)

The human epidermal growth factor receptor 2 (*Her2/neu* or *c-erbB-2*) is a gene that is over expressed in 20-30 % of all invasive breast carcinomas which mediates growth, differentiation and survival of the tumor cells. It is routinely measure because its predictive value for response to Herceptin (*trastuzumab*) (30). The over expression of Her2/neu has a negative impact respect to time-free from disease and survival (31).

1.3.6 New subtypes classification

Lately a molecular subtype classification of breast cancer (*or Sørli and Perou subtypes-classification*) which subdivided the tumors according to a global gene expression profile has been used for prognosis and therapeutic purposes (32-34):

- Luminal A (mostly ER positive with low proliferation rate and grade tumors)
- Luminal B (mostly ER positive with high proliferation rate and grade tumors)
- Basal-like subtype (triple-negative tumors: ER, PR and Her2/neu negative)
- Her2/neu positive (over expressed)
- Normal breast-like subtype (*still unclear if it is a subtype or a poorly sampled tissue*)

2 BREAST DISEASE DIAGNOSIS

The so called “triple assesment” or “triple diagnosis” of a patient with a breast disease (both asymptomatic and symptomatic) still remains widely accepted as the first management steps in order to confront the disease.

This assesments combine the interaction of: 1) clinical breast examination (CBE), including axillas and areas related, 2) breast imaging diagnostic methods (mammography, ultrasound and magnetic resonance imaging of the breast), and 3) percutaneous biopsy (fine-needle aspiration biopsy or FNAB, core biopsy, vacuum-assisted biopsy or VAB).

2.1 BREAST IMAGING DIAGNOSTIC METHODS

2.1.1 Mammography

Digital x-ray mammography is still the first chosen imaging method for breast cancer diagnosis, which can be divided into screening and clinical mammography, depending if the woman is asymptomatic or not (clinical mammography even solve the abnormalities found at screening mammography).

Screening mammography was introduced in Sweden in 1986 and finally implemented in the whole country as a national program in 1997. The interval period between screening examination are every 18 month from 40 to 49 years-old and every 24 months from 50 to 74 years-old (35), with an attendance rate of aproximatly 72% in the invited group (36). The rates of sensitivity for breast cancer detection in screening mammography varies between 74-95% and their specificity between 89-99% (37). However, mammography alone can not detect all the malignancies (38).

2.1.2 Ultrasound

Ultrasound or ultrasonography (US) of the breast is the most used method as a complement to clinical mammography (39); specially in those patients with radiographically dense breasts, where mammography is unable to detect abnormalities in many occasions. The combination of this two imaging methods increase the detection rate of breast abnormalities, both in specificity and sensitivity (40). This means that in many cases ultrasound is the first method of choice for undetermined clinical and/or mammographic findings (41, 42).

Berg et al. (43) described in 2008 that adding a single screening ultrasound to mammography will yield an additional 1.1 to 7.2 cancers per 1000 in a group of women with elevate risk of getting the disease. Unfortunately this study also yielded to an increased number of false positive biopsies. On the other hand, Stavros described a Negative Predictive Value (NPV) in excess of 99% when mammography is negative and ultrasound shows normal tissue (ACR BI-RADS 1) to cause palpable abnormalities

(44). Ultrasound (2D B-mode), as a single method, is proved not to be a screening tool in the detection of breast cancer (45).

Local staging of breast cancer when talking about the regionally lymph nodes status (axilla, internal mammary chain area, fossa infra-clavicularis and supra-clavicularis areas) is one of the most clinically relevant use of the ultrasound, since lymph node status is consider the single most important prognostic factor (46).

In addition, ultrasound has become increasingly popular for guiding percutaneous interventional procedures (FNAB, core biopsy, VAB) in the breast and areas related, pre-operative localization of lesions, drainage of abscess, seromas and cysts (47, 48).

2.1.3 Magnetic resonance imaging

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) as well as non-contrast MRI has emerged in the last decades as the most sensitive complementary tool for the diagnosis of breast cancer.

Due to its relative high sensitivity (94-100% for invasive breast carcinoma and 50-80% for carcinoma in-situ) but low specificity, integrating both morphologic and dynamic criteria (65-79%) (49-52) the indication of breast MRI are limited to:

- Assessment before breast conservation surgery, where breast MRI shows a higher sensitivity than any other imaging method in detecting multiple malignant invasive foci (ductal and lobular type), ipsilateral and contralateral, in breasts with scattered or heterogeneous and extremely dense fibroglandular tissue (53, 54).
- Annual screening of patients with risk of hereditary breast cancer, which includes: BRCA mutations, first-degree relative of BRCA carrier but untested, lifetime risk 20-25% or greater dependent on family history, radiation to chest between age 10-30 years, Li-Fraumeni syndrome and first degree relatives Cowden and Bannayan-Riley-Ruvalcaba syndrome and first degree relatives (55).
- Detection of unknown primary tumor in the breast, where metastases are diagnosed but a primary tumor site cannot be identified (CUP syndrome or Carcinoma Unknown Primary syndrome) (56, 57).
- Evaluation of the response in neoadjuvant chemotherapy setting (58, 59).
- Evaluation of breast implants integrity (60).

3 CONTRAST-ENHANCED ULTRASOUND (CEUS)

“The challenge presented to ultrasound imaging technology by the advent of microbubble contrast agents is to create a method that detects the echoes from bubbles in preference to the echoes from tissue.”

Contrast-enhanced ultrasound or CEUS is a combination of the pharmacokinetics of contrast agents (or microbubbles-based ultrasound contrast agents), the signal processing of these agents and the contrast-specific imaging modalities. One of the main objectives of this method is to gather vascular information (from large vessels to capillaries) in different organs or tumors by increasing the signal intensity from blood.

The phenomena of microbubbles as a “contrast agent” was first describe by Gramiak and Shah in 1968 (61) when the microbubbles effect was obtained in the aorta as an increase of the backscattering of blood during cardiac catheterization followed by an injection of saline solution. Small cavitation bubbles that were formed due to the injection of saline solution produced the enhanced echoes phenomena. Other authors like Bove and Ziskin in 1969 (62) and Kremakau et al. in 1970 (63) described the same type of phenomena. From that time on, the efforts of developing a clinical and relevant use of microbubbles-based ultrasound contrast agents in diverse organs were enormous.

While Doppler methods (color and power) has been used to assess vascularity and blood flow, it can only demonstrate vessels larger than $\geq 200\mu\text{m}$ in diameter , with signals above the noise level and with flow velocity higher than the tissue motion. On the other hand, Contrast-Enhanced Ultrasound consists of a pool of small bubbles (microbubbles), free flowing in the blood stream, generating enhanced echoes which are detected by contrast-specific imaging modalities. This provides the information from the microvasculature, in the order of 5-10 μm in diameter (capillary system), with a flow velocity inferior than the tissue motion (1mm/sec).

3.1 THE CONTRAST AGENT (MICROBUBBLES)

“Contrast agent differs fundamentally from tissue”

The commercial development of contrast agent for ultrasonography began in the 80’s by different laboratories around the world. This contrast agent consisted of small air or gas filled micro-spheres with sizes like the normal erythrocytes (7-8 μm) or even less; one of the important key role for their transcapillary stability.

Microbubbles contrast agent is prepared under controlled conditions outside the body and injected though a vein where they are taken up into the blood stream and transported to the region under investigation (64).

A second generation contrast agent was used for the studies in this thesis, consisted of microbubbles containing an inert and hydrophobic gas (SF₆ sulphur hexafluoride) stabilized by a thin and flexible monolayer shell of phospholipids (Sono Vue® from Bracco, Milano, Italy) (65). These characteristics of the microbubbles slows the gas diffusion into the blood, increased stability/persistence in the bloodstream and resistance to external pressures which prevents the bubbles either to dissolve, burst or to coalesce to form larger bubbles (66).

By increasing the acoustic power of the sound field, the microbubbles start to oscillate in a certain way which is the primary source of the high scattering strength of the contrast agent (67, 68). This oscillation generates a signal with two special characteristics: (a) it contains a spectrum of frequencies other than the transmitted single frequency called “harmonics” (b) the oscillation is non-symmetrical or “non-linear” since expansion of the microbubbles is higher than the compression. The “non-linear” behavior of the microbubbles differs strongly from the “linear” signal from the tissue where compression is higher than expansion. This difference is called: contrast-to-tissue ratio (CTR).

The second generation microbubbles used nowadays range in a size between 1-10µm with a mean size of 2.5µm and 95% of them smaller than 5µm. Its physicochemical characteristics (soft flexible shell and high stability) make possible the oscillation even a very low MI imaging (mechanical index ≤ 0.1), allowing continuous real time assessment of the whole enhancement period using bolus injection or continuous infusion. As a blood pool agent (distributed within the whole blood volume) the microbubbles do not diffuse into the interstitial space like the contrasts agents used in CT or MRI, making it a purely intravascular. It has persistence in time up to 20 minutes, both in macro and microvasculature, excellent toleration (low solubility in blood for the gaseous phase) and very few contraindications (severe cardio-respiratory disease). Microbubbles-based agents are isotonic to human plasma and are eliminated through the respiratory system (69).

3.2 THE CONTRAST-SPECIFIC IMAGING MODALITY

“Detection methods for contrast agent differs from imaging methods for tissue”

The success of CEUS depends very much on the ability to detect the presence of contrast agent; either in blood or tissue.

Initially, microbubbles-based contrast agents was used to increase the vascular signal at Doppler ultrasonography, accomplished by increasing the vascular signal and improving the color and power Doppler US assessment or just to detect the presence of contrast agent in order, for instance, to delineate the left ventricle (70, 71). Nowadays, both clinical use and detection methods had developed.

3.2.1 Harmonic Imaging

Harmonic Imaging (or real time contrast harmonic imaging) is a protocol that relies on information from harmonic echoes, but not from the fundamental echo. To exploit the

high level of harmonics reflected by a microbubble-base agent, the echo signal is filtered with a band-pass filter to extract the second harmonic from the non-linear response. This modality in which the ultrasound receiver is tuned to detect preferentially echoes that are multiples or submultiples of the frequency of the emitted sound, has been shown to provide a substantial increase in the conspicuity of bubbles when present in high dilution in tissue. Using the second harmonic, a much larger difference between tissue echo levels and contrast agent echo level is found than when using the fundamental echo. Compared to the CTR at the fundamental, harmonic imaging will significantly improve the CTR (72). The result is that harmonic imaging removes artefacts from the normal tissue so that the contrast is resolved from the background.

3.2.2 Pulse Inversion

Pulse Inversion is a two pulse technique in which a regular broadband ultrasound pulse and a phase inverted copy of this pulse are alternately sent into the medium. Once these pulse is sent and due to the existence of a non-linear effect in propagation of the pulse through the medium and as a response from the contrast agent, there is a suppression of the odd harmonics in the response (based on cancellation of the odd function) and hence improve the CTR at the second harmonic (part of the frequencies that were not present in the excitation signal are suppressed in favor of frequencies that show better discrimination between contrast and tissue) (73).

3.2.3 Mechanical Index

Mechanical Index (MI) is the power output (insonation power) being deposit or transmitted in a structure; in the CEUS case, to the contrast agent. The MI should not be confused with the gain or time gain control (TGC) which simply control the setting of the receive amplifier and do not change the transmitted power (74).

3.3 THE KINETIC PARAMETERS

“Kinetic: from the Greek ‘Kineticós’: pertaining to or cause by motion...”

The uptake and elimination of the contrast agent in neofomed pathologic vasculature show different kinetic characteristic when compared benign with a malignant tumor. This is due to vascular properties like: vascular density, permeability, presence or not of shunts.

