Quantitative Ultrasound Evaluation of Tumour Cell Death Response in Locally Advanced Breast Cancer Patients Receiving Chemotherapy

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Running Title: Ultrasound Visualization of Cancer Treatment Response

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Statement of Translational Relevance

Current methods of assessing patient responses to cancer treatment are clinically based on physical measurements of the tumour. Tumour size reduction frequently requires several months of treatment administration and in some cases tissue diminishment is not present despite treatment response. In contrast, functional imaging methods that probe tumour physiology may be used to detect tumour responses from days to weeks after starting therapy. This study indicates, for the first time, that conventional-frequency quantitative spectroscopic ultrasound can be applied to non-invasively monitor the effects of chemotherapy cancer treatment in locally advanced breast cancer patients. We demonstrate that quantitative ultrasound techniques can be used to detect and evaluate responses of cancer patients within weeks after starting therapy. This ultrasound based functional imaging can therefore facilitate the practice of personalized medicine for cancer patients.
Abstract

Purpose: Quantitative ultrasound techniques have been recently demonstrated to be capable of detecting cell death through studies conducted on *in vitro*, and *in vivo* models. This study investigates for the first time the potential of early detection of tumour cell death in response to clinical cancer therapy administration in patients, using quantitative ultrasound spectroscopic methods.

Experimental Design: Patients (n=24) with locally advanced breast cancer received neo-adjuvant chemotherapy treatments. Ultrasound data was collected prior to treatment onset and at 4 times during treatment (weeks 1, 4, and 8, and pre-operatively). Quantitative ultrasound parameters were evaluated for clinically responsive and non-responding patients.

Results: Results indicated that quantitative ultrasound parameters demonstrated significant changes for patients who responded to treatment, and no similar alteration was observed in treatment-refractory patients. Such differences between clinically and pathologically determined responding and non-responding patients were statistically significant (p<0.05) after four weeks of chemotherapy. Responding patients demonstrated changes in parameters related to cell death with, on average, an increase in mid-band fit and 0-MHz intercept of 9.1 ± 1.2 dB and 8.9 ± 1.9 dB, respectively, whereas spectral slope was invariant. Linear discriminant analysis revealed a sensitivity of 100% and a specificity of 83.3% for distinguishing non-responding patients, by the fourth week into a course of chemotherapy lasting several months.

Conclusion: This study reports for the first time that quantitative ultrasound spectroscopic methods can be applied clinically to evaluate cancer treatment responses, non-invasively. The results form a basis for monitoring chemotherapy effects and facilitating the personalization of cancer treatment.
Introduction

Breast cancer is the most frequent form of cancer diagnosed in women, second only to non-melanoma forming skin cancers (1). Increases in awareness coupled with the rate and efficacy of mammogram screenings has increasingly allowed for the detection of breast cancers. One third of newly diagnosed cases have detected the presence of breast malignancies in the initial stages with tumours less than 1 cm in size (2). Although these trends do indicate a positive direction with regard to disease detection, there still remains a significant fraction of the population in which diagnosis is not made until later stages, with 5-20% of newly diagnosed cases being classified as locally advanced breast cancer (LABC) (3,4), and with even greater numbers outside of North America. Locally advanced breast cancer typically comprises all stage III and a subset of stage IIB (T3N0) tumours and are diagnosed as tumours which are frequently greater than 5 cm, involving the chest wall and/or classified as inflammatory breast cancer. Due to the progression of the disease and high risk for metastatic spread, LABC patients typically have poor long-term survival rates (five-year survival rate of 55%, approximately) in comparison to the early stages patients(4).

Current methods of assessing patient responses to cancer therapy are based upon ascertaining physical measurements of the tumour during treatment. These methods include clinical examination, X-ray mammography, conventional ultrasound imaging and magnetic resonance imaging (MRI). Physical examination by palpation is commonly employed by clinicians; however this method of tumour assessment is subjective (5). During the course of the last several decades, X-ray mammography has made considerable improvements in the detection of breast cancers but has not shown significant promise in tracking tumour response during treatment. MRI is commonly employed for assessing the end result of treatment in LABC patients as it
provides high resolution images allowing clinicians to fairly accurately measure tumour volume. However it remains a costly imaging modality to employ. Another limitation stems from the fact that these methods attempt to ascertain patient response to treatment by determining the physical size of the tumour. Tumour size reduction, however, frequently requires several weeks to months of treatment administration and in some cases mass diminishment is not present despite a cytotoxic treatment response (6).

Changes in tumour size with treatment are the late cumulative result of early micro-structural changes in tumour cell morphology often due to cell death, starting to take place within hours to days after treatment administration. This then provides the possibility of assessing the early effects of a treatment on a tumour to monitor therapy efficacy, in advance of changes to the overall tumour volume. Thus the advent of a non-invasive functional method to monitor patient responses to therapy would be advantageous in order to guide the customization of chemotherapy or other cancer therapies on an individual patient basis. Alternatively, such imaging methods could also facilitate a switch to an early salvage therapy for patients whose responses to the current therapy are determined to be limited.

