Chemical Exchange Saturation Transfer MRI for Detection of Cell Death
in Breast Cancer Xenografts

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Detecting cell death after chemotherapy could help optimize treatment regimens and improve outcomes. Breast cancer xenografts (MDA-MB-231) were scanned before and after chemotherapy to investigate parameters of chemical exchange saturation transfer (CEST) MRI that can differentiate regions of cell death from viable tumour. The CEST effect at 0.5 μT saturation amplitude was measured using the magnetization transfer ratio (MTR) at 1.8 and -3.3 ppm frequency offsets. An MTR cutoff of 0.12 at 1.8 ppm was able differentiate between viable tumour and cell death regions (p<0.0001) and the detected patterns of cell death closely matched those detected with ISEL staining. Using this cutoff, the mean increase in cell death index (± standard error of the mean) after chemotherapy was 4±4%, 10%±7%, 10±8%, and 4±9% at 4, 8, 12, and 24 h, respectively. These results suggest that CEST MRI can detect cell death from chemotherapy in breast cancer.
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Chapter 1
Background

1.1 Introduction

As with any medical procedure, treatments for cancer come with associated risks of side effects. These side effects are caused by the cytotoxicity of modern treatments like radiotherapy and chemotherapy. Because the primary mode of cell death is through DNA damage, rapidly dividing tissue is more sensitive to these therapies than is more indolent tissue. With careful dose titration, healthy cells can be allowed to recover while cancerous cells are unable to do so leading, hopefully, to eradication of the cancer and ultimate cure.

Many cancers, including locally advanced breast cancer and rectal cancer, are now routinely treated with so-called “neoadjuvant” regimens whereby some treatments (such as chemotherapy and/or radiotherapy) are given prior to surgery, instead of afterwards as in more traditional treatment methods. These neoadjuvant treatments may be given to attempt to shrink the tumour to permit a less extensive, and therefore less debilitating, surgery to be performed. For example, a combination of neoadjuvant chemotherapy and radiation therapy for rectal cancer increases the chance that a sphincter-sparing surgery can be performed, which significantly improves patients’ quality of life after treatment. Neoadjuvant treatment is also associated with less disease recurrence compared to administering chemotherapy and radiation post-operatively. Similarly, neoadjuvant chemotherapy given for breast cancer can increase the likelihood that a breast-conserving surgery can be pursued, although survival has not yet been shown to improve with neoadjuvant chemotherapy compared with post-operative administration. Methods to predict the ultimate tumour response to these neoadjuvant therapies early in the treatment course (or even in advance of beginning treatment) would permit ineffective therapies to be switched for more effective ones, increasing the chance of cure and avoiding potentially debilitating side effects with no clinical benefit.

Monitoring or predicting the response of cancer during and after the course of treatment could also provide prognostic information for expected survival after completion of therapy. Large
studies have shown that breast cancer patients who achieve a complete pathological response to neoadjuvant chemotherapy (defined as no presence of cancer either in the breast or regional lymph nodes at the time of surgery) experience significantly improved survival compared with patients who have residual cancer after neoadjuvant chemotherapy.\textsuperscript{5-7} This association is considered to be robust enough that ongoing clinical trials are now using complete response as a surrogate endpoint for survival to shorten the time it takes to obtain and present clinical data. As well, given good survival outcomes after complete response to chemotherapy, randomized trials are now investigating whether post-operative radiotherapy can be safely omitted in patients who achieve a complete response to neoadjuvant chemotherapy.

Standard magnetic resonance imaging (MRI) techniques, such as T1 and T2-weighted sequences, can measure tumour size and demonstrate macroscopic changes in tissue structure, but they lack the ability to detect physiologic data or microscopic changes in tumour composition.\textsuperscript{8} Traditionally, monitoring response of cancer to therapy is done by physical exam and/or radiographically: the tumour is initially measured prior to treatment, such as via physical examination, ultrasound, or MRI. The treatment is then administered, which can last up to 4-6 months for modern chemotherapy regimens, with additional time added for the patient to recover from side effects and to allow for immunologic removal of the dead tissue. Afterward, a repeat test is performed and the size is compared to measurement obtained before treatment; if the tumour has shrunk then a response (or non-response) is defined using criteria such as the Response Evaluation Criteria in Solid Tumours (RECIST).\textsuperscript{9} Monitoring the patient for onset of new signs and symptoms may also suggest that disease has spread (for example, new skin changes or pain). Clearly, many months may elapse before a definite determination of response can be established using tumour size measurements. If the treatment ends up being ineffective, the patient may have suffered toxicity for little benefit and tumour proliferation in the meantime may have reduced or even eliminated the chance of cure.\textsuperscript{10}