In general the neovascularization in malignant tumors is prone to be highly permeable causing rapid uptake and elimination of contrast agents while benign tumors show gently enhancement and elimination kinetics. Nevertheless there is still an overlap in the kinetic criteria between benign and malignant foci, probably related to the heterogeneity of breast tumors (75).

The kinetic parameters used for our studies were:

- **Wash-in (initial slope)** defines the upslope of the kinetic curve to peak signal intensity.
- **Peak intensity signal (SI_{peak})** is the point of maximum signal intensity at certain time.
- **Time-to-peak intensity signal (T_{peak})** is the time elapsed between the first appearance of contrast agent and maximum signal intensity value of the kinetic curve.
- **Wash-out** is an established quantitative parameter that is used to describe the down-slope of kinetic curves. It gives the relative signal intensity decrease from the peak to the endpoint of the curve.
- **Mean transit time (MTT)** is the average time the contrast agent at a given dose spends in the tumor vessels.

The curve analyses performed in our studies were both **Qualitative** in study I (visual assessment concerning the shape of the curve) and **Quantitative** in studies II to IV (contrast agent concentration as a function of time).

4 AIMS OF THE THESIS

The general aim of this thesis was to evaluate the kinetic value of real-time harmonic imaging Contrast-Enhanced Ultrasound (CEUS) in breast tumors as a differential diagnostic tool and correlated it with prognostic factors in invasive breast carcinomas. Since there are no clear guidelines concerning the optimal technical investigation procedures of CEUS in the breast, the secondary aim of this thesis was to investigate the optimal methodological approach.

The aims by study were:

- I. To evaluate which method is to prefer between bolus and continuous infusion of equal doses of contrast agent, in order to achieve the best technique to gather vascular information of breast tumors.

- II. To investigate whether kinetic parameters of real-time harmonic CEUS imaging could be used to differentiate between benign and malignant breast tumors.

- III. To compare different doses of injected contrast agent in order to establish an optimal dose for the diagnosis of invasive breast cancer using real-time harmonic CEUS.

- IV. To correlate real time harmonic CEUS kinetic parameters with traditional and molecular prognostic factors in invasive breast cancer.

5 MATERIAL AND METHODS

Participants

An approval of the Regional Ethics Committee was obtained for the next following four studies (2005/448-3173). All patients were informed about each study procedure and the contraindications of the method, and gave their oral consent.

All participating patients in these studies were evaluated between March 2007 and September 2009.

The inclusion criteria for all four studies were age of 18 years or more, with palpable/non-palpable lesion/s in the breast or axilla detected primarily either with mammography, ultrasound or both.

The exclusion criteria in all four studies were pregnancy or lactation, known allergy to sulphur hexafluoride (contrast agent) and severe cardio-pulmonary disease.

Investigation procedures common for all four studies

All mammography images were performed with analogue equipment (Siemens Mammomat 3000 Nova - Erlangen, Germany; Planmed Sophie - Helsinki, Finland and GE Senographe 800T – Milwaukee Wisconsin., USA) using the standard three views (CC at 0°, MLO at 45°, LM at 90°) and some additional when needed (spot compression, magnification or supplementary views). The fundamental ultrasonography examination in B-mode in the studies were performed with a Philips iU22 (Philips Medical Systems - Eindhoven, The Netherlands) using a high resolution multifrequency linear probe L17-5 MHz and special modalities SonoCT (real-time compound imaging scanning, crossbeam scanning) and XRES (speckle, noise and clutter reduction imaging). Scanning with power Doppler were performed in all tumors as a standard procedure, with the intention of getting an adequate and optimal detection of vascularity that then was used as the scan plane for real time CHI ultrasound.

All CEUS were performed with the real time grey-scale contrast harmonic imaging (CHI and fundamental imaging side by side, except in Study I) with the same equipment (Philips iU22 , software Vision 2005-2009) using a multifrequency L9-3 MHz linear probe, especially design for this purpose.

A low mechanical index was used for our studies and did not exceed the value of 0.07 (values were always between 0.06 and 0.07). This provided sufficient tissue cancellation (disappearance of the B-mode parenchyma) with maintenance of adequate depth penetration. Also made the microbubbles oscillate in a non-linear way, without bursting them.

Evaluation of images common for all four studies

All CEUS studies were saved in the ultrasound hard disc system (native data) and then transferred to a PC for further post-processing images quantitative analyses with an advanced ultrasound quantification software (Q LAB 5.0-7.0™, Philips Medical Systems - Seattle WA, USA) in order to acquire the kinetic curves for all the studies. This computer-assisted program, with the help of a “whole tumor ROI” delineating every tumor, allowed acquisition of time (sec) / signal intensity (db) curves. In our studies, kinetic curves acquired in the first 50 seconds after the appearance of contrast, were used for statistical analysis.

In addition, the wash-in and wash-out patterns of the contrast agent were evaluated by a qualitative (Study I)/quantitative (Studies II-IV) assessment of the images. Those prospective image analyses were performed by two experienced radiologist: Ariel Saracco (Studies I-IV) and Botond Szabó (Studies II - IV).

Statistical analyses for the studies

- *For Study I:*

Statistical significance was established at a P-value of <0.05. Pair sample test was used.

- *For Studies II to IV:*

For all 3 studies statistical significance was established at a P-value of <0.05, whereas “trend” towards statistical significance was defined as P-value of >0.05 and <0.1 for all studies.

In Study II, statistical analysis of standardized time-signal intensity curves and to determine which of the kinetic parameters that were significantly different between the benign and malignant groups was made with the non-parametric Wilcoxon rank-sum test for independent samples (the test is equivalent to the Mann-Whitney U-test).

In Study III, each conventional and model-based kinetic parameters measured within the same lesion at different doses (at the dose of 1.2 and 2.4 mL in Group A, and also at the dose of 2.4 and 4.8 mL in Group B) were statistically correlated using a non-parametric test (Spearman rank correlation). The assumption was that the greater the similarity between time-intensity curves obtained at different doses, the stronger the correlation with kinetic parameters will be. Due to the number of statistical tests performed on the same dataset, significance level (α) was adjusted with the Bonferroni method to avoid Type I errors.

In Study IV Spearman’s rank correlation test was performed to identify relationships between continuous and ordinal variables. Pearson’s correlation was used for the comparison of two interval variables. One-way analysis of variance was applied to correlate nominal data with continuous variables. Dichotomous prognostic factors were

correlated with kinetic parameters using the Wilcoxon rank-sum test (which is equivalent to Mann-Whitney U test).

For all 3 studies the software package Mathematica[®], version 8 (Wolfram Research Inc., Champaign, IL, USA) was used for curve fitting. Data extraction, calculation of parameters and statistical analysis was performed using the R statistical language and environment for Linux (version 2.10.1).

5.1 STUDY I

5.1.1 Material

A total of 29 patients (29 women; mean age 54 year-old; range: 24-73) with 29 palpable or non-palpable lesions/ findings in the breast or axilla were evaluated for this first study. Eighteen lesions were scored like clear malignant as ACR BI-RAD 5 and eleven like benign/probably benign as ACR BI-RADS 2-3 either with mammography, ultrasound or both. Fine needle aspiration biopsy or core biopsy were obtained from all findings confirming the categorical scoring given before:

- ACR BI-RADS 5 (15 invasive ductal carcinomas, 2 invasive tubular carcinomas and 1 invasive lobular carcinoma)
- ACR BI-RADS 2-3 (6 fibroadenomas, 1 intramammary lymph node, 1 axillary lymph node and 3 benign non-typical fibroadenoma).

All 18 malignancies and some of the benign lesions (6 fibroadenomas) were additionally correlated with the histopathology of the surgical specimen. The size of the 29 lesions/findings varied between 7 and 43 mm (mean 25); the 18 scored ACR BI-RADS 5 with sizes between 7 and 23 mm (mean 15) and the 11 scored ACR BI-RADS 2-3 with sizes between 7 and 43 mm (mean 36) (Table 1).

	BI-RADS 5	BI-RADS 2,3	Mean size (mm)	Mean age (y)
IDC	15			
ILC	1		15 (7-23)	
ITC	2			54 (24-73)
FA		6		
IMLN		1	36 (7-43)	
ALN		1		
BNFA		3		

Table 1. IDC (invasive ductal carcinoma), ILC (invasive lobular carcinoma), ITC (invasive tubular carcinoma), FA (fibroadenoma), IMLN (intra mammary lymph node), ALN (axillary lymph node), BNFA (benign non-typical fibroadenoma)

5.1.2 Methods and investigation procedures

Second generation MB (microbubbles) contrast agent (Sono Vue[®], Bracco – Milano, Italy) was reconstituted by the addition of 5mL sterile saline solution and then injected through a 18G catheter and a three-way connector via the antecubital vein. The first

injection was performed as a continuous infusion of 2.4 mL of MB during 60 seconds (0.04 mL/sec) with an automatic assistant pump. The examination was recorded as a video clip from the start of the infusion and for 120 seconds. After 10 minutes, a second injection of 2.4 mL of MB was administrated manually as a bolus in 2 seconds followed by a flush of 10cc saline solution. The examination was also recorded from the start of the bolus and for a 120 seconds period. During the contrast harmonic imaging, a low dynamic MI of 0.06-0.07 was used in both cases, as well as a scanning with minimal compression of the breast, to avoid bursting the MB. The dose of 2.4 ml contrast agent was used as recommended in the literature.

5.2 STUDY II

5.2.1 Material

A total of 95 patients (95 women; mean age 55 year-old; range: 29-76) with 96 palpable or non-palpable lesions/ findings in the breast or axilla were evaluated for this second study. Seventy five lesions were scored like clear malignant as ACR BI-RAD 5 and twenty one like benign/probably benign as ACR BI-RADS 2-3 either with mammography, ultrasound or both. Fine needle aspiration biopsy or core biopsy were obtained from all findings confirming the categorical scoring given before:

- ACR BI-RADS 5 (57 invasive ductal carcinomas, 4 invasive tubular carcinomas, 12 invasive lobular carcinomas, 1 invasive mucinous carcinoma and 1 invasive papillary carcinoma)
- ACR BI-RADS 2-3 (11 fibroadenomas, 1 intramammary lymph node, 1 axillary lymph node and 8 benign non-typical fibroadenoma).

All 75 malignancies and some of the benign lesions (8 fibroadenomas) were additionally correlated with the histopathology of the surgical specimen. The size of the 96 lesions/findings varied between 4 and 48 mm (mean 18.60); the 75 scored ACR BI-RADS 5 with sizes between 4 and 48 mm (mean 18.33) and the 21 scored ACR BI-RADS 2-3 with sizes between 7 and 43 mm (mean 18.76) (Table 2).

	(75) BI-RADS 5	(21) BI-RADS 2,3	Mean size (mm)	Mean age (y)
IDC	57			
ILC	12			
ITC	4		18.33 (4-48)	
IMC	1			
IPC	1			55 (29-76)
FA		11		
IMLN		1	18.76 (7-43)	
ALN		1		
BNFA		8		

Table 2. IDC (invasive ductal carcinoma), ILC (invasive lobular carcinoma), ITC (invasive tubular carcinoma), IMC (invasive mucinous carcinoma), IPC (invasive papillary carcinoma) FA (fibroadenoma), IMLN (intra mammary lymph node), ALN (axillary lymph node), BNFA (benign non-typical fibroadenoma)

5.2.2 Methods and investigation procedures

The contrast agent was reconstituted as in Study 1. An injection of 2.4 mL of MB through a 21G catheter and a three-way connector via the antecubital vein was administrated manually as a bolus in 2 seconds followed by a flush of 10cc saline solution. The examination was recorded from the start of the bolus and for a 120 seconds period. A low MI of 0.06 was used in all cases as Study 1 and for the same purpose.