Research into the potential of ultrasound to monitor non-invasively the effects of cancer therapy administration was initially conducted using high-frequency (20-50 MHz) ultrasound and demonstrated its capacity to detect changes in tissue microstructure associated with a variety of cancer therapies in in vitro, in situ, and in vivo models (7–14). Initial in vitro observations indicated increases in tissue echogenicity in acute myeloid leukemia (AML) cells exposed to the chemotherapeutic agent cisplatin (7,8). Further research used quantitative ultrasound techniques such as spectroscopic analysis of radiofrequency data and statistical analysis of the signal envelope to quantify the effects of treatment in a variety of concentration and exposure
dependant experiments (9,10). To date, high-frequency ultrasound has been used to detect apoptotic cell death resulting from photodynamic, X-ray radiation and ultrasonically activated anti-vascular micro-bubble therapies in a variety of in vivo mouse models (12–14). These studies demonstrated up to 16-fold maximal increases in observed backscatter signal intensity accompanied by increases in spectral parameters such as spectral slope, 0-MHz intercept, and mid-band fit (MBF) quantitative parameters which can be related to effective scatterer size, acoustic concentration, and both, respectively (15,16). Similar techniques have been applied previously in a variety of other ultrasound tissue characterization applications, such as the differentiation of mammary carcinomas and sarcomas from benign fibroadenomas, and the diagnoses of cardiac and liver abnormalities, and prostate cancer (17–20).

High-frequency 20-50 MHz ultrasound benefits from an increased imaging resolution (30-80 μm) when compared to clinically employed conventional-frequencies of 1 to 20 MHz (80 μm - 1.5 mm). However, it suffers from a limited tissue penetration depth, restricting its use to superficial tissue sites near the skin surface. On the other hand, while the application of conventional-frequency ultrasound carries a decreased imaging resolution, it benefits from a substantive tissue penetration capacity. Such an advantage potentially allows to non-invasively monitoring the effects of therapy on deeper malignant tissues such as breast, kidney and liver cancerous tumours. This has motivated a number of recent studies into the capacity of conventional-frequency ultrasound in the detection of cancer treatment effects, effectively laying the groundwork for this clinical evaluation (21–23).

In this study, the potential of conventional-frequency ultrasound has been investigated for the first time, to non-invasively monitor the effects of clinical cancer treatment in patients. Locally advanced breast cancer patients receiving neo-adjuvant chemotherapy were monitored during
their treatment. Conventional-frequency (~7 MHz) quantitative spectroscopic ultrasound data was collected prior to treatment, as well as at weeks one, four and eight during the course of treatment, and within one week prior to the modified mastectomy surgery. Based on their ultimate clinical and pathological response to the chemotherapy, patients were classified into two groups of treatment response: responders and non-responders. Patient responses were assessed based upon tumour diminishment and levels of cellularity. Analysis of ultrasound data employed linear regression analysis of the normalized power spectrum using a sliding window approach, in addition to statistical quantification of spectral parametric images. Results indicated statistically significant differences in the mid-band fit change between patients responding to treatment and non-responders, after four weeks of treatment. Changes in the 0-MHz intercept exhibited similar trends between two groups of patients, where statistically significant differences were revealed after four weeks of treatment. The spectral slope parameter was found to be invariant in both groups. Statistical analysis of spectral parametric images also indicated changes which resulted in a statistically significant difference between two groups, after eight weeks of treatment.

This study thus indicates for the first time that conventional-frequency ultrasound can be potentially employed as a non-invasive technique to monitor patient responses to clinical cancer therapies within weeks of their start. This work forms a basis for clinical application of these methods to detect and evaluate cancer patient responses to the treatments, which can consequently facilitate treatment customization, or even the early switch to salvage therapy.
Materials and Methods

Study Protocol and Data Collection

This study was conducted with research ethics approval and was open to all women with locally advanced breast cancer aged 18 to 85 meeting study criteria. Ultrasound data was acquired at 5 times during the patients’ course of treatment. The first scan was acquired immediately prior to the start of chemotherapy which was used as a baseline of comparison for subsequent scans. The following three scans were acquired during the first, fourth and eighth week of treatment, with a fifth scan acquired within one week prior to the modified radical mastectomy surgery, typically occurring four to six weeks after the course of treatment was completed.

All the ultrasound data in this study were collected by the same sonographer following standardized protocols for data acquisition. Ultrasound data was acquired with patients lying supine with their arms above their heads. Conventional B-Mode and radiofrequency ultrasound data were acquired using a Sonix RP, (Ultrasonix Vancouver, Canada) system utilizing a L14-5/60 transducer transmitting pulses with a 50% duty factor over 50 ns, exciting transducer elements with a frequency of 10 MHz, and resulting in a frequency bandwidth with a centre frequency of ~7 MHz. The transducer focus was set at varying depths depending on individual patient circumstances and digital radiofrequency data acquired with a sampling frequency of 40 MHz. Scan focal depths remained consistent for individual patients throughout the study. Breast regions selected for ultrasound scanning were directed by an oncologist, who determined acquisition scan planes via physical examination of the patient. Data was acquired in a single continuous sweep over the tumour volume in order to provide context regarding changes in
localization and dimensionality of the tumour across visits. Scans of individual tumour regions were also acquired at approximately 1 cm increments across the tumour volume.