Some studies have been carried out involving mid-treatment imaging to detect early changes in tumour size and adjust treatment according, but anatomic imaging retains the downside of requiring significant time before adequate treatment assessment can take place.\textsuperscript{11} One such study performed serial contrast-enhanced MRI imaging on 33 patients undergoing three cycles of neoadjuvant chemotherapy before initiation of therapy, after the first cycle and just prior to
surgery. That study suggested that a measured reduction of 8.8% or lower after the first cycle of chemotherapy in the sum of largest diameters of the tumour as measured on MRI could be used as a cutoff to identify patients who will likely not respond to chemotherapy. However, among the 10 patients in this cohort who had an early size reduction of 8.8% or less, 2 patients eventually became responders. The fact that contrast enhancement can be seen both in viable tumour and in post-chemotherapy sclerosis could contribute to the unreliability of anatomical monitoring methods. Response prediction methods that are more reliable and become apparent earlier in the treatment course are needed.

1.2 Physiologic Imaging for Treatment Response Monitoring

1.2.1 Non-MRI-Based Techniques

Physiologic imaging techniques have been studied in an attempt to detect changes earlier and predict response to therapy earlier in the treatment course than anatomic methods. One such modality is positron emission tomography (PET), which is based on the introduction of a positron-emitting radionuclide into the body. When a positron is emitted, it rapidly annihilates with a nearby electron, leading to emission of a pair of gamma ray photons in anti-parallel directions. An array then detects the arrival of these photon pairs and, based on the arrival of many different photon pairs at the array, computes the location of emission. If the radionuclide is attached to a molecule with biological activity or which tends to accumulate in a given area of the body, the flow or distribution of the molecule can be tracked by the PET technique.

The most common PET radiotracer is 18-fluoro-deoxyglucose (18F-FDG), which accumulates in active tumour through increased metabolism and consequent increased glucose uptake compared to normal tissue. It is commonly used for staging and restaging solid tumours including gastrointestinal, gynecological, breast, and head and neck malignancies. PET is highly reproducible using the standard uptake value (SUV) parameter to accurately assess tumour glucose uptake. Treatment response monitoring using 18F-FDG PET has suggested a decrease in tumour SUV, which is significantly greater in breast cancer patients who ultimately responded to chemotherapy than those who did not respond after two courses of chemotherapy and
possibly even as early as the first cycle. Response prediction using FDG-PET has been demonstrated for both local disease (which is potentially curable) and metastatic disease. Early changes in SUV with FDG-PET correlate with ultimate response as assessed by both histopathology and conventional size-based measurements using computed tomography (CT) imaging or MRI. Response and survival prediction by changes in FDG-PET uptake have also been demonstrated for esophageal and ovarian cancers.

While FDG-PET is well established in the diagnosis and staging of cancer, concerns regarding sensitivity, specificity and image resolution have arisen since treatment-induced inflammatory responses can cause transient increases in tracer uptake mimicking residual disease. At present, standard FDG-PET is neither sufficiently sensitive nor does it have sufficient resolution to serve as a primary assessment modality for malignancies such as breast cancer. As a result, alternate PET methods have been studied to counteract this effect. The cellular proliferation marker 3’-[F-18]fluoro-3’-deoxythymidine (FLT) accumulates in proliferating cells as it is an analogue of the DNA nucleoside thymidine. A clinical study of 14 patients with breast cancer (metastatic or localized) demonstrated changes in FLT uptake that correlated with ultimate response to treatment measured anatomically via CT. However, FLT-PET is still in the developmental stages and is not widely available.

Ultrasound is a non-invasive imaging technique with a wide range of clinical applications. Standard B-mode ultrasound imaging is useful for visualizing tumours in the breast, prostate, liver, and other areas, but image quality is variable between machines and is dependent on operator skill to obtain high quality images. Tumour measurements and response prediction with such techniques also suffer from the limitations of other anatomical measurement methods, namely the delay between tumour response and measureable changes in size. Quantitative parameters of tumour backscatter properties are independent of the machine operator and consistent between machines, and so represent a promising treatment prediction modality. In preclinical models, quantitative ultrasound (QUS) parameters such as average acoustic concentration (AAC) correlated with cell death after chemotherapy while ultrasound backscatter and spectral slope have been demonstrated to specifically correlate with apoptosis. An early clinical study of 24 patients with locally advanced breast cancer using conventional frequency ultrasound (~7 MHz) used parameters such as mid-band fit and 0-MHz
intercept and reported 100% sensitivity and 83% specificity in discriminating responders from non-responders by the fourth week of neoadjuvant chemotherapy. A more recent clinical study of 100 patients undergoing neoadjuvant chemotherapy for breast cancer demonstrated that QUS parameters estimating the diameter of acoustic scattering elements within the tumour as well as their concentration and spacing between them can predict ultimate treatment response to neoadjuvant chemotherapy with greater than 80% accuracy as early as 1 week into therapy. Those patients classified as responders on the basis of QUS had significantly improved survival compared with patients classified as non-responders.