5.3 STUDY III

5.3.1 Material

A total of 51 patients (51 women; mean age 58.94 year-old; range: 35-81) with 51 palpable or non-palpable lesions in the breast were evaluated for this their study. The study population was divided into two groups; Group A where we compared the bolus doses of 1.2 mL vs. 2.4 mL in the same patient (26 women with 26 lesions comprising 52 examinations; mean age 63.69 year-old; range 38-81) and Group B where we compared the bolus doses of 2.4 mL vs. 4.8 mL, also in the same patient (25 women with 25 lesions comprising 50 examinations; mean age 54 year old; range 35-71). All 51 lesions were scored like highly suspicious malignant as ACR BI-RADS 5 lesions, detected primarily either with mammography, ultrasound or both. Fine needle aspiration biopsy or core biopsy were obtained from all lesions confirming the categorical scoring given before:

- ACR BI-RAD 5: 42 invasive ductal carcinomas, 8 invasive lobular carcinomas and 1 invasive mucinous carcinoma.

The 26 lesions in Group A were: 22 invasive ductal carcinomas, 3 invasive lobular carcinomas and 1 invasive mucinous carcinoma. The 25 lesions in Group B were: 20 invasive ductal carcinomas and 5 invasive lobular carcinomas. All 51 malignancies were additionally correlated with the histopathology of the surgical specimen.

The size of the 51 lesions varied between 4 and 48 mm (mean 22.03). The 26 lesions in Group A were on a range between 4 and 48 mm (mean 17.11) and the 25 lesions in Groups B were on a range between 8 and 45 mm (mean 27.16) (Table 3).

	(51) BI-RADS 5	Mean size (mm)	Mean age (y)
Group A (26)			
IDC	22		
ILC	3	17.11 (4-48)	63.69 (38-81)
IMC	1		
Group B (25)			
IDC	20	27.16 (8-45)	54 (35-71)
ILC	5		

Table 3. IDC (invasive ductal carcinoma), ILC (invasive lobular carcinoma), IMC (invasive mucinous carcinoma)

5.3.2 Methods and investigation procedures

The contrast agent was reconstituted as in Study 1. The injection of MB was performed through a 21G catheter and a three-way connector via the antecubital vein.

In Group A, an injection of 1.2 mL of MB was first administrated manually as a bolus (2 seconds) followed by a flush of 10cc saline solution. The examination was recorded in a contrast side/side imaging mode loop from the start of the bolus and for a 120 seconds period. After 10 minutes and the corroboration of no contrast agent were neither in the lesion nor in the vessels after the bursting of the microbubbles, a second injection of 2.4 mL of MB was administrated manually as a bolus (2 seconds) followed by a flush of 10cc saline solution. The examination was also recorded in the same way as described above

In Group B, an injection of 2.4 mL of MB was first administrated manually as a bolus in 2 seconds followed by a flush of 10cc saline solution. Recording procedure did not differ from that in Group A. After 10 minutes and the corroboration of no contrast agent were neither in the lesion nor in the vessels after the bursting of the microbubbles, a second injection of 4.8 mL of MB was administrated manually as a bolus (2 seconds) followed by the same procedure as in Group A. A low MI of 0.06 was used in all cases as Study 1 and for the same purpose.

As mentioned before, in order to make a comparison between time-signal intensity curves at different contrast agent doses, conventional and model-based parameters were analyzed with a non-parametric correlation test (Spearman rank correlation). Due to the number of statistical tests performed on the same dataset, significance level (α) was adjusted with the Bonferroni method to avoid Type I errors.

5.4 STUDY IV

5.4.1 Material

A total of 74 patients (74 women; mean age 58 year-old; range: 37-81 years) with 75 breast tumours were evaluated for this study. All seventy five lesions were scored like clear malignant as ACR BI-RAD 5 either with mammography, ultrasound or both. Fine needle aspiration biopsy or core biopsy were obtained from all lesions confirming the categorical scoring given before:

- ACR BI-RADS 5 (57 invasive ductal carcinomas, 4 invasive tubular carcinomas, 12 invasive lobular carcinomas, 1 invasive mucinous carcinoma and 1 invasive papillary carcinoma).

The sizes of the 75 lesion varied between 4 and 45 mm (mean 18.36). Of the 75 lesions included, 35 were palpable and 40 were impalpable. Asymptomatic patients were recruited from screening mammography and patients with palpable lesions from symptomatic breast clinic. All patients underwent ultrasound examination of the axilla. Pathological axillary lymph nodes found on ultrasound were confirmed with imaging

guided FNAB. All patients with clinically positive nodal status underwent axillary lymph node dissection, whereas sentinel node biopsy was performed in patients with clinically negative axilla.

Histopathologic analysis of the 74 patients with 75 malignant lesions showed invasive ductal carcinoma not otherwise specified in 57 patients (76%), invasive lobular carcinoma in 12 (16%), invasive tubular carcinoma in 4 (5.3%), invasive mucinous carcinoma in 1 (1.3%) and invasive papillary carcinoma in 1 (1.3%). According to the Elston-Ellis grading system, there were 17 (22.6%) grade-I, 42 (56%) grade-II and 16 (21.3%) grade-III tumours. There were 7 (9.3%) ER negative, 21 (28%) PR negative, and 6 (8%) Her2/neu positive tumours.

Estrogen (ER) and progesterone receptor (PR) status of the tumours were classified as: tumours into 0=negative, 1+=low, 2+=intermediate and 3+=highly positive groups. In our institution the cut-off value of 1% was used, 0=defined as ER/PR negative – less than 1% positive invasive tumour nuclei in the sample tested with IHC. The categories 1+, 2+ and 3+ were all considered as positive (more or equal to 1% positive invasive tumour nuclei in the sample tested with IHC).

Her2/neu immunohistochemical staining was classified as 0=negative, 1+=low, 2+=intermediate or 3+=highly positive. Tumours with 2+ or 3+ staining were considered as Her2/neu positive, and tumours with 0 or 1+ were negative in this study. Fluorescent in-situ hybridization (FISH) was performed for samples with Her2/neu 2+ and 3+ staining (in 19 cases), which confirmed the amplification of Her2/neu-gene in 6 (31.5%) cases. Her2/neu receptor analysis was performed according to the American Society of Clinical Oncology/College of American Pathologists (ASCO-CAP) guidelines from 2010.

Of the 74 patients, ultrasound identified pathological lymph nodes in the ipsilateral axilla in 28 cases (the patient with two foci of cancer had negative axilla). Of these 28 cases, lymph nodes were palpable in 25 cases, and impalpable in 3 cases. The axilla was found to be negative on physical examination and ultrasound in 46 patients. Among these, sentinel node biopsy revealed 4 additional cases of axillary lymph node involvement. Altogether there were 32 malignant tumours with associated histologically proven axillary lymph node metastasis (Table 4).

	(75) BI-RADS 5	Mean size (mm)	Mean age (y)
IDC	57		
ILC	12		
ITC	4	18.36 (4-45)	58 (37-81)
IMC	1		
IPC	1		
Grade I,II,III	17/42/16		
ER (-)	7		
PR (-)	21		
Her2/neu (+)	6		
Ax. metastasis	32		

Table 4. IDC (invasive ductal carcinoma), ILC (invasive lobular carcinoma), ITC (invasive tubular carcinoma), IMC (invasive mucinous carcinoma), IPC (invasive papillary carcinoma), ER (estrogen receptor), PR (progesterone receptor)

5.4.2 Methods and investigation procedures

Done as in Study 2.

6 RESULTS AND DISCUSSION

6.1 STUDY I

The method of choice on how to administer the contrast agent varies depending on the organ to be studied. Infusion of microbubbles is used in echocardiography where large vascular structures and cardiac cavities are evaluated, while bolus administration is the preferred technique for other organs.

The evaluation of tumors, for example, need a pool of contrast in order to their vascular net be enhanced and studied (kinetic parameters).

In our study the qualitative assessment of the curves after bolus administration of contrast agent provided sharply demarcated tumors with clear visible wash-in and wash- out patterns, thanks to the administration of contrast agent as a pool.

The continuous infusion with the same doses of contrast agent was not able to show a clear wash-in/wash-out in all 29 cases (*Fig.3a, b and 4a, b*)

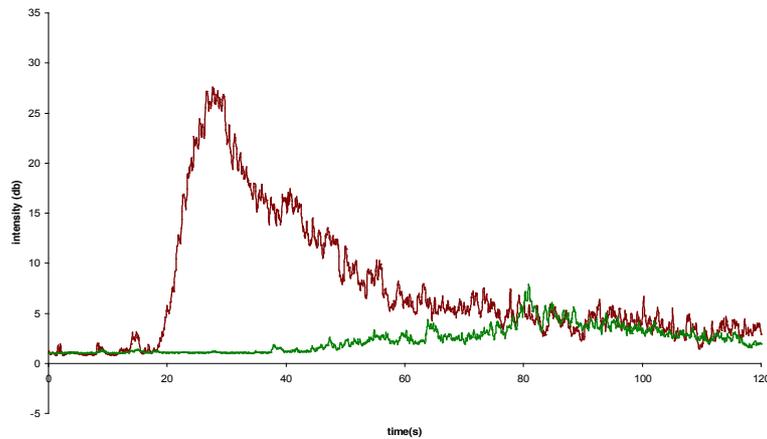


Fig.3a. Invasive carcinoma example; bolus (red curve) compared to continuous infusion administration of contrast agent (green curve).

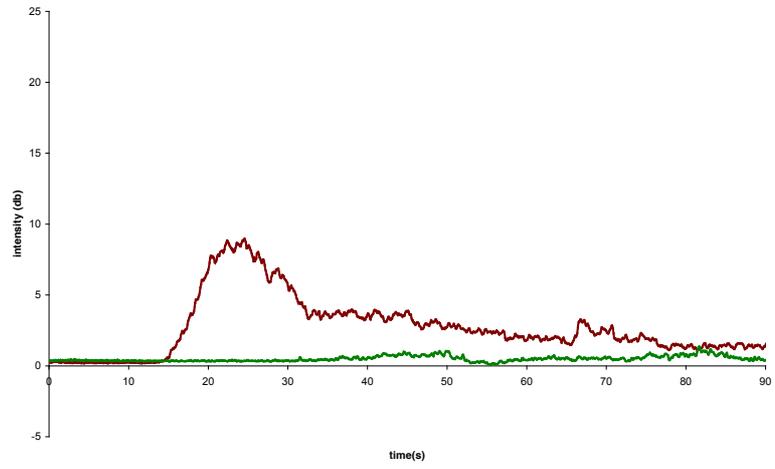
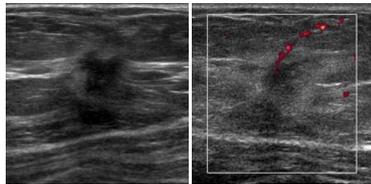
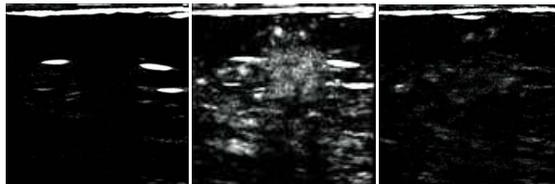


Fig.3b. Fibroadenoma example; bolus (red curve) compared to continuous infusion administration of contrast agent (green curve).