Prior to therapy, all patients underwent a core needle biopsy to confirm a cancer diagnosis, where information regarding the tumour grade and histological subtype were recorded. Pre-treatment imaging of patients included MRI of the breast to determine initial tumour size and to perform a metastatic workup as necessary as part of the institutional standard of clinical care for such patients. Patients were followed clinically by oncologists who remained blinded to the study results. Physical Examination was conducted at each time and tumour size was assessed by clinicians. Post-treatment MRI scans of the breast were also acquired immediately before patient surgery to measure residual tumour size. Following surgery, patient mastectomy specimens were mounted on whole-mount (24) 5"x7" pathology slides digitized using a confocal scanner (TISSUEscope™, Huron Technologies, Waterloo, ON) at 2 micron resolution. All cases were examined by the same pathologist (J.Z.), who provided information regarding residual tumour size, grade, extent of cellularity, histological subtype, and tumour response. Patient responses were categorized based upon changes in overall tumour volume determined clinically during the treatment, in addition to the residual tumour cellularity. Patients were considered responders if there was a decrease in tumour size of 30% or more, and included patients which were deemed to have a complete pathological response to treatment (no residual invasive carcinoma) (25). Conversely, patients were deemed to be non-responders if there was less than a 30% decrease in tumour size and included patients with progressive disease in which the tumour volume increased despite treatment (25). In cases where the tumour cellularity was very low (overall volume of viable tumour cells), the patient was considered as a responder as well, even if the diminishment in the physical tumour size was less than 30%. In contrast, in cases where tumour
cellularity was very high, the patient was considered a non-responder, even if shrinkage in the physical tumour size was approximately 30%.
Data Analysis

Ultrasound radiofrequency data analysis was performed using linear regression analysis of the normalized power spectrum (7–15,17–20). Ultrasound data was analyzed across all acquired planes through the scan volume which included identifiable tumour regions. Analysis parameters were reported from data within a region of interest (ROI) located at the tumour central area which was consistently positioned at the transducer focal depth, typically accounting for approximately two third of the tumour area in cross-sectional plane.

The power spectrum was calculated using a Fourier transform of the raw radiofrequency data for each scan line through the whole field of view of the ultrasound data (described further below). In order to remove the effects of system transfer functions, transducer beam-forming, and diffraction artefacts, in addition to acting as a mechanism of depth related attenuation correction, data were normalized using a sliding window analysis with the power spectrum obtained from an agar embedded glass bead phantom model, modified from (26), with properties similar to those of breast tissue. Phantom data was acquired for each setting used during patient data acquisition, including variations in image gain and focal depth. Linear regression analysis was performed within the -6 dB window centered at the transducer central frequency, which was determined from a calibration pulse, to generate a best-fit line. Parameters subsequently reported included the mid-band fit (MBF), the spectral slope, and the corresponding 0-MHz intercept (16,27–29). Parameters were determined for each of the scan planes collected per patient visit and subsequently averaged across the tumour volume.

Specifically, spectral parameters for each scan plane were determined through averaging on the parametric images generated using a sliding window analysis on a pixel by pixel basis. Each sliding window was normalized separately to a reference curve obtained from the same region of
the phantom, with equivalent location and size. This was carried out in order to more accurately account for the effects of attenuation and beam diffraction across the region of interest especially in larger tumours. Statistical analysis was carried out on parametric images via probability density function estimation of histogram of the MBF intensity, by fitting a generalized gamma distribution (10).

Comparison of each patient’s data during treatment was conducted using her corresponding data acquired prior to the treatment administration onset, as the baseline. The values of each quantitative parameter for clinically and pathologically determined responders and non-responders were compared independently for each time. Statistical analysis using a t-test (unpaired, two-sided, 95% confidence) was carried out to assess if patients demonstrating statistically significant changes in the quantitative ultrasound parameters correlated to the ultimate treatment response. Discriminant analysis (PASW Statistics 18, SPSS, Inc., Chicago, IL) was used to determine which quantitative parameter better discriminate between responders and non-responders. Sensitivity and specificity were calculated to measure the performance of the ultrasound treatment-response classification method in comparison to clinically and pathologically determined responses.
Results

Patient Characteristics and Clinical/Pathological Treatment Response

Characteristics of the participating patients, as well as their tumour properties, and the treatments administrated are summarized in Table 1A. The patients had an average age of 47 years (SD=9.5, range: 33-72). The average size of the largest tumour dimension was 8.1 cm (SD=2.9, range: 3-13). Among the 24 patients, eleven had tumours with positive estrogen and/or progesterone receptors (ER/PR+), while nine had a Her-2-Neu positive (HER2+) status. The majority of patients received combined anthracycline and taxane based chemotherapy.