Although optical imaging is an older concept, having been studied for breast cancer as early as 1929, it has recently been revived as new technological and computing advances have promised improvements in sensitivity and specificity. Near-infrared diffuse optical spectroscopy (IR DOS) measures near infrared absorption and derives functional images of oxyhemoglobin, deoxyhemoglobin and water concentration, which correlate in general with levels of angiogenesis, tumour proliferation, and hypoxia. Optical spectroscopy thus provides potential for non-invasive functional image without the need for exogenous contrast. While breast cancers have been shown to contain up to twice the hemoglobin concentration as normal breast tissue, some benign neoplasms such as fibroadenomas may have similar optical characteristics as malignant tumours. Small clinical studies have shown significant differences in the concentration of oxy- and deoxyhemoglobin as well as water between breast cancer patients who respond to neoadjuvant chemotherapy and those who do not respond. One study of 10 breast cancer patients demonstrated differences in some optical parameters (scattering power and concentrations of oxyhemoglobin and deoxyhemoglobin), but not in water content, between responders and non-responders 4 weeks after chemotherapy. Another study of 11 patients with breast cancer suggested that DOS can predict eventual response to neoadjuvant chemotherapy with 100% specificity and sensitivity only 1 week after treatment start.
1.2.2 MRI-Based Techniques

Morphological MRI (such as T1 and T2-weighted imaging) can show tumour size and macroscopic tumour characteristics.\textsuperscript{8} In a comparison with clinical examination, mammography, and ultrasound, morphological MRI has shown better agreement with pathological response.\textsuperscript{29} However, morphological MRI may tend to overestimate that response, as shown in one study in which MRI-based response assessment was discordant with all pathologic non-responders while it showing better agreement with patients who did respond to treatment.\textsuperscript{30}

Magnetic resonance spectroscopy (MRS) can help diagnose breast cancer based on the detection of choline-containing compounds, which may correlate with active cell replication. Several small prospective trials have assessed MRS as a predictive marker for tumour response. In one trial of 20 women receiving doxorubicin-based neoadjuvant chemotherapy, patients underwent an MRS scan before the start of treatment as well as after 1-2 cycles and after 4 cycles of neoadjuvant chemotherapy. Patients were then categorized as responders or non-responders based on change in tumour size as assessed by gadolinium-enhanced MRI scanning. At the first follow-up (after 1-2 cycles of chemotherapy), significant reductions in both tumour size and the amount of MRS-detected choline-containing compounds were seen for patients characterized as responders to therapy while no significant change was seen for the non-responder group. This study also concluded that metabolic changes, as measured by MRS, were greater than changes in morphological size, suggesting that MRS-detected changes may become evident sooner, allowing for earlier prediction of ultimate response using these techniques.\textsuperscript{31} Another study of 16 women with breast cancer suggested that MRS may be able to detect significant changes and predict ultimate response as early as 24 h after chemotherapy administration.\textsuperscript{32} Pre-clinical work has suggested that MRS can be used to monitor changes in tumour lactate levels which can act as an early indicator of response.\textsuperscript{33} Despite this early promise, the applicability of MRS to clinical situations is potentially limited, however, by poor spatial resolution and limited availability and expertise in the technology by clinical radiologists.\textsuperscript{11,34,35}

Dynamic contrast-enhanced (DCE) MRI can produce high-resolution images depicting perfusion and the permeability of capillaries within a tumour.\textsuperscript{36} DCE-MRI can differentiate between non-vascularized fibrosis and viable tumour.\textsuperscript{37,38} DCE-MRI parameters have also been shown to be
superior predictors of breast cancer response to chemotherapy than tumour size. One study of thirty women undergoing neoadjuvant chemotherapy for breast cancer found that DCE-MRI assessment after two cycles of chemotherapy yielded 93% accuracy in predicting patients who would achieve pathological complete response. Another study found 94% sensitivity and 82% specificity with DCE-MRI when assessed after two cycles of chemotherapy. Tumour size measured by MRI did not predict response in this study. A larger study of 188 breast cancer patients investigated whether response prediction using DCE-MRI would vary based on the subtype of breast cancer as defined by hormone and HER2-neu receptor status. This study found that DCE-MRI was an effective predictor of ultimate response for hormone receptor negative breast cancer (HER2-neu positive or negative), but not for hormone receptor positive cancers.