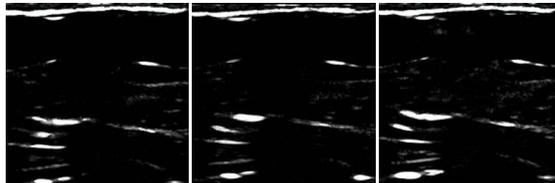
Invasive carcinoma B-mode/Doppler



CEUS

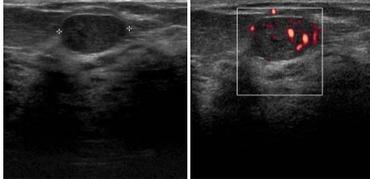


Bolus injection



Continuos Infusion

Fibroadenoma B-mode/ Doppler



CEUS

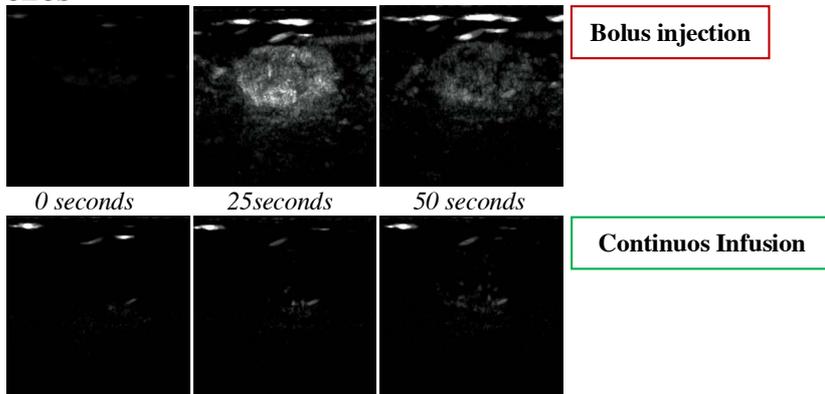
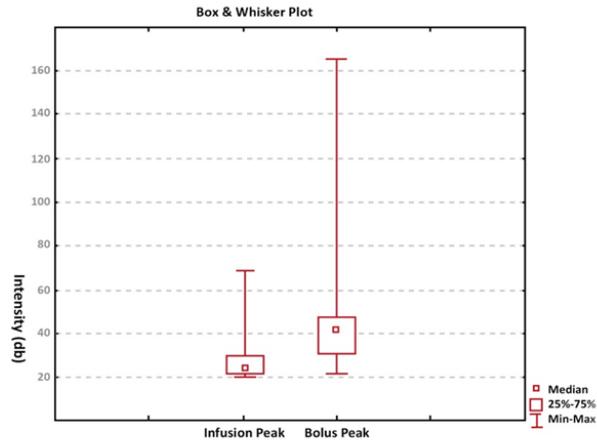


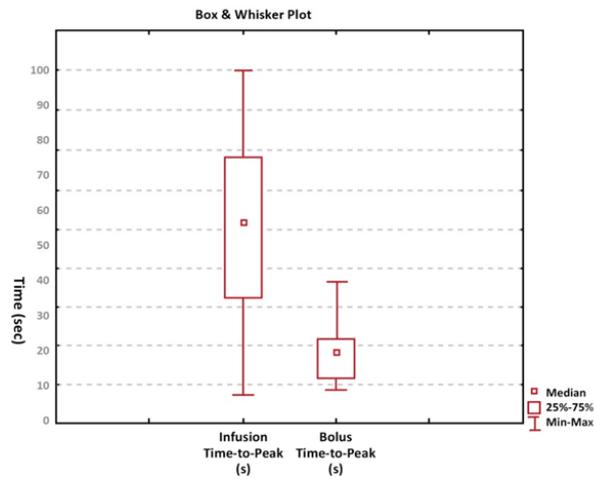
Fig.4 a, b. Invasive carcinoma and Fibroadenoma examples with CHI CEUS; comparison of bolus with continuous infusion at 0, 25 and 50 seconds.

The mean peak intensity signal was higher with bolus administration of the contrast agent (26.16 ± 28.26 db; mean \pm SD) than infusion (10.52 ± 13.48 db) (Pair sample test; $p < 0.001$) (Fig. 5a).

The time-to-peak intensity was shorter with bolus administration (17.64 ± 6.74 sec; mean \pm SD) than infusion (64.72 ± 17.45 sec) (pair sample test; $p < 0.001$) (Fig. 5b). Statistical significance was established at a P-value of < 0.05 in all cases.



a)



b)

Fig.5a, b. Box and Whisker plots showing differences in intensity peaks (db) and time to peaks (sec) between bolus and continuous infusion of contrast agent.

The results from this study demonstrate that bolus administration of MB as a pool of contrast agent provides shorter time to peak intensity and higher peak intensity values, as well as clear delineated wash-in and wash-out curves, which are important features in the characterization of the vascularity of tumors (neovascigenesis) for diagnostic and prognosis purposes (76, 77).

The time/intensity curves obtained with bolus administration of the contrast agent are more reliable for this purpose. The aim of studying tumors with contrast enhanced dynamic imaging, like CEUS, MRI or CT, is mainly to gather information about the wash-in and wash-out patterns of these lesions, in order to be able to further characterize them. In breast tumors, only bolus administration of contrast agent gave appropriate delineation of these two phenomena.

Both quantitative and qualitative evaluation of the examination revealed bolus injection as a preferable method of contrast agent administration while imaging breast tumors with CEUS.

6.2 STUDY II

Initial slope, peak signal intensity, time-to-peak and wash-out ratio values were calculated from the raw data of time - signal intensity curves, in all 96 cases. From the kinetic parameters a significant difference was found between the benign and malignant lesions in *time-to-peak* (Wilcoxon rank-sum statistic $W=422$, $p\text{-value}=0.00127$) and *wash-out ratio* W_{50} ($W=1309$, $p\text{-value}=0.000004$). The mean time-to-peak was 9.3 sec (SD=2.46) for malignant and 14.6 sec (SD=8.46) for benign lesions, respectively. The mean signal drop from the peak to the signal intensity measured at 50 sec was 85% (SD=0.09) for malignant, and 66% (SD=0.16) for benign lesion, respectively.

There was no statistically significant difference in the absolute values of *peak signal intensity* ($W=767$, $p\text{-value}=0.85$) and *initial slope* ($W=837$, $p\text{-value}=0.67$). The mean values and standard deviation (SD) for peak signal intensity were 20.06 (SD=22.82) for malignant and 29.53 (SD=38.53) for benign lesion and for the initial slope were 2.54 (SD=4.01) for malignant and 3.08 (SD=4.27) for benign lesions. The most significant difference between standardized benign and malignant curves was found at 21 sec ($p\text{-value}=5 \times 10^{-9}$, Fig 6), but statistical significance was reached in the range of 14-50 sec. Because of the significance of this time point, similarly to W_{50} , wash-out ratio was also determined at 21 seconds.

$$W_{50} = \frac{SI_{peak} - SI_{50}}{SI_{peak}} * 100 \quad W_{21} = \frac{SI_{peak} - SI_{21}}{SI_{peak}} * 100$$

Where W_{21} is the wash-out ratio, which shows the decrease from the peak (SI_{peak}) to the signal intensity measured at 21 seconds (SI_{21}). The statistical difference in W_{21} between benign and malignant groups was confirmed (Wilcoxon rank-sum statistic $W=1444$, $p\text{-value}=6 \times 10^{-9}$).

Figure 7 shows the ROC curves with AUC values for the wash-out ratios W_{21} and W_{50} . Statistical significance was established at a P-value of <0.05 in all cases.

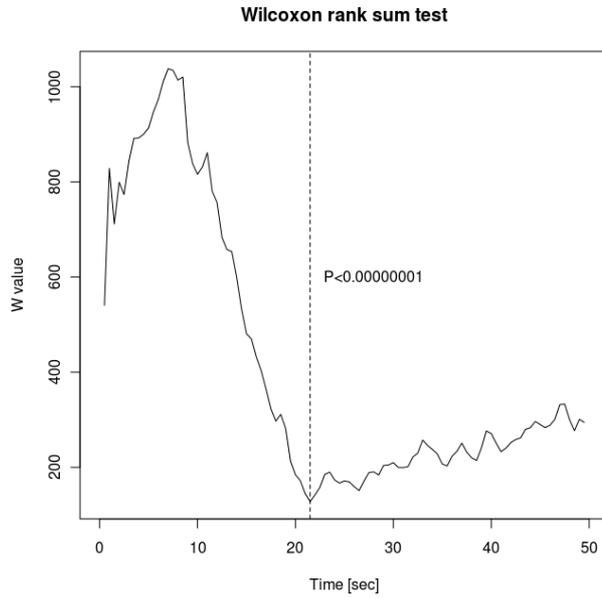


Fig.6. Comparison of benign and malignant time-signal intensity curves standardized to their peak values using Wilcoxon rank-sum test. W-statistic plotted against time of acquisition.

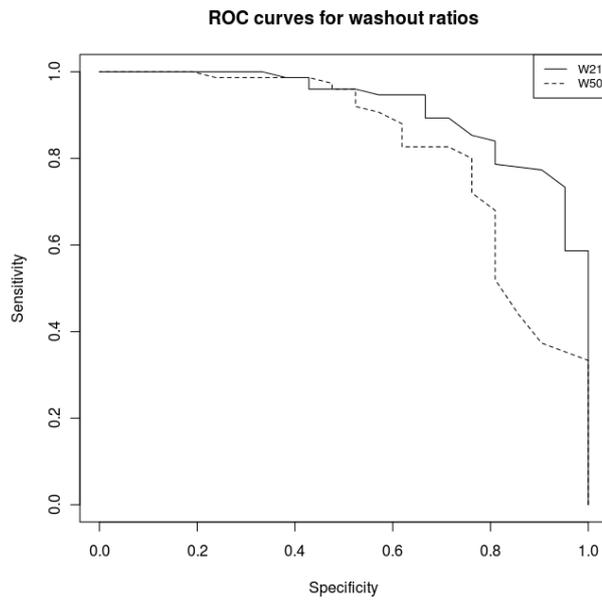


Fig.7. Receiver Operating Characteristic (ROC) curves for two different wash-out ratios W_{21} and W_{50} showing diagnostic accuracy in differentiation between benign and malignant breast tumors. The area under the curve (AUC) was 0.9171 for W_{21} and 0.8308 for W_{50} , respectively. Our results indicate that wash-out measured at 21 s has the highest diagnostic accuracy for the diagnosis of breast cancer with contrast-enhanced ultrasound.

This study has showed that Real time Harmonic Contrast Enhanced Ultrasound as a kinetic tool in the breast can detect significant differences between benign and malignant tumors, when evaluating two main parameters: time-to-peak and wash-out ratio. Both parameters have been observed to be earlier/faster in malignancies when compared with benign tumors. Balleyguier et al. (78) observed the same phenomena in a similar study, when they describe a faster wash-in and an immediate (early) wash-out after peak enhancement in malignant lesions. However, histology and/or cytology are still the most reliable methods to differentiate between malignant and benign lesions in breast tumor diagnosis. In spite of that, there is always need to develop other methods, less invasive but as reliable as the ones mentioned above.

During the breast tumors evaluation, regardless of their histology, it was found that CEUS and breast DCE-MRI have had certain similarities in the kinetics area (79, 80). These similarities are in two curve parameters: time-to peak and wash-out ratio. The analysis of enhancement kinetics is achieved by visually assessing the real time dynamic images and by measuring the signal intensity in a ROI of the lesion that yield the kinetic curve (time-signal intensity curve). When evaluating breast cancer (mass-like invasive tumors), both CEUS and DCE-MRI show that there is an earlier intense peak and faster elimination of the contrast media when compared with benign tumors.