Clinical/pathological responses of the patients are given in Table 1B. Patients 1, 4, 5, 7, 8, 10, 13, 14, 15, 16, 18, 19, 20, 21, 23, and 24 had either a complete pathological response, or had substantial reductions in their tumour size along with decreases in tumour cellularity, and were categorized as responders. In the case of patients 2, and 17 the reduction in physical tumour or mass size was less than 30%, however residual tumour cellularity was very low and these patients were clinically/pathologically classified as responders. Patients 6, 9, 11, 12, and 22 were non-responses. They demonstrated progressive disease, or their tumour size only slightly changed during treatment. In the case of patient 3, whereas the reduction in tumour size was slightly more than 30%, the tumour cellularity in the residual mass remained very high. As such, this patient was also classified as a non-responder clinically and pathologically.
Quantitative Ultrasound Evaluation of Treatment Response

Representative ultrasound B-mode images, and the spectral parametric images, acquired from a responding patient with locally advanced breast cancer tumour prior to the start of chemotherapy onset, and after four weeks of treatment, are presented in Figure 1A. An overall increase in the ultrasound spectral backscatter power was detectable within the tumour region, as also presented in the MBF and 0-MHz parametric images. Normalized power spectra and generalized gamma fits on the histograms of the MBF intensity for the representative tumour region is also presented in Figure 1B. Figure 1C demonstrates representative 0-MHz parametric images corresponding to non-responder and responding patients, acquired from the same nominal breast tumour regions during treatment. Whereas considerable changes were visualized over the course of treatment in the parametric images that correspond to the responding patients, no such striking change was observed in the case of the non-responding patients. Representative light microscopy images of whole-mount histopathology obtained following modified radical mastectomy surgery are given in Figure 2, for a responding and a non-responding patient. Contrary to the case of the responding patient, the whole-mount histopathology sample corresponding to the non-responding patient indicates a large compact residual mass in the mastectomy specimen, in both haematoxylin and eosin (H&E) stained slide and the slide stained to highlight positive estrogen receptor (ER+) areas. There were differences also observed in the trend of their corresponding MBF parameters, measured over the course of treatment for these patients. Whereas the responding patient exhibited an average increase of up to ~19 dBr (logarithmic unit for normalized power relative to a reference phantom) in MBF at weeks 4 to 8 of chemotherapy, the non-responding patient did not demonstrate such a considerable change (only up to ~5 dBr) in this quantitative parameter during the course of treatment.
Average data obtained from treatment responding and non-responding patients over the course of treatment is presented in Figure 3. The results indicate a substantial increase in the ultrasound spectral backscatter power acquired from the patients that responded to the treatment. Mean increases in MBF in responding patients were measured as 3.5 ± 1.1 dBr, 9.1 ± 1.2 dBr, 8.6 ± 1.4 dBr, 1.2 ± 2.3 dBr in the first, fourth, eighth week of treatment, and pre-operatively, respectively. Results from patients who did not respond to chemotherapy were measured as 0.3 ± 1.9 dBr, 1.9 ± 1.1 dBr, 3.3 ± 1.5 dBr, -1.2 ± 4.3 dBr in the first, fourth, eighth week of treatment, and pre-operatively, respectively. The two patient populations exhibit a statistically significant difference (unpaired t-test, two-sided, $\alpha=0.05$) in changes of MBF, at week 4 ($p=0.005$) and with less significance at week 8 ($p=0.046$) of chemotherapy. Results obtained for the 0-MHz intercept parameter followed similar trends. Mean increases of 4.0 ± 1.4 dBr, 8.9 ± 1.9 dBr, 10.8 ± 2.4 dBr, 2.4 ± 2.1 dBr were measured in positively responding patients, in the first, fourth, eighth week of treatment, and pre-operatively, respectively. Results corresponding to the non-responding patients were measured as -1.3 ± 1.4 dBr, 1.6 ± 0.9 dBr, 1.4 ± 2.7 dBr, 0.6 ± 3.2 dBr in the first, fourth, eighth week of treatment, and pre-operatively, respectively. Statistical analysis of the 0-MHz intercept data indicated a statistically significant difference in the changes observed in the two patient populations, at the fourth ($p=0.041$) and eighth ($p=0.046$) week of treatment. The spectral slope parameter remained almost invariant within the two patient populations and was not shown to be a statistically significant parameter.

Statistical analysis of the MBF parametric images employing the generalized gamma probability density function also indicated increases in the parameters examined. Results for the generalized gamma $\alpha$ parameters (which can be linked to the effective scatterer cross-section (30)) implied increases of 3.0 ± 0.5 A.U. (arbitrary unit), 6.0 ± 1.3 A.U., 14.8 ± 3.0 A.U., 4.0 ±
1.2 A.U. in positively responding patients in the first, fourth, eighth week of treatment, and preoperatively, respectively. Non-responding patients had measured increases of 2.3 ± 0.8 A.U., 4.1 ± 1.7 A.U., 3.5 ± 1.9 A.U., 3.3 ± 0.9 A.U. in the first, fourth, eighth week of treatment, and preoperatively, respectively. Statistical analysis performed on the changes observed in the generalized gamma $a$ parameter indicated a statistically significant difference in the eighth week of treatment ($p=0.046$). The changes in the generalized gamma $c/v$ parameter (which can be linked to the effective scatterer number density (30)) were not found to be statistically significantly different between the two treatment groups.