Notable limitations of DCE-MRI include the need for injected contrast, delays of up to several weeks following chemotherapy to allow sufficient changes to be seen and the relative difficulty with image analysis which makes it not well-suited for routine clinical use. Studies have shown variable sensitivity and specificity of DCE-MRI for evaluation of cancer, questioning the reliability of the technique in applications like breast and prostate cancer.

Cost and difficulty with administration of contrast agents has led to discovery of endogenous contrast mechanisms capable of creating high quality images without the need for injection of exogenous contrast media. Diffusion-weighted MRI (DW-MRI) measures change in the Brownian motion of water within tumour tissue. Since tumours cells restrict diffusion of fluid in tissue, a decrease in cellularity (such as caused by cytotoxic therapy like chemotherapy or radiation) should lead to an increase in diffusion, which can be represented by the apparent diffusion coefficient (ADC). Thus, ADC values increase after cytotoxic therapy. Study of ADC in liver metastases after chemotherapy treatment suggests that ADC values in tumours that ultimately responded to treatment significantly differ from baseline as early as 4 or 11 days after treatment (depending on the analysis used), while those that do not respond never show a difference compared to baseline values. Other studies have confirmed that ADC changes measured as early as after the first chemotherapy cycle can predict ultimate tumour response in breast and rectal cancer.
However, ADC is sensitive to different types of tissue changes, not only those related to cell death. Edema or inflammation, which may be induced in normal tissues by cancer treatments, also affect ADC, limiting its utility as a cancer treatment monitoring technique. As well, many new molecular targeted agents such as tyrosine kinase inhibitors and angiogenesis inhibitors are primarily cytostatic in action, arrest cell development and proliferation rather than directly killing cells. This lack of cytotoxicity and subsequent decrease in overall cellularity may inhibit the ability of diffusion-weighted imaging to predict response to such new treatment methods. Paradoxical findings of decreased ADC in rectal tumours that respond to chemotherapy have also been reported, perhaps suggesting a lack of consistency in response between tumour sites or even individual tumours.

As described above, significant effort has been made to develop imaging-based monitoring techniques for tumour response. While early trials of several such modalities have shown promise, most published trials are based on small samples of tumours and questions of sensitivity, specificity, inter-rater reliability, and spatial resolution remain. Other drawbacks include the requirement for injectable contrast media before each scan, which is expensive, can be associated with potentially severe reactions, and requires additional time from the patient and staff. As well, standard T1 and T2-weighted techniques lack the ability to show microscopic or functional changes in the tumour microenvironment and optimal tumour visualization with T1-weighted imaging requires injection of gadolinium-based contrast agents, increasing costs and requiring clinical monitoring for sensitivity reactions. However, early tumour changes to therapy, including cellular swelling and blood flow restrictions, may cause transient increases in ADC values on DWI, limiting the sensitivity of DWI to predict ultimate tumour response early in a course of treatment. A modality which does not require contrast injection but is sensitive and specific to early cellular changes predictive of cell death across a range of different cancer histologies would offer significant advantages for treatment response monitoring.
1.3 Chemical Exchange Saturation Transfer MRI

Chemical exchange saturation transfer (CEST) MRI is a novel metabolic imaging technique that reflects changes in the tumour microenvironment, such as those brought about by cell death\(^2,3\) (apoptosis or necrosis) or changes in cellular metabolism.\(^4-6\) It thus presents a promising method for detecting tumour growth and predicting response to treatment.\(^2\) The CEST effect derives from mobile hydrogen nuclei (commonly referred to as protons) in solute molecules exchanging rapidly (correlation time \(t_c \sim 10^{-10}\) s)\(^7\) with those in water molecules within a solution either through direct exchange or by the nuclear Overhauser effect (NOE).\(^2\) For \textit{in vivo} systems such as tumours, the main solutes are proteins, which present a variety of chemical microenvironments for the protons, such as amide, amine, or aliphatic groups among others.\(^4,8\) However, because water represents the vast majority of the molecules present in a tumour, most of the protons that contribute to this signal are present on water molecules. When placed in an external magnetic field (such as is present in an MRI scanner), a net magnetization is created in these nuclei.