With CEUS, malignant mass-like invasive breast tumors often show early strong contrast enhancement and wash-out phenomenon, in a visually chaotic pattern. This indicates rapid perfusion. On the other hand, slow increase of contrast uptake and wash-out, in a neater visual pattern, is clearly observed in benign lesions (81). In our kinetic study we analyzed the behavior of the contrast agent, in both malignant and benign tumors, only during 50 sec. The reason for this is that during the initially visual assessing of the procedure, it was a clear cut-off in both time-to –peak and in the elimination of contrast agent between malignant and benign tumors at early time. Zhao et al. had showed with CEUS that the enhancement velocity or “slope of enhancement” is one of the diagnostic criteria to differentiate cancer from benign lesions as the first ones exhibits faster enhancement (82). Many of the tumors in our study showed the same kinetic behavior (*Fig.8 and 9*).

A problem that we faced while analyzing these criteria was that even though the slope of enhancement was faster in cancer tumors, the differences between this group and benign tumors was not statistically strong enough. The reason for this might be a limitation of the method or physiological conditions while performing CEUS; some tumors, especially among the benign ones, experienced several peaks of high amount of contrast agent before reaching the peak-intensity (*Fig. 7*). This condition forced us to standardize some of the wash-in curves with the results described above. Maybe the most useful clinical tool we observed is the fact that after 21 seconds the malignant tumors tends to eliminate more than 50% of the total amount of contrast, while the benign tumors tends to eliminates less than 50 % (mean values are 69% respective 36% elimination of contrast at 21 seconds).

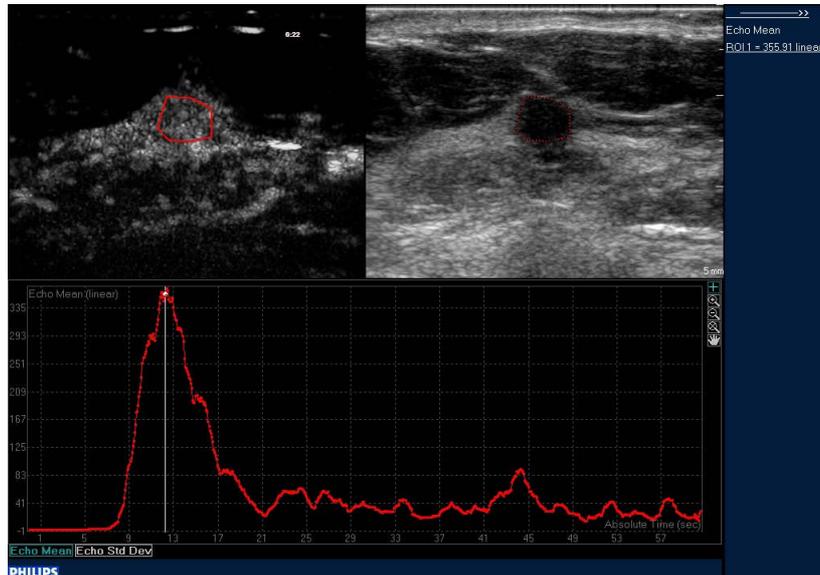


Fig.8. Contrast-enhanced ultrasound image of a 7 mm invasive ductal carcinoma, showing enhancement curve with early peak. Time-to-peak measured 12 seconds, wash-out ratio was 65% at 50 seconds (W50), and 57% at 21 seconds (W21).

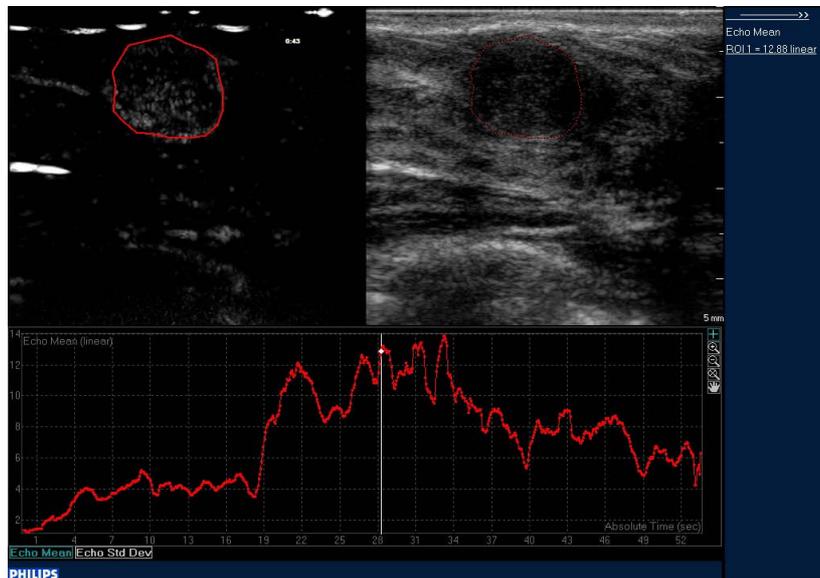


Fig.9. Contrast-enhanced ultrasound image of a 12 mm fibroadenoma, enhancement curve with delay peak and wash-out. The wash-out ratio is much less than 50% at 21seconds.

The pathophysiology behind the two observed curve parameters remains speculative but the faster enhancement/elimination of contrast media in cancer tumors strongly suggests the presence of a high number of arterio-venous shunts with rapid in/outflow (83-85). This speculative observation might be used, in the future, to correlate the behavior of the contrast media with the grading of tumors and their prognosis (86). The clue to understand all those different behaviors of any contrast agent in a tumor is that we are indirectly watching the process of angiogenesis. Angiogenesis is the formation of new vessels and maybe the result of a physiologic (wound healing) or pathologic process (tumor growth) (87).

Another factor is the fact that neo-vessels in and around a tumor differ from normal capillaries. This is proved since their layer is thinner and lacks pericytes and the biochemical constitution of the basal membrane increases its permeability. Unlikely with what happens in breast DCE-MRI, the physiology behind CEUS kinetics when evaluating enhancement and wash-out, it is exclusively related with the same vessel properties, but permeability since one of the most important characteristics of the microbubbles (contrast agent in CEUS) is that they are purely vascular; endothelial permeability does not affect the pool of contrast at any time (88). This may explain why the main two kinetics characteristics observed in our study and described previously occurs during the first 50 second of the incoming contrast agent to the areas in concern. CEUS shows the vascular and neo-vascular net; there is no diffusion effect to outer spaces than just the pool of contrast agent inside the neo-capillaries.

This exclusive property/ behavior of the contrast agent in CEUS compared with DCE-MRI might potentially become the leading method in the follow-up of diverse neoadjuvant therapies in advanced breast cancer. Even though in this study we were able to find certain kinetics differences between benign and malignant tumors, it should be remember that the study population was chosen on the basis of typically benign and malignant morphological appearance by mammography and ultrasound.

6.3 STUDY III

Comparison between doses of 1.2 and 2.4 mL (Group A)

Comparison of *conventional kinetic parameters* showed statistically significant correlation with peak signal intensity (SI_{peak} , P-value=0.001) and initial slope (S_i , P-value=0.001). There was however no significant correlation with wash-out parameters (W_{21} and W_{50}) and time-to-peak showed only a trend towards significance (T_{peak} , P-value=0.04)

Among the *model-based parameters*, area under the curve (AUC, P-value=0.0001) and curve maximum (C_{max} , P-value=0.0008) showed statistically significant correlation. Model based time-to-peak showed a trend towards significance (t_p , P-value=0.008). There was no significant correlation with other model based parameters like μ , σ , mean transit time (MTT), and wash-out parameters (W_{21m} , W_{50m}).

Comparison between doses of 2.4 and 4.8 mL (Group B)

Comparing *conventional kinetic parameters* showed that there was statistically significant association in terms of peak signal intensity (SI_{peak} , P-value<0.00001), initial slope (S_i , P-value<0.00001), and wash-out ratios (W_{21} , P-value<0.0001; W_{50} , P-value=0.01). Time-to-peak showed a trend towards significance (T_{peak} , P-value=0.03). With regard to the *model-based quantitative parameters*, statistically significant correlation was found in the following: area under the curve (AUC, P-value<0.00001), lognormal model parameter μ (P-value=0.0007), lognormal model parameter σ (P-value<0.0001), mean transit time (MTT, P-value<0.0001), model-based wash-out ratios (W_{21m} , P-value=0.0002; W_{50m} , P-value=0.0001), time-to-peak (t_p , P-value=0.005) and curve maximum (C_{max} , P-value<0.00001).

Each conventional and model-based kinetic parameters measured within the same lesion at different doses (at the dose of 1.2 and 2.4 mL in Group A, and also at the dose of 2.4 and 4.8 mL in Group B) were statistically correlated using a non-parametric test (Spearman rank correlation). The assumption was that the greater the similarity between time-intensity curves obtained at different doses, the stronger the correlation with kinetic parameters will be. Due to the number of statistical tests performed on the same dataset, significance level (α) was adjusted with the Bonferroni method to avoid Type I errors. Statistical significance was established at a P-value of <0.05 in all cases.

It had been discussed before that the optimal way to evaluate invasive breast carcinomas using CHI and CEUS is with a 2 second bolus injection of 2.4 mL microbubbles solution (89, 90). Evaluating time/intensity curves clear cut-off values demonstrated that the bolus injection is the method of choice (91). But there is still a need for comparative studies in order to identify the correct injected dose.

Analyzing the lesions both from a qualitative point of view (patterns of enhancement) and quantitative (kinetics patterns) Liu et al in 2008 and Zhao et al. in 2009 (92, 93) used 2.4 mL dose of MB as a bolus to differentiate benign from malignant lesions in the breast. The same dose of 2,4 mL of contrast agent was used by Wang et al. in 2011 (94) in the study of different histopathological types of breast cancers.

On the other hand, Sorelli et al. in 2010 (95) used a dose of 4.8 mL MB in order to evaluate breast tumors in hope to find an additional benefit over the conventional triple assessment when differentiating between benign and malignant neoplasms. This high dose of MB was also applied by Balleyguier et al. in 2008 (96), where they suggest a dose of 5 mL contrast agent in order to detect small vessels, arguing that a half dose was clearly insufficient for studying breast tumors.

Recently two studies were published evaluating different doses of injected agent. Wan et al. 2012 and Du et al 2012 (97, 98) used a double injection technique of MB: first a high dose of 3,6 mL for the quantitative acquisition time for kinetics analysis,

and after waiting some minutes to avoid any cumulative effect of contrast agent a second injection of 1,2 mL dose of contrast agent for MFIA (micro flow imaging analysis-qualitative analysis) during the same examination was performed.

Wan et al. using this combined doses (3,6 mL respective 1,2 mL of contrast agent for quantitative respective qualitative analysis), found that for the quantitative analysis with a higher dose than 2,4 mL has the potential to differentiate benign from malignant lesions, but it has not yet improved the final diagnostic accuracy. Even though the kinetic parameters analyses were statistically significance, their predictive capability is not yet reliable enough for routine clinical use. The logistic regression analysis showed that they did not enhance qualitative parameters and the final diagnostic accuracy was not improved.

On the other hand, Du et al. using the same combined doses methodology of 3,6 mL and 1,2 mL for the same quantitative/qualitative purposes, found that a higher dose than 2,4 mL of CEUS for quantitative purposes together with conventional US have a better diagnostic performance than either method alone and displays good agreement with MRI in the differentiating benign from malignant breast lesions.