Linear discriminant analyses (Figure 4) were performed to evaluate the separability of the two patient populations based on their clinical/pathological response to the treatment, using the changes in the quantitative ultrasound parameters measured in fourth and eighth week of treatment. Results are summarized in Table 2. For the linear combination of MBF and 0-MHz intercept, the analyses resulted in sensitivities (percentage of non-responders identified correctly) and specificities (percentage of responders identified correctly) of 100.0% and 83.3% at week 4, and 100% and 66.7% at week 8, respectively. Figure 4 demonstrates the scatter plots of the patient data in the MBF and 0-MHz intercept feature plane where the determined borders of the treatment response classes has been demonstrated by dashed lines. The plots demonstrate separations on responders versus non-responding patients at weeks 4 and 8 that were statistically significant ($p=0.02$), and approaching statistical significance ($p=0.08$), respectively.
Discussion

The results presented in this study demonstrate for the first time that conventional frequency ultrasound may be clinically used to evaluate patient responses to cancer therapy regimens, non-invasively. This study monitored 24 women through the course of their neo-adjuvant chemotherapy treatment. Changes in the quantitative ultrasound spectral parameters were measured for each patient over the course of treatment. In contrast to the clinical/pathological non-responding patient population, considerable increases were observed in the patient population which responded to the treatment, according to the clinical/pathological reports. The fact that patients who responded poorly to treatment exhibited slight increases in tumour echogenicity (implied by small increases in MBF and 0-MHz intercept parameters) is not surprising, as it is likely due to the fact that there was some limited response to chemotherapy which was detected. Statistically significant differences were exhibited between the two treatment response populations by the determined spectral and statistical parameters, after four and eight weeks of treatment, respectively, but not as early as one week. This reflects observations in the clinic in which patients with an ultimately positive response to treatment may exhibit changes in tumour morphology on the macroscopic level within the first few weeks of treatment.

Linear discriminate analysis conducted suggested a favourable separability of two treatment response populations using quantitative ultrasound parameters acquired at weeks 4 and 8 of chemotherapy. The combination of MBF and 0-MHz intercept parameters distinguished between clinical responders and non-responding patients with 100% sensitivity and 83.3% specificity at week 4, and 100% sensitivity and 66.7% specificity at week 8. These promising results imply that quantitative ultrasound spectral parameters can be applied for the early prediction of
ultimate treatment response in patients undergoing cancer-targeting therapies. Such an early prediction could be used to facilitate the critical decision of switching to a more effective therapy for the treatment refractory patients, early during the course of treatment.

Changes in the quantitative ultrasound spectral parameters from the baseline are expected to mainly show the development of response (apoptotic cell death) for each patient (as further discussed below). At the eighth week of treatment, the non-responding patients may exhibit a late slight response to the treatment. In addition, a number of partial responders may have their tumour cells repopulated in partial regions demonstrating small response levels. As such having a relatively less separability between responding and non-responding patient populations can be expected. Results obtained in this study (Figure 3) demonstrated a lesser separability between these two patient populations prior to surgery (pre-op). This may be happening due to the fact that at this time, the neo-adjuvant chemotherapy has been stopped for several weeks, and thus minimal cell death is expected. Also the complete pathological responders, who have no residual tumour left in ultrasound scans, are not expected to show response and are excluded from the analysis at that time.

Previous in vitro and in vivo investigations of ultrasound-based cell death detection support the results presented in this study. The previous studies include those investigations where apoptosis was induced in cells and normal-tissues using a variety of modalities, and were analyzed using ultrasound data (7–14). It was demonstrated, that nuclear condensation by the induction of apoptotic death can result in increases in ultrasonic backscatter signal intensity, which is consistent with observations in this study. One might anticipate that measurable changes in backscatter characteristics from micron-sized particles are not expected at low-frequencies, essentially because of the weak scattering strength of small-size scatterers. However, bulk
changes in tissue associated with tumour cell death are principally related to alterations in ensembles of cells and nuclei smaller than the wavelength of the ultrasound in the low- to mid-frequency range (near 10 MHz). Cell death introduces significant alterations in nuclear structures within these ensembles, in addition to cellular changes in elasticity and viscosity as well as density (13). Acoustic properties of such ensembles are influenced by all of these alterations, affecting ultrasound backscatter characteristics, consequently. The potential scatterers are about 10 times smaller than the interrogating wavelength (10-30 µm versus 100-200 µm), with sizes closer to those that predominate in the Rayleigh scattering regime (related to $f^4$, where $f$ is frequency) (31). Speckle patterns still forming at these low frequencies also suggest that several sub-resolution scatterers contribute to the detected signals. Another factor which can also influences ultrasound backscatter characteristics is the spatial organization of cellular-based scatterers (32) which can be altered with cell death. Banihashmei et al. demonstrated that these sub-resolution scatterers can affect ultrasound at low-frequency with cell death (13) and evidence for the role of cell death related nuclear changes is summarized there.