This contrast mechanism is intrinsic to the cell, and thus no injectable contrast medium is required, reducing costs and eliminating the requirement of medical oversight of an injection process and possible allergic reactions. CEST is also a promising modality for cell death detection because it is sensitive to small changes in metabolite concentration, as the exchange of protons between water and dissolved metabolites creates a large pool of protons that amplify the CEST effect.\(^9\)

When a pulse of radiofrequency (RF) energy is introduced to the system, protons whose resonant frequency matches that of the RF pulse will absorb that energy (termed “saturation”), resulting in attenuation of the net magnetization signal. The solute protons can exchange with those in water multiple times during saturation, so a large pool is created of protons which retain the magnetic properties they possessed when bound to protein and which amplifies the contributions from the solute molecules to the net magnetization. If no protons resonate at the frequency of the RF pulse, no energy is absorbed and the measured magnetization remains unchanged; this is defined as the reference signal strength \(S_0\).\(^2\)
The magnetization transfer ratio (MTR) is a simple metric which combines the contributions from all contrast mechanisms, including CEST and the magnetization transfer contrast (MTC) phenomenon, that can be manipulated by way of radiofrequency (RF) energy absorption and saturation effect. The MTR is defined as

\[
MTR = 1 - \frac{S}{S_0}
\]

where \(S\) is the measured strength of the MRI signal at a specific frequency offset of RF saturation and \(S_0\) is the strength of the MRI signal when no RF saturation is applied. The ratio \(\frac{S}{S_0}\) is called the “normalized signal” value. By varying the RF pulse frequency and recording the net magnetization as a function of frequency, a spectrum known as the CEST spectrum (also known as a Z-spectrum) is generated. Changes in relative concentrations of different solutes and chemical groups, including contribution from large semisolid macromolecules from MTC, can then be characterized and used to differentiate different tissue types. Figure 1 shows a graphical representation of the MTR measurement on a sample Z-spectrum.

The RF absorption and subsequent signal attenuation creates negative peaks in the Z-spectrum which are characteristic of the different absorption frequencies of chemical groups as well as a large peak centered around the resonant frequency of water. The central frequency of a Z-spectrum is the resonant frequency of bulk water, given by the formula

\[
f_0 = \frac{\gamma}{2\pi} B_0
\]

where \(f_0\) is the resonant frequency, \(\gamma\) is the gyromagnetic ratio, and \(B_0\) is the magnetic field strength. However, the resonant frequency of a given proton species, measured in hertz, is dependent on the magnetic field strength of the MRI scanner being used.
Figure 1: Graphical representation of the value of the magnetization transfer ratio (MTR).
This figure shows the MTR value at the 2 ppm offset of a sample Z-spectrum.

Resonant frequencies are given in units of parts per million (ppm), which is independent of MRI field strength, and given by the formula

\[ \Delta = \frac{f_0 - f_{0,\text{ref}}}{f_{0,\text{ref}}} \times 10^6 \]

where \( \Delta \) is the frequency offset (in ppm), \( f_0 \) is the resonant frequency of interest, and \( f_{0,\text{ref}} \) is the resonant frequency of a reference compound. Contrary to MRS (which uses tetramethylsilane as the reference), the convention in CEST analysis is to use water as the reference, so the frequency offset of water is defined as 0 ppm.

In cancerous tissue, the main contributors to the observed peaks within the Z-spectrum are from proteins. While CEST is sensitive to protein content and tissue pH,\(^{56}\) previous research suggests negligible change in tumour pH after treatment, so changes in the Z-spectrum should reflect changing protein concentration and thus be predictive of ultimate treatment response.\(^8\)
Specifically, the main contributors are protons found in amine groups in guanidine groups (e.g., in creatine)\textsuperscript{62} which resonate at a frequency offset around 2 ppm, amide groups from the peptide backbone (3.5 ppm), and aliphatic groups in both the side chains and backbone (-3.5 ppm).\textsuperscript{52,63}

The CEST imaging technique has many of the characteristics of an ideal treatment response mechanism. First, because of the signal amplification effect due to the transfer of protons from water to solute, CEST MRI should be extremely sensitive to small changes in tumour composition, allowing for very early detection of cell death and response to treatment. Second, the quantitative nature of CEST MRI analysis should make analysis mostly independent of the user and operator skill. Third, because it is MRI-based, the lack of ionizing radiation eliminates the risk of inducing second cancers as seen with CT imaging. CEST does not require injection of exogenous contrast, obviating the risk of allergic reactions and the need to monitor for them. And finally, standard assessment and treatment algorithms for many cancers (such as breast, rectal, head and neck, cervix, and prostate) already involve MRI so adding CEST imaging to these pathways is relatively simple and would not significantly increase costs or time.