According to the literature above the spectrum of contrast doses varies from 1,2 mL up to 5 mL of contrast agent when talking about CEUS as a diagnostic and differential diagnostic tool for breast diseases.

In the presented study of invasive breast tumors in order to find the optimal single dose of injected agent we applied a kinetic analysis, comparing the inferior contrast dose of 1,2 mL according to some previous studies in the literature, our double established required dose of 2,4 mL and a superior double dose of 4,8 mL.

Applying the kinetic analysis from our previous study, we could show that the dose of 2.4 mL of contrast agent established significant kinetic features differences when compared between benign and malignant lesions in: *time-to-peak* signal intensity (t_p) and *wash-out ratios*: W_{21} and W_{50} (99). Malignant tumors exhibited faster in- and outflow (wash-in and wash-out) of contrast agent, which appears a typical characteristic for this type of lesions.

In the present study we reproduced those “malignant dynamic enhancement features” obtained with a bolus injection of 2.4mL of contrast agent in two different groups of patients with invasive breast tumors. In Group A, we compared the established dose of 2.4 mL of contrast agent with 1.2 mL and in Group B, we did the same but compared with 4.8 mL of contrast agent; with an equivalence hypothesis of “one dose is as effective as another”.

Our results showed that there is stronger and significant correlation in reproducing the malignant dynamic enhancement features among the patients in the group, where we compared the established dose of 2.4 mL with a higher dose of 4.8 mL of contrast agent

(Group B); and we found much less correlations between the dynamic parameter rates using a lower dose in the group where we compared the established dose of 2.4 mL with 1.2 mL (Group A). This correlates the fact that the dose of contrast agent may need to be on the high side to compensate for the lower sensitivity when working at 7-9 MHz in small parts tissues tumors, like in the breast (100).

Correlations are seen in both conventional and model-based calculations, making it clear that these statistical finding are probably not incidental (*Tables 5, 6*). In Table 6, it is seen that the correlation with the area under the curve (AUC) and the curve maximum (C_{\max}) is independent of the doses in use.

When we compared the figures of the two different groups we could observe that Group A (1.2 mL vs 2.4 mL) and Group B (2.4 mL vs 4.8 mL) shared some kinetic features like: peak intensity, initial slope, AUC and C max. We interpret this as the contrast agent is just arriving inside the tumors in a similar way. In Group B (2.4 mL vs 4.8 mL) we observed more and statically stronger correlation between conventional and model base kinetic parameters. Most of the malignant wash-in and wash-out parameters show correlation in Group B (*wash-out ratios, W_{21m} and W_{50m} , time-to-peak signal intensity (t_p) and mean transit time (MTT)*); whereas only the dose related wash-in parameters showed correlation in Group A (*time-to-peak signal intensity (t_p)*). The kinetic interpretation of these results is that when using the two higher doses, the contrast agent stays for a longer time and in a more stable and reliable manner inside the tumors making the calculation and interpretation of typical malignant kinetics features possible (101). The use of 1.2 mL of contrast agent may evolve to a higher rate of measurement error since this dose is more sensitive to physiological factors like patient's weight and heart rate and/or factors like different tumors size and histopathologic types.

These features may be considered as limitation of the study together with small sample population, where further studies with larger population of both benign and malign lesions should be considered to confirm our results.

Also the fact of the existence of different contrast agent manufacturers, the use of different probe's strengths and diversity of quantification softwares, should be taken in consideration when compared this study with other similar in the interpretation of the results.

Detailed results for the comparison of conventional parameters are shown in Table 5; the comparison of quantitative model-based parameters is shown in Table 6. Figures 10 a-d illustrates the raw and fitted time-signal intensity curves in two separate patients with the contrast doses of 1.2, 2.4 and 4.8 mL.

Table 5. Comparison of conventional contrast-enhanced ultrasound parameters in 51 patients with 51 lesions. Numbers are mean values with standard deviation in brackets. Significance level was calculated with the Bonferroni method and established at $\alpha=0.01$, P-values calculated with Spearman's rank correlation test (statistically significant correlation indicated with italic).

	<u>GROUP A</u> Comparison of 1.2/2.4 ml doses*			<u>GROUP B</u> Comparison of 2.4/4.8 ml doses**		
	1.2 mL	2.4 mL	P-value	2.4 mL	4.8 mL	P-value
SI _{peak}	22.39 (18.17)	37.70 (26.35)	<i>0.001</i>	4.24 (4.78)	6.31 (7.21)	<i>2.03 x10⁻⁶</i>
S _i	248.71 (207.58)	501.86 (576.36)	<i>0.001</i>	46.36 (48.39)	62.02 (72.66)	<i>2.09 x10⁻⁶</i>
W ₂₁	64.52 (17.45)	64.69 (15.76)	0.07	70.09 (15.45)	60.53 (15.79)	<i>2.84 x10⁻⁵</i>
W ₅₀	82.79 (9.81)	82.25 (9.75)	0.61	83.77 (6.87)	80.09 (8.25)	<i>0.01</i>
T _{peak}	9.94 (3.53)	9.03 (2.29)	0.04	9.74 (2.55)	10.5 (1.97)	0.03

Conventional parameters: SI_{peak}: peak (maximum) signal intensity, S_i: initial slope – the upslope of the curve, W₂₁ and W₅₀: conventional wash-out parameters measured at 21 s and 50 s, T_{peak}: time to peak.

* 26 women with 26 lesions comprising 52 examinations

** 25 women with 25 lesions comprising 50 examinations

Table 6. Comparison of quantitative model-based contrast-enhanced ultrasound parameters in 50 patients with 50 lesions. Numbers are mean values with standard deviation in brackets. Significance level was calculated with the Bonferroni method and established at $\alpha=0.00625$. P-values are calculated using Spearman's rank correlation test, statistically significant correlation indicated with italic.

	GROUP A Comparison of 1.2/2.4 ml doses*			GROUP B Comparison of 2.4/4.8 ml doses**		
	1.2 mL	2.4 mL	P-value	2.4 mL	4.8 mL	P-value
AUC	696.96 (1065)	1075.92 (1235)	<i>0.0001</i>	61.27 (60.85)	128.56 (140.87)	<i>2.2 x10⁻⁶</i>
M	3.02 (1.01)	2.921(0.97)	0.44	2.70 (0.55)	2.82 (0.48)	<i>0.0007</i>
Σ	1.10 (0.46)	1.11 (0.52)	0.29	0.99 (0.41)	1.04 (0.29)	<i>6.7 x10⁻⁵</i>
MTT	380.43 (1262)	307.13 (1227)	0.58	52.53 (86.99)	46.95 (71.36)	<i>1.14 x10⁻⁵</i>
W _{21m}	52.25 (22.26)	55.59 (19.62)	0.58	58.37 (19.11)	45.81 (16.37)	<i>0.0003</i>
W _{50m}	81.56 (15.01)	83.97 (11.01)	0.91	87.08 (9.51)	83.64 (9.51)	<i>0.0001</i>
t _p	5.59 (3.22)	4.84 (2.32)	0.008	5.37 (2.53)	5.83 (2.88)	<i>0.005</i>
C _{max}	19.81 (15.47)	34.82 (27.41)	<i>0.0008</i>	3.68 (4.36)	5.32 (6.20)	<i>1.9 x10⁻⁶</i>

Parameters determined by the lognormal model: AUC=area under the time-signal intensity curve, μ =lognormal model parameter for horizontal scaling of the curve (increasing μ will result in slower wash-in and wash-out), σ =lognormal model parameter determining the skewness of the curve, MTT=mean transit time, W_{21m}=model based wash-out ratio at 21 sec, W_{50m}=model based wash-out ratio at 50 sec, t_p=model based time-to-peak, C_{max}=curve maximum.

* 25 women with 25 lesions comprising 50 examinations (one lesion {No 20} was excluded due to failed curve fitting)

** 25 women with 25 lesions comprising 50 examinations

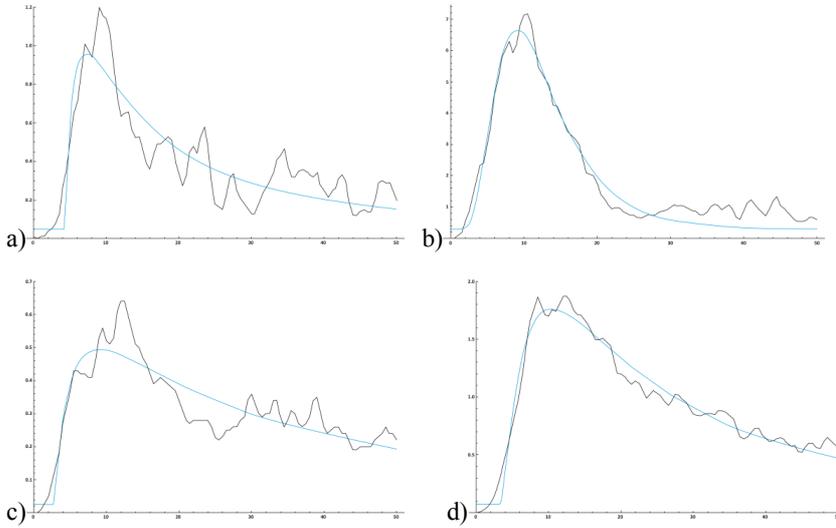


Fig.10 a-d. Raw and fitted enhancement curves using the lognormal function in two separate patients. a) shows the enhancement curves in a 79 year-old patient, using a contrast dose of 1.2 mL, with the conventional parameters of $SI_{peak}=1.2$, $T_{peak}=9$ s, $W_{21}=72\%$, $W_{50}=83\%$; the enhancement curve of the same lesion is shown on b) with the contrast dose of 2.4 mL, $SI_{peak}=7.2$, $T_{peak}=10.5$ s, $W_{21}=68\%$, $W_{50}=92\%$; c) shows the enhancement curves in a 68 year-old patient, using a contrast dose of 2.4 mL, with the conventional parameters of $SI_{peak}=0.6$, $T_{peak}=12$ s, $W_{21}=56\%$, $W_{50}=66\%$; the enhancement curves of the same lesion is shown on d) with the dose of 4.8 mL, and the parameters of $SI_{peak}=1.8$, $T_{peak}=8.5$ s, $W_{21}=39\%$, $W_{50}=69\%$

One involuntary limitation we had experienced when evaluating the average raw peak signal intensities (Table 5) is that although all the peak intensities are higher with higher dose, inside each individual group (the peak intensity values are higher with 2.4 mL in Group A, and with 4.8 mL in Group B), there is no logical correlation between those two groups (the peak values are higher in group A where we compared lower doses than in Group B where we compared higher doses). This can be explained by different settings in the ultrasound devices (gain) in each group (102). But importantly, all settings were exactly the same when investigating the individual patient.

6.4 STUDY IV

Statistically significant correlation was found between model based time-to-peak (t_p) and tumour grade (Spearman's rank correlation, P-value=0.023); t_p appears to be shorter in more aggressive tumours (Figure 11). The difference in t_p was statistically significant between PR negative and PR positive tumours; t_p was found to be significantly shorter in the PR negative group (Wilcoxon rank sum statistic, $W=394$, P-value=0.042). The comparison of t_p with axillary node status showed that t_p is shorter in node positive tumours, after replacing an outlier (case 39) with mean this difference was statistically significant (Wilcoxon rank sum statistic, $W=897$, P-value=0.025, Figure 12). The model based wash-out ratio, measured at 21 sec (W_{21m}) was found to be significantly higher in the ER negative than the ER positive tumours

(Wilcoxon rank sum statistic, $W=350$, $P\text{-value}=0.042$). Similar correlation was found between W_{21m} and PR status, W_{21m} appeared to be significantly greater in PR negative than PR positive tumours (Wilcoxon rank sum statistic, $W=756$, $P\text{-value}=0.026$) (Figure 13a-b).