In this study changes in the MBF and the 0-MHz intercept followed general trends experimentally observed for higher ultrasound frequencies. Effects of larger scattering structures can affect the spectral slope and its invariance suggests than both small and large scattering structures play a role at these frequencies. We have previously demonstrated slope changes but only at high ultrasound frequencies (>20 MHz) when small scattering structures change their size (13). Attenuation was accounted for in this study by a sliding window normalization process against a tissue-mimicking phantom under similar scan settings for every scan. In addition, the 0-MHz intercept, sensitive to the concentration of acoustic scatterers was determined parametrically for scans as it is believed to be free of attenuation effects (16).
Applications of other non-invasive imaging modalities for cancer treatment response monitoring have been investigated in previous studies, including those based on positron emission tomography (PET), magnetic resonance imaging, or diffuse optical imaging and spectroscopy (DOI/DOS) (6,33–35). Unlike PET and MRI based methods, the ultrasound method here relies on inherent contrast changes arising from changes in acoustical properties as cancer cells die, hence does not need the injection of any external contrast agents. Diffuse optical spectroscopy has recently been used to demonstrate the capability of distinguishing between treatment responsive and non-responding patients at the fourth week of chemotherapy (34). However, its lower resolution may lead to uncertainties for determining the tumour boundaries, specially in the case of smaller tumours. Ultrasound has the advantages of low cost, rapid imaging speed, high resolution, and portability and can access tumour location not easily visualized with that modality.

In conclusion, this study indicates for the first time that quantitative conventional-frequency ultrasound can be clinically used to monitor treatment response in patients receiving cancer-targeting chemotherapy. Obtained results indicate that contrary to the case of treatment-refractory patients where quantitative ultrasound parameters were relatively invariant the quantitative ultrasound parameters demonstrated a considerable change for the patients who responded to treatment. Statistically significant differences were found after four to eight weeks of chemotherapy onset. Parameters were also found to have a favourable sensitivity and specificity to identify patients with poor ultimate response to the therapy, early following the treatment onset. As such this work is a substantial forward step towards clinical application of quantitative ultrasound as early surrogate of ultimate treatment response for cancer patients. Such a surrogate may facilitate personalized cancer therapy where an inefficient treatment
regimen for a particular patient is switched to a more effective one, early after the therapy initiation, or early salvage treatment is undertaken. Nevertheless, investigations on larger cohorts of patients will be required in order to assess the efficacy of the proposed technique for distinguishing between sub-types of treatment response, and to further evaluate the robustness of the technique in clinic.
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References


Table and Figure Captions

Table 1- A: Patient Characteristics. B: Ultimate responses of patients according to the clinical and pathological reports.

Table 2- Results of the discriminant analyses performed at weeks 4 and 8.

Figure 1- A: Representative data from a large breast tumour before starting the neo-adjuvant chemotherapy (first row), and after 4 weeks of treatment (second row). The columns from left to right demonstrate ultrasound b-mode, and parametric images of MBF, 0-MHz intercept, and spectral slope, respectively. The scale bar is ~1 cm, and the colour map represents a scale encompassing ~50 dBr for MBF and 0-MHz intercept, and ~15 dBr/MHz for the spectral slope. B: Normalized power spectra (left) and generalized gamma fits on the histograms of the MBF intensity (right) for the tumour region. C: Representative parametric images of 0-MHz intercept from a non-responding patient (first row), as well as from two patients which responded to the treatment (second and third rows). The data for each patient was acquired from the same nominal regions, prior to treatment, as well as at weeks 1, 4, and 8 during treatment, and pre-operatively from left to right, respectively. The scale bar represents ~1 cm. The colour bar represents a scale encompassing ~80 dBr.
**Figure 2**- Representative data obtained from a non-responder (left) and a responding patient (right). Both patients were initially confirmed with an ER+ status according to the pathology reports on core biopsy samples. A-C: Light microscopy images of whole-mount histopathology slides obtained following modified radical mastectomy surgery. The scale bars are ~1 cm in A and B, and ~200 µm in C. A: H&E stained slides. B: The immunohistochimistry-stained slides highlighting ER+ areas. C: high magnification images of the areas marked with rectangles in B. Contrary to the case of the responding patient, the whole-mount pathology sample corresponding to the non-responder patient indicates a large compact residual mass in the mastectomy specimen. D: Results for the MBF measured in the same patients over the course of treatment. Data were measured prior to treatment onset, at weeks 1, 4 and 8 during treatment and preoperatively.

**Figure 3**- Average data obtained from treatment responding and non-responding patients during treatment, for the MBF, 0-MHz intercept, and spectral slope, in addition to a and c/ν parameters of the generalized gamma fit of the histogram of the MBF intensity. Data were measured prior to treatment onset, at weeks 1, 4 and 8 during treatment and preoperatively. Blue lines display results obtained from patients who were clinically/pathologically categorized as non-responders, whereas red lines display results obtained from responding patients. Error bars represent ± one standard error.