Clinically, CEST MRI can differentiate between regions of viable tumour and radiation necrosis within the brain.\textsuperscript{53,63} In a preclinical model, Sagiyama et al.\textsuperscript{64} used amide proton transfer (APT) imaging, which is a CEST-based imaging protocol specifically focused on the amide region of the Z-spectrum (3.5 ppm). After administration of temozolomide (TMZ), a standard alkylating chemotherapy agent used to treat glioblastoma multiforme (GBM), to mice with tumours grown using a human GBM cell line, the strength of the APT signal was found to decrease after one dose of TMZ while it continued to increase in untreated controls. This study suggested that APT (and other chemical exchange-based imaging techniques) can be sensitive to changes in tumours induced by chemotherapy. In another pre-clinical study, Zhou et al.\textsuperscript{63} used amide proton transfer (APT) imaging, which is a CEST-based imaging protocol specifically focused on the amide region of the Z-spectrum (3.5 ppm) to study human GBM xenografts. This study demonstrated large differences in APT signal between areas of viable tumour identified by conventional T1-weighted contrast-enhanced MRI imaging and both radiation-induced brain necrosis and normal brain tissue. The difference in APT signal between normal brain tissue and radiation necrosis was much smaller than for tumour-necrosis or tumour-brain signals, but this difference was still statistically significant. APT signal changes were observed early after radiation therapy (3 and 6
days after treatment) while other imaging techniques like T1, T2, and DW-MRI showed no change at these time points.

In a clinical study, Mehrabian et al.\textsuperscript{53} studied human patients with brain metastases from a variety of primary cancers (6 breast, 5 lung, 3 kidney, 2 melanoma) previously treated with stereotactic radiosurgery. As defined by the mean MTR values, the largest separation between CEST signals of regions of necrosis and regions of tumour progression were seen in the amide and NOE (aliphatic) regions of the CEST spectra, suggesting that CEST can differentiate between tumour and necrosis in the setting of brain metastases. CEST has also been studied as a method to predict response of brain metastases undergoing stereotactic radiosurgery treatment.\textsuperscript{65} In this study, changes in CEST parameters within the tumour in the aliphatic and amine regions of the Z-spectrum at 1 week after treatment correlated with ultimate change in tumour size at 1 month after treatment. CEST can also differentiate progression of glioma from pseudoprogression, a benign phenomenon which mimics the MRI characteristics of glioma progression after chemoradiotherapy.\textsuperscript{66}

A preliminary clinical study of 3 patients with locally advanced breast cancer has shown an increase in APT signal in a patient whose cancer did not respond to neoadjuvant chemotherapy (as demonstrated by ultimate growth in tumour size), while the APT signal decreased in a patient who ultimately developed a complete tumour response.\textsuperscript{8} A preliminary report of CEST MRI for breast cancer patients\textsuperscript{67} compared CEST results at 1.2 – 1.8 ppm with results from DCE-MRI. In 3 of the 6 patients in this cohort, high CEST signal correlated well with tumour identified using DCE-MRI and CEST signal values were higher in tumour than in surrounding fibroglandular tissue.

These preclinical and clinical studies suggest that CEST can be used to detect changes induced by chemotherapy and predict ultimate response to therapy. However, much of this previous work has focused on the CEST characteristics of exchanging protons from protein amide groups (i.e., APT imaging), although differences may be evident in other regions of the Z-spectrum.

Building on this work, differences in Z-spectrum characteristics may be evident between viable tumour tissue and areas of cell death. Differences between tumour and other tissue types should
also be detectable for other cancer types, such as breast cancer. The work described here sought
to study the CEST characteristics of breast cancer xenografts and determine if CEST (either in
the APT region or elsewhere in the Z-spectrum) can differentiate between viable tumour and
necrosis in a murine breast cancer model. Such differences could help form the basis for future
CEST-based cell death detection and treatment response monitoring protocols for breast cancer.

1.4 Structure of the Thesis

The purpose of the work described in this thesis is to characterize the CEST parameters of breast
cancer xenografts. In particular, the goal is to define differences in the CEST spectra of viable
tumour tissue and regions of cell death. Such differences could be studied to develop methods for
distinguishing viable and dead tissue and to detect onset of cell death early in the treatment
course in an attempt to predict ultimate treatment response and guide clinical decision making.