Kinetic parameters C_{max} (curve maximum, Wilcoxon rank sum statistic, $W=923$, $P\text{-value}=0.012$) and AUC (area under the curve Wilcoxon rank sum statistic, $W=904$, $P\text{-value}=0.02$) were also showed significant correlation with axillary node status. Although it should be noted that lower C_{max} and AUC values were associated to positive axillary status. Typical enhancement curves of low and high-grade invasive breast cancers are shown on Figure 14 a-b.

Notable trends towards statistical significance were observed in the following correlations: between the lognormal parameter μ and PR status of tumors (μ appears to be decreased in PR negative tumors; Wilcoxon rank sum statistic, $W=417$, $P\text{-value}=0.079$), between wash-out ratio W_{50m} and ER status (W_{50m} appears to be greater in ER negative tumors; Wilcoxon rank sum statistic, $W=330$, $P\text{-value}=0.095$), between MTT and ER status (MTT appears to be decreased in ER negative tumors; Wilcoxon rank sum statistic, $W=140$, $P\text{-value}=0.075$).

The correlation between contrast-enhanced ultrasound kinetic parameters derived from a lognormal model and prognostic factors is shown in Table 9. For further comparison, mean values of kinetic parameters in different prognostic groups are show in Table 10.

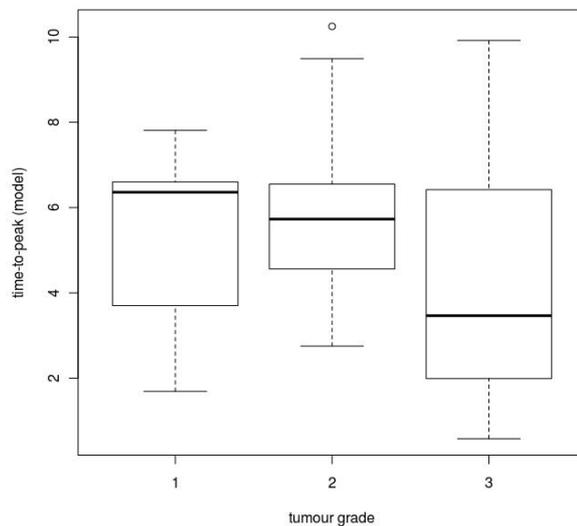


Fig.11. Boxplot showing the relationship between model based time-to-peak (t_p) and tumor grade (Spearman's rank correlation, $\rho=-0.26$, $P\text{-value}=0.023$). Shorter time-to-peak (t_p) represents more aggressive tumor.

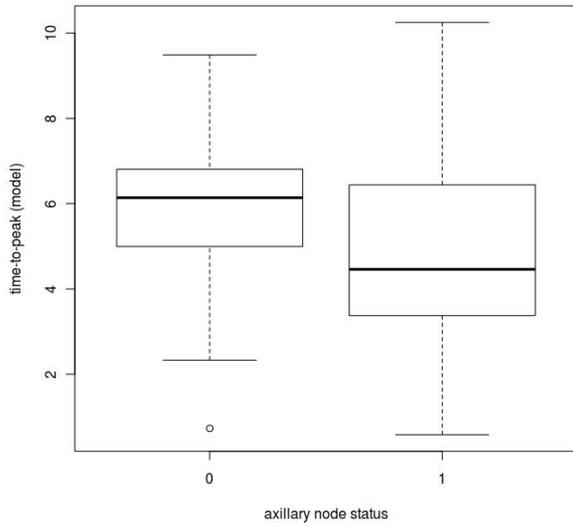
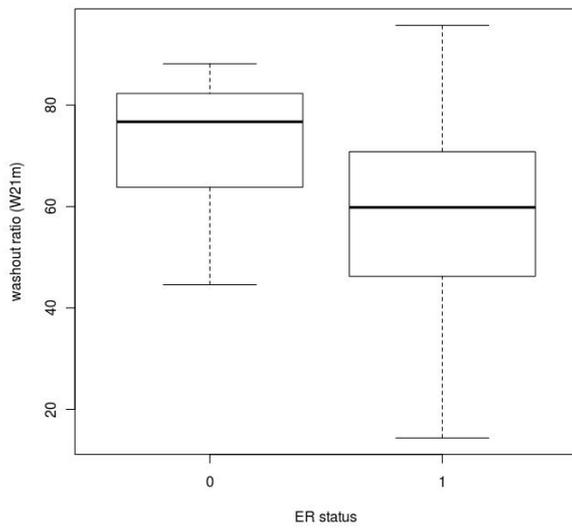
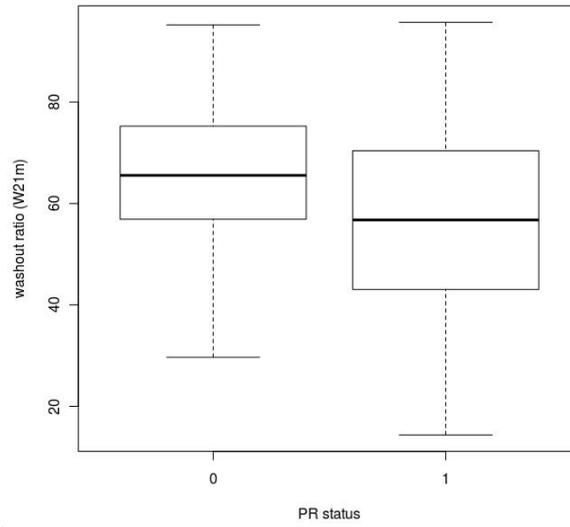


Fig.12 . Boxplot showing the relationship between model-based time-to-peak (t_p) and axillary lymph node status. Time-to-peak (t_p) is significantly shorter in node positive tumors (after replacing an outlier with mean, Wilcoxon rank sum statistic $W=897$, $P\text{-value}=0.025$).



a)



b)

Fig.13 a-b. Boxplot showing the relationship between W_{21m} (model-based wash-out ratio at 21 sec) and ER/PR-status. W_{21m} is significantly greater in ER and also in PR negative tumors (for ER status, Wilcoxon rank sum statistic $W=350$, P -value=0.042, for PR status $W=756$, P -value=0.026).

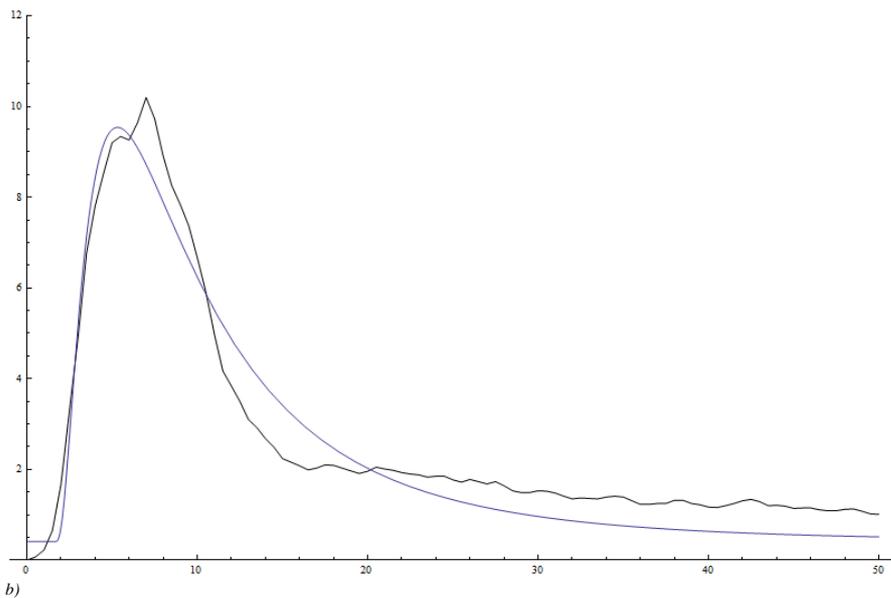
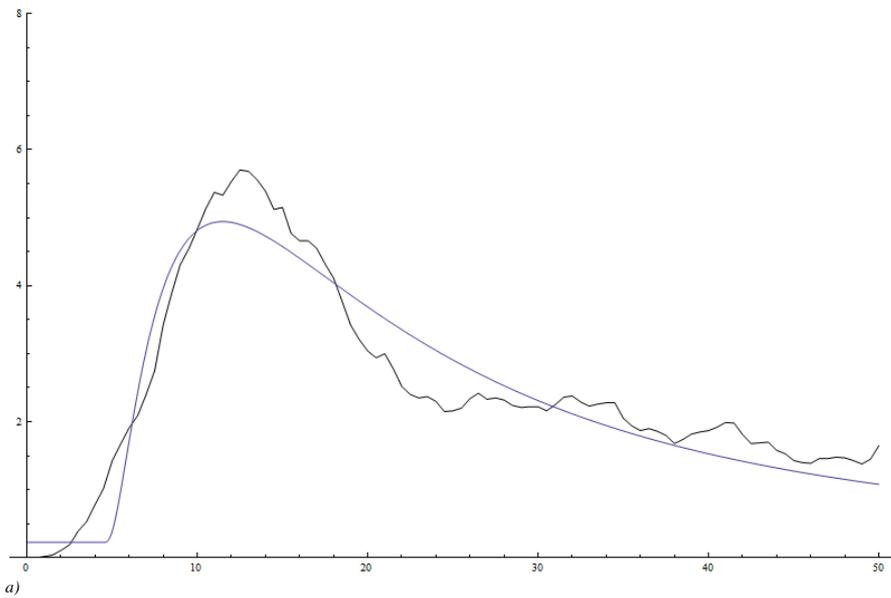


Fig.14a-b. The lognormal function was used for mathematical modeling of time-signal intensity curves in contrast-enhanced ultrasound of breast cancer. (a) A grade I, ER positive, axillary status negative – typically low grade invasive cancer (case #46) showing relatively slower wash-in and lengthened wash-out. (b) A grade III, ER negative, axillary node positive – typically high grade invasive cancer (case #66) showing faster wash-in and accelerated wash-out.

	Histological type ^a	Tumour size ^b	Tumour grade ^c	ER status ^d	PR status ^d	Her2 status ^d	Axillary node status ^d
C_{max}	0.108	0.199	0.404	0.792	0.186	0.145	0.012
C_{max-sd}	0.716	0.577	0.786	0.090	0.087	0.271	0.662
AUC	0.125	0.354	0.308	0.591	0.086	0.130	0.020
μ	0.163	0.491	0.954	0.172	0.079	0.145	0.936
σ	0.176	0.660	0.207	0.193	0.684	0.667	0.692
t_p	0.796	0.244	0.023	0.906	0.042	0.519	0.025*
W_{21m}	0.797	0.614	0.547	0.042	0.026	0.287	0.1765
W_{50m}	0.297	0.316	0.692	0.095	0.342	0.353	0.811
MTT	0.368	0.841	0.575	0.075	0.392	0.296	0.810

Table 9. Correlation of contrast-enhanced ultrasound kinetic parameters derived from a lognormal model with prognostic factors.