**Figure 4**- Scatter plot of the MBF and 0-MHz intercept data acquired at A: week 4 and B: week 8 of the chemotherapy treatment. Responsive and non-responding patients have been classified in the feature plane via a linear discriminant analysis, where the determined border of classes has been demonstrated by a dashed line.
Figure 2

A

B

C

D

18
12
6
0
-6
-12

18
12
6
0
-6
-12

Pre-Tx

Week 4

Week 8

Post-Tx

NR

R
Figure 4

Graph A: Scatter plot showing the relationship between 0-MHz intercept (dBr) and mid-band fit (dBr) for NR and R categories.

Graph B: Scatter plot similar to Graph A, but with different data points and scales.
**Table 1**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Menopausal status</th>
<th>Pre-treatment Tumor dimensions (AP x ML x Sl in cm)</th>
<th>Histology</th>
<th>Grade</th>
<th>ER/PR</th>
<th>Her-2-neu</th>
<th>Neoadjuvant Treatment</th>
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<td>5.4 x 5 x 2.3</td>
<td>ductal</td>
<td>N/A</td>
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<td>+</td>
<td>FEC + paclitaxel, trastuzumab</td>
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<td>+</td>
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<td>Epirubicin, docetaxel</td>
</tr>
<tr>
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<td>7</td>
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<td>+</td>
<td>Docetaxel, carboplatin, trastuzumab</td>
</tr>
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<td>4 x 5</td>
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<td>N/A</td>
<td>+</td>
<td>+</td>
<td>AC + docetaxel, trastuzumab</td>
</tr>
<tr>
<td>5</td>
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<td>7 x 8</td>
<td>ductal</td>
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<td>+</td>
<td>-</td>
<td>AC + paclitaxel</td>
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<td>N/A</td>
<td>+</td>
<td>+</td>
<td>AC + docetaxel, paclitaxel, trastuzumab</td>
</tr>
<tr>
<td>7</td>
<td>33</td>
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<td>2</td>
<td>+</td>
<td>-</td>
<td>AC + docetaxel</td>
</tr>
<tr>
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<td>2</td>
<td>+</td>
<td>-</td>
<td>AC + paclitaxel</td>
</tr>
<tr>
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<td>4.4 x 3.4</td>
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<td>3</td>
<td>-</td>
<td>-</td>
<td>AC + paclitaxel</td>
</tr>
<tr>
<td>10</td>
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<td>2</td>
<td>+</td>
<td>-</td>
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<tr>
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<td>3</td>
<td>-</td>
<td>-</td>
<td>AC cisplatinum, gemcitabine platinum</td>
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<td>3</td>
<td>-</td>
<td>-</td>
<td>AC + paclitaxel</td>
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<td>3</td>
<td>-</td>
<td>+</td>
<td>Docetaxel, trastuzumab</td>
</tr>
<tr>
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<td>49</td>
<td>Premenopausal</td>
<td>3 x 2.4 x 3</td>
<td>ductal</td>
<td>3</td>
<td>+</td>
<td>-</td>
<td>AC + paclitaxel, trastuzumab</td>
</tr>
<tr>
<td>15</td>
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<td>2</td>
<td>+</td>
<td>-</td>
<td>AC + paclitaxel, trastuzumab</td>
</tr>
<tr>
<td>16</td>
<td>49</td>
<td>Premenopausal</td>
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<td>2</td>
<td>+</td>
<td>-</td>
<td>FEC + docetaxel</td>
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<td>9 x 6.6 x 6</td>
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<td>+</td>
<td>-</td>
<td>AC + paclitaxel</td>
</tr>
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<td>18</td>
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<td>Premenopausal</td>
<td>13 x 12</td>
<td>ductal</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>AC + docetaxel</td>
</tr>
<tr>
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<td>36</td>
<td>Premenopausal</td>
<td>12.5</td>
<td>ductal</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>AC + paclitaxel</td>
</tr>
<tr>
<td>20</td>
<td>72</td>
<td>Postmenopausal</td>
<td>10 x 8</td>
<td>ductal</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>AC + paclitaxel</td>
</tr>
<tr>
<td>21</td>
<td>72</td>
<td>Premenopausal</td>
<td>8 x 10</td>
<td>ductal</td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>AC + paclitaxel</td>
</tr>
<tr>
<td>22</td>
<td>47</td>
<td>Premenopausal</td>
<td>7.9 x 4.1 x 5.5</td>
<td>ductal</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>AC + paclitaxel</td>
</tr>
<tr>
<td>23</td>
<td>57</td>
<td>Premenopausal</td>
<td>6.3 x 4.1 x 7.4</td>
<td>ductal</td>
<td>N/A</td>
<td>-</td>
<td>+</td>
<td>AC + paclitaxel, trastuzumab</td>
</tr>
</tbody>
</table>