Chapter 2 describes the main experiment dedicated to studying CEST parameters of viable
tumour tissue and necrosis within breast cancer xenografts and to developing a CEST-based
method to differentiate between the two. Groups of xenografts were scanned with a CEST
protocol before chemotherapy and at a variety of times afterwards (ranging from 4 to 24 h). The
CEST data were then compared with ISEL stained histology specimens to determine appropriate
MTR values to differentiate cell death from viable tumour and to study the time course of cell
death response to chemotherapy.

Although previous experiments have studied CEST parameters after radiotherapy treatments,
chemotherapy represents the most common treatment modality given for breast cancer prior to
surgery. Detecting response (or non-response) to neoadjuvant chemotherapy would provide the
most relevant information for prognosis and treatment selection in the locally advanced breast
cancer setting. For this reason, CEST parameters before and after chemotherapy administration
were studied in the experiments described here. Chapter 3 summarizes the relevant findings of
the study and suggests future work that can expand on the results.
Chapter 2
Chemical Exchange Saturation Transfer MRI to Assess Cell Death in Breast Cancer Xenografts at 7T

2.1 Introduction

Locally advanced breast cancer, generally defined as tumours which are i) larger than 5 cm, ii) invading skin or the chest wall, or iii) have matted or multiple involved axillary lymph nodes, is an aggressive form of cancer associated with poor survival. Modern treatment approaches increasingly use chemotherapy before surgery (“neoadjuvant chemotherapy”) followed by surgery and then radiotherapy. Although chemotherapy can shrink or even eliminate cancer within breast and lymph nodes on microscopic examination, recurrence is common even after maximal treatment. However, the degree of response to chemotherapy has been shown to correlate with survival outcomes.

Standard assessment of tumour response to therapy involves anatomical measurements of tumour size before and after therapy, typically using magnetic resonance imaging (MRI) or ultrasound. Unfortunately, the cancers of some patients do not respond well to chemotherapy, which may lead to 4-6 months of ineffective treatment associated with potentially significant side effects such as alopecia, nausea/vomiting, long-term cardiac toxicity, and weakness or numbness in the hands and feet. A tumour may also grow and/or metastasize during this time, decreasing or even eliminating the chance of cure. A method to detect response to chemotherapy either prior to treatment or early in a course of treatment could potentially improve outcomes for these patients while sparing side effects during ineffective therapy. One way to predict such a response would be to detect cell death, such as through apoptosis or necrosis, quickly after administration of cytotoxic therapy. Those tumours whose cells are not killed by cytotoxic treatment may require additional or alternate treatments to achieve adequate cytotoxicity. Similarly, cell death processes such as apoptosis or necrosis may not be the only physiological responses undergone by a tumour receiving cytotoxic therapy. The totality of these changes and the subsequent changes in the chemical microenvironment within the tumour may be detectable by functional
imaging techniques, and such changes can be correlated with ultimate tumour response and patient survival.

As previously described, various imaging techniques have been studied for their ability to identify changes in tumours early during treatment and to predict tumour response and patient outcomes. However, the various limitations to these methods suggests that a superior alternative would be beneficial. CEST MRI possesses many characteristics of such an alternative. Previous work has demonstrated that it can predict tumour response and differentiate between regions of active tumour and apoptotic and necrotic tissue in lung cancer xenografts. CEST has also been shown to successfully evaluate treatment in patients with brain metastases and differentiate between tumour progression and radiation-induced necrosis.53

Because of both the limitations of other imaging techniques for clinical response monitoring and the promise of CEST MRI imaging for these purposes, a proof-of-principle study was conducted to study the CEST properties of MDA breast cancer xenografts both before and after treatment with chemotherapy. Next, the CEST properties of a larger sample of breast cancer xenografts were characterized using MTR to determine methods for differentiating viable tumour tissue and cell death. These methods were then used to study the time dependence of the tumour response to chemotherapy.
2.2 Methods

2.2.1 Animal Model

Tumours were grown by injecting 100 µL of solution containing up to $5 \times 10^6$ MDA-MB-231 tumour cells (American Type Culture Collection, Manassas, VA; henceforth referred to as "MDA") into the hind legs of CB-17 SCID mice (Charles River Laboratories, Canada, Saint-Constant, QC).

Animal care protocols were approved by the local Animal Care Committee at Sunnybrook Research Institute. Mice were anesthetized during scanning by inducing anesthesia with 3-4% isoflurane. Thereafter, respiratory rate was monitored by a pneumatic pillow. Isoflurane concentration was titrated to maintain a breathing rate of 60-90 breaths per minute; generally, 1-2% isoflurane concentration was sufficient to maintain this rate. Temperature was monitored with a probe placed in constant contact with the skin of the mouse’s stomach. Constant external temperature was maintained using a warm water circulating bath on the ventral surface of the mouse’s thorax and abdomen.

Due to the known propensity for MDA xenografts developing necrotic cores, tumours were scanned when they reached approximately 5 mm in diameter as evaluated by measuring the visible tumour using calipers. Doxorubicin (50 mg/m$^2$) and paclitaxel (100 mg/m$^2$) chemotherapy was used, as these drugs form the basis of standard, modern, clinical neoadjuvant chemotherapy regimens. The chemotherapy was administered via tail vein catheter immediately after completion of each pre-chemotherapy scan. Tumours were rescanned at a pre-determined time (4, 8, 12, or 24 h) after chemotherapy injection. Scans were timed such that the desired time point (e.g. 4 hours after chemotherapy injection) occurred at some point during the CEST scan, as illustrated in Figure 2.
Figure 2: CEST MRI pulse sequence and timing of scans.

A) CEST MRI pulse sequence. The line labelled “RF” shows radiofrequency pulse application. The lines labelled $G_{\text{slice}}$, $G_{\text{phase}}$, and $G_{\text{read}}$ show the imaging gradients. 
*Adapted from: K. L. Desmond, “Endogenous Chemical Exchange Saturation Transfer: Quantitative Modelling and Application in Cancer.”*

B) Timing of scans. Each tumour was scanned before chemotherapy, and then either at 4, 8, 12, or 24 h after chemotherapy injection. The scan was arranged such that the appropriate time after injection occurred between the beginning of the first CEST scan and the end of the second CEST scan.
Immediately after completion of the post-chemotherapy scan, animals were sacrificed under anesthesia by cervical dislocation. Tumours were excised, leaving the skin overlying the tumour and a layer of muscle underneath the tumour intact. The tumour was cut in half at the point of largest diameter. The proximal portion of the tumour tissue was fixed in 10% formalin and then transferred to a solution of 70-80% ethanol for storage until processing; the distal portion was frozen in liquid nitrogen and stored in a -80°C freezer for future retrieval. The proximal portion of the tumour was sectioned into 5 μm slices and the largest (closest to the cut surface) slices were stained with hematoxylin and eosin (H&E) for morphological identification and in situ end labeling (ISEL) assay for identification of cell death (apoptosis and necrosis). As apoptosis and necrosis both stain positively (dark purple) using ISEL, this report will refer to regions stained by ISEL as regions of “cell death.”

High-magnification images of the ISEL-stained histology slides were obtained using a Leica DC100 microscope with a 40x objective and a Leica DC100 camera connected to a 2-GHz PC running Leica IM1000 software (Leica GmbH, Wetzlar, Germany). Using the ImageJ program (National Institutes of Health, Bethesda, MD), the entire tumour area and the region of cell death staining using ISEL (dark purple staining) were delineated and the cell death index (CDI) was calculated by

\[
CDI = \frac{A_{\text{cell death}}}{A_{\text{tumour}}}
\]

Where \( A_{\text{cell death}} \) is the area of the cell death region and \( A_{\text{tumour}} \) is the area of the entire tumour.
2.2.2 MRI

Animals were imaged before and after chemotherapy injection on a 7T preclinical MRI system (BioSpec 70/30 USR, Bruker BioSpin, Billerica, MA). A volume coil was used for transmission and a 20-mm diameter surface coil was used for reception. The tumours were positioned at the isocenter of the magnet for optimal shimming. A high-resolution, T2-weighted Rapid Acquisition with Relaxation Enhancement (RARE) image\textsuperscript{58} (RARE factor 8, TR/TE = 2500/50 ms) was acquired with 11 slices and the tumour volume identified to perform field map-based shimming using Bruker’s Map Shim functionality. A correction to account for spatial inhomogeneity in the $B_0$ field was also performed.\textsuperscript{31}

The MRI sequence used began with a single rectangular off-resonance RF pulse of 490 ms which was followed by a single slice 2D FLASH sequence with TR/TE = 501/3.1 ms at a resolution of 0.31 mm $\times$ 0.31 mm $\times$ 1 mm and a matrix size of 64 $\times$ 64. Saturation pulse amplitudes of 0.5 $\mu$T was used. Measurements were made at frequency offsets between -1800 Hz (-6 ppm) and 1800 Hz (6 ppm) in increments of 30 Hz between -180 Hz (-0.6 ppm) and 180 Hz (0.6 ppm) and increments of 90 Hz outside this region. Reference images at 200 kHz offset were interleaved every 5 offsets throughout the acquisition to correct for signal drift. While previous signal drift reports showed exponential