Numbers are P-values (cor=Pearson's correlation coefficient, W=Wilcoxon rank sum statistic, a= analysis of variance - interval vs. nominal data, b= Pearson's correlation - interval vs. interval data, c= Spearman's rank correlation - interval vs. ordinal data, d= Wilcoxon rank sum test - equivalent to Mann-Whitney U-test / interval vs. dichotomous data)

Parameters determined by the lognormal model: C_{max} =curve maximum=peak signal intensity, C_{max-sd} =curve maximum standardized to AUC, AUC=area under the time-signal intensity curve, μ =lognormal model parameter for horizontal scaling of the curve (increasing μ will result in slower wash-in and wash-out), σ =lognormal model parameter determining the skewness of the curve, t_p =model based time-to-peak, W_{21m} =model based wash-out ratio at 21 sec, W_{50m} =model based wash-out ratio at 50 sec, MTT=mean transit time

a Analysis of variance, interval vs nominal data

b Pearson's correlation, interval vs interval data

c Spearman's rank correlation, interval vs ordinal data

d Wilcoxon rank sum test, equivalent to Mann-Whitney U test / interval vs dichotomous data

*correlation after replacing an outlier (case 39, 103.86) with mean (6.69)

	Tumour grade			ER status		PR status		Her2 status		Axillary node status	
	I	II	III	neg.	pos.	neg.	pos.	neg.	pos.	neg.	pos.
C_{max}	13.42 (15.26)	19.05 (19.06)	10.48 (13.78)	14.59 (15.73)	16.08 (17.69)	11.79 (14.21)	17.55 (18.39)	15.41 (17.41)	22.05 (17.94)	19.8 (18.61)	10.75 (14.38)
C_{max-sd}	0.054 (0.031)	0.066 (0.039)	0.059 (0.029)	0.08 (0.037)	0.06 (0.035)	0.073 (0.04)	0.057 (0.032)	0.063 (0.036)	0.046 (0.027)	0.062 (0.037)	0.061 (0.032)
AUC	673 (1364)	466 (603)	392 (950)	375 (617)	510 (919)	281 (486)	581 (999)	450 (821)	1043 (1492)	586 (953)	378 (804)
μ	2.89 (0.93)	2.59 (0.47)	2.8 (0.83)	2.41 (0.59)	2.73 (0.69)	2.47 (0.46)	2.79 (0.74)	2.67 (0.64)	3.11 (1.04)	2.65 (0.57)	2.78 (0.81)
σ	0.98 (0.38)	0.87 (0.32)	1.99 (0.5)	0.8 (0.37)	0.98 (0.4)	0.95 (0.35)	0.97 (0.41)	0.95 (0.38)	1.09 (0.59)	0.92 (0.33)	1.02 (0.47)
t_p	11.22 (23.94)	5.89 (1.76)	4 (2.66)	5.61 (2.47)	6.8 (12.12)	4.68 (2.18)	7.47 (13.52)	6.77 (12.04)	5.85 (2.7)	5.79 *	4.87 *
W_{21m}	54.87 (21.4)	58.86 (19.94)	59.94 (18.93)	71.66 (16.14)	56.8 (19.81)	66.19 (15.42)	55.07 (20.67)	58.88 (19.87)	50.23 (20.04)	55.72 (20.68)	61.49 (18.57)
W_{50m}	81.25 (21.63)	87.79 (9.21)	86.87 (7.43)	91.04 (7.91)	85.6 (13.24)	89.57 (6.78)	84.76 (14.44)	86.37 (13.09)	83.08 (11.06)	86.5 (11.25)	85.57 (15.02)
MTT	71.89 (101.2)	29.11 (29.81)	174.1 (461.3)	27.8 (40.89)	74.06 (232.2)	28.81 (34.85)	85.66 (259.3)	46.94 (74.05)	331.89 (750.4)	39.14 (58.74)	110.8 (331)

Table 10. Comparison of contrast-enhanced ultrasound kinetic parameters derived from a lognormal model with prognostic factors (numbers are mean values with standard deviation in brackets).

Parameters determined by the lognormal model: C_{max} =curve maximum=peak signal intensity, C_{max-sd} =curve maximum standardized to AUC, AUC=area under the time-signal intensity curve, μ =lognormal model parameter for horizontal scaling of the curve (increasing μ will result in slower wash-in and wash-out), σ =lognormal model parameter determining the skewness of the curve, t_p =model based time-to-peak, W_{21m} =model based wash-out ratio at 21 sec, W_{50m} =model based wash-out ratio at 50 sec, MTT=mean transit time

*values were calculated after replacing an outlier (case 39, 103.86) with mean (6.69)

Multivariate analysis

Kinetic parameters found to be significant at the exploratory univariate tests were used in the multivariate models. Two models were constructed for binary logistic regression: the first model was included time-to-peak (t_p) and wash-out ratio W_{21m} , in which the dependent variable was PR status; whereas in the second model, t_p , area under the curve (AUC) and curve maximum (C_{max}) were tested against axillary node status. No multivariate models were made for tumor grade and ER status, because there was only one statistically significant correlation in each dataset.

In the first model, W_{21m} was retained in the equation and proved to be an independent predictor of PR status (Wald z-statistic=-2.11, P-value=0.034), but t_p was rejected from the model. In the second regression model, where axillary node status was the dependent variable, C_{max} retained its significance (Wald z-statistic=-2.15, P-value=0.031). Kinetic parameters t_p and AUC were rejected from the model.

Angiogenesis is a normal physiological process in embryonic vascular development, but also plays an important role in wound healing, and reproduction in health. Abnormal regulation of angiogenesis can occur in a number of pathological conditions including malignancies, inflammatory and autoimmune diseases (103).

First Folkman hypothesized in 1971 that the growth and spread of malignant tumors are highly dependent upon the formation of new vessels (104), angiogenesis became one of the most extensively researched topics in biomedical science since tumor expansion facilitates the release of several angiogenic growth factors, of which vascular endothelial growth factor (VEGF) is the most often studied. These newly formed pathological microvessels in tumors are largely different from normal capillaries. This vascular network shows perivascular detachment, irregular shape, abnormal caliber, and the lining is composed of fenestrated endothelial cells. These changes usually lead to altered perfusion, increased permeability, and lacking regulatory processes of normal vessels.

Modern radiology provides a number of imaging methods that are capable of non-invasive assessment of tumor-associated angiogenesis. In breast imaging, DCE-MRI has emerged as a powerful supplementary tool for the detection and evaluation of cancer (105). Gadolinium based contrast agents are intravascular and extracellular in distribution, therefore enhancement kinetics are influenced by both tumor perfusion and permeability of microvessels. Unlike MR contrast media, microbubble contrast agents used in CEUS remain in the vasculature leading to somewhat different time-signal intensity curves, but basic perfusion patterns are compatible to that of DCE-MRI. This allows CEUS to evaluate angiogenesis quantitatively (106), furthermore CEUS kinetics can discriminate benign from malignant breast lesions similarly to MRI (107, 108). It is difficult to quantify tumor neovascularization, but microvessel density (MVD) and VEGF expression have been repeatedly proposed as a prognostic marker for breast cancer (109, 110). Similarly to DCE-MRI, CEUS imaging also has the ability to yield insight into tumor-associated angiogenesis, but contrast-enhanced ultrasound parameters are not affected by changes in vascular permeability and appear to be related more closely to MVD than VEGF expression (111).

In our study, quantitative CEUS kinetic parameters measured in invasive breast cancers were compared with different traditional and immunohistochemical prognostic variables that are used routinely in clinical practice. We observed that tumors with shorter time-to-peak were more likely to have higher histological grade, negative PR status and metastatic involvement of the axillary lymph nodes. Wash-out ratio measured at 21 sec after the appearance of contrast was also significantly associated to ER and PR status. There were further notable trends towards statistical significance in other comparisons: decreased lognormal parameter μ , increased wash-out ratio measured at 50 sec and shorter MTT are likely to be associated with established predictors of poorer prognosis. According to our findings, increased tumor perfusion - that is shown by faster wash-in and accelerated wash-out - appears to be an indicator of worse prognosis in invasive breast cancer.

At univariate analysis there were two significant correlations that are difficult to explain: model based curve maximum (C_{\max} , peak intensity) and area under the curve (AUC) also showed association with axillary node status, although boxplots showed that both C_{\max} and AUC were lower in tumours with positive than negative nodal status. We assume that these correlations are incidental and presumably due to the relatively small sample size. Multivariate statistics showed that wash-out ratio at 21 sec is a stronger predictor of PR status than time-to-peak (t_p). Time-to-peak was rejected from the second multivariate model as well, where the dependent variable was axillary status, and C_{\max} was shown to be independently related to axillary status. Again we believe that our small sample size and relatively weak correlations at univariate analysis had a great influence on multivariate analysis, therefore definitive conclusions cannot be drawn from the multivariate tests.

Owing to the lack of similar published research in the field of CEUS, we can only compare our findings with studies that focused on the prognostic value of MRI enhancement kinetics. We can assume that the amplitude of MRI signal is mainly affected by vascular permeability, but the early appearance of peak enhancement with faster elimination of contrast is strongly related to increased tumor perfusion even in DCE-MRI. In this aspect, our results are in accordance with previously presented data. In a previously done MRI study (112), shorter time-to-peak enhancement was associated with higher tumor grade, Her2/neu and ER status; and also, wash-out type enhancement curve showed association with increased proliferation activity as assessed by Ki-67. Another study demonstrated that early peak enhancement on MRI was correlated with negative ER expression (113).

We can hypothesize that the more aggressive a tumor is, the higher its angiogenic activity will be to support its faster expansion. Increased new vessel formation in high grade tumors may lead to higher MVD, which in turn affects the signal at DCE-MRI and CEUS. Furthermore, faster development of microvessels may also result in a chaotic pathological vascular pattern, which possibly influences the formation of more arterio-venous shunts as well. As a result of these factors, blood perfusion will be increased allowing a contrast bolus to flow quickly through tumor microvasculature on both DCE-MRI and CEUS imaging.

7 CONCLUSIONS

- I. Bolus injection is the method of choice for administration of microbubbles contrast agent for real-time contrast-enhanced ultrasound (using harmonic imaging) in the investigation of breast tumors for further evaluation of kinetic parameters.
- II. The evaluations of kinetic parameters of real-time contrast-enhanced ultrasound (using harmonic imaging) can evolve into a new non-invasive option for differentiate malignant from benign breast lesions. Further investigation in a larger population including different ACR BI-RADS lesions, should be performed to corroborate the reliability of the method.
- III. In terms of diagnostic features, the optimal way to evaluate kinetic features of invasive breast tumors using real-time contrast-enhanced ultrasound harmonic imaging is with a bolus injection of contrast agent of either 2.4 mL or 4.8 mL.
- IV. Invasive breast carcinomas exhibiting earlier peak enhancement and fast elimination of microbubbles contrast agent at contrast-enhanced ultrasound are found to be associated with established predictors of poor prognosis. These promising results suggests that CEUS has potential to determine the biological behavior of invasive breast cancers non-invasively, and may also help to identify future clinical application areas for this new imaging method.

8 OVERALL CONCLUSION

This thesis demonstrates that kinetic parameters of real-time harmonic contrast-enhanced ultrasound (CEUS) with a bolus injection of either 2.4 or 4.8 mL contrast agent has potential for diagnosis, differential diagnosis and prognosis of invasive breast cancer tumors.

9 FUTURE DEVELOPMENT

CEUS in the breast is in constant development.

From the oncology perspective could develop into a new method to evaluate early response to neoadjuvant therapy in patients with locally advanced breast cancer, where diminish of the vascular net is to be expected.

From the breast cancer surgical perspective, sentinel lymph node may be identified and localized before surgery after the intra-lymphatic administration of microbubbles, allowing staging of the disease much less invasive (114).

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