**AC:** Adriamycin and Cytoxan  
**FEC:** Fluorouracil (5FU), epirubicin and cyclophosphamide

**Table 2**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Residual tumor dimensions (AP x ML x Sl in cm)</th>
<th>Notes</th>
<th>Clinical / Pathological Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N/A</td>
<td></td>
<td>Good</td>
</tr>
<tr>
<td>2</td>
<td>7 x 5 x 3</td>
<td>Carcinoma with mucinous features; Very low cellularity</td>
<td>Good</td>
</tr>
<tr>
<td>3</td>
<td>2.7 x 2.5 x 2.4</td>
<td>Tumor cellularity remains very high</td>
<td>Poor</td>
</tr>
<tr>
<td>4</td>
<td>1.6 x 0.8 x 0.5</td>
<td>Good response</td>
<td>Good</td>
</tr>
<tr>
<td>5</td>
<td>N/A</td>
<td>Complete pathological response</td>
<td>Good</td>
</tr>
<tr>
<td>6</td>
<td>3 x 6.4 x 3.5</td>
<td>High grade invasive ductal carcinoma</td>
<td>Poor</td>
</tr>
<tr>
<td>7</td>
<td>N/A</td>
<td>Complete pathological response</td>
<td>Good</td>
</tr>
<tr>
<td>8</td>
<td>1.4 x 1 x 1</td>
<td>Small volume of invasive tumor remaining</td>
<td>Good</td>
</tr>
<tr>
<td>9</td>
<td>11.4</td>
<td>Extensive residual disease</td>
<td>Poor</td>
</tr>
<tr>
<td>10</td>
<td>N/A</td>
<td>Complete pathological response, with only fibrous tumour bed remaining</td>
<td>Good</td>
</tr>
<tr>
<td>11</td>
<td>6.5 x 3 x 7.3</td>
<td>Invasive ductal carcinoma remaining</td>
<td>Poor</td>
</tr>
<tr>
<td>12</td>
<td>All the breast</td>
<td>Residual tumor took up all the breast; no response</td>
<td>Poor</td>
</tr>
<tr>
<td>13</td>
<td>N/A</td>
<td>Good response</td>
<td>Good</td>
</tr>
<tr>
<td>14</td>
<td>2 x 1.5 x 1</td>
<td>Complete pathological response, with only in situ disease remaining</td>
<td>Good</td>
</tr>
<tr>
<td>15</td>
<td>0.2 x 0.2</td>
<td>Complete pathological response, with only in situ disease remaining</td>
<td>Good</td>
</tr>
<tr>
<td>16</td>
<td>3.5 x 2.3 x 1.3</td>
<td>Only in-situ carcinoma with one focus of micro-invasion (&lt;=0.1 cm)</td>
<td>Good</td>
</tr>
<tr>
<td>17</td>
<td>6.5</td>
<td>Exceedingly low cellularity, thus overall tumor volume is also very low</td>
<td>Good</td>
</tr>
<tr>
<td>18</td>
<td>2.9 x 2 x 1.5 &amp; 2 x 1.5 x 1</td>
<td>Tumour cellularity is low</td>
<td>Good</td>
</tr>
<tr>
<td>19</td>
<td>8 x 7.5 x 6</td>
<td>Good reduction in the tumor size</td>
<td>Good</td>
</tr>
<tr>
<td>20</td>
<td>N/A</td>
<td>Complete pathological response</td>
<td>Good</td>
</tr>
<tr>
<td>21</td>
<td>6.5 x 5.5</td>
<td>Good reduction in the tumor size</td>
<td>Good</td>
</tr>
<tr>
<td>22</td>
<td>12.5 x 4.5 x 3.5</td>
<td>No definite response</td>
<td>Poor</td>
</tr>
<tr>
<td>23</td>
<td>N/A</td>
<td>No residual invasive carcinoma in the breast, only lymphovascular invasion remaining</td>
<td>Good</td>
</tr>
<tr>
<td>24</td>
<td>N/A</td>
<td>Complete pathological response, only scattered in situ component remaining</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td></td>
<td>Week 8</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------</td>
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<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
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<td>Mid-band Fit</td>
<td>100%</td>
<td>72.2%</td>
<td>83.3%</td>
</tr>
<tr>
<td>0-MHz Intercept</td>
<td>100%</td>
<td>77.8%</td>
<td>83.3%</td>
</tr>
<tr>
<td>Slope</td>
<td>66.7%</td>
<td>38.9%</td>
<td>50%</td>
</tr>
<tr>
<td>Parametric MBF GG $a$</td>
<td>66.7%</td>
<td>38.9%</td>
<td>100%</td>
</tr>
<tr>
<td>Parametric MBF GG $c/v$</td>
<td>66.7%</td>
<td>66.7%</td>
<td>66.7%</td>
</tr>
<tr>
<td>Mid-band Fit &amp; 0-MHz Intercept</td>
<td>100%</td>
<td>83.3%</td>
<td>100%</td>
</tr>
</tbody>
</table>
Quantitative Ultrasound Evaluation of Tumour Cell Death Response in Locally Advanced Breast Cancer Patients Receiving Chemotherapy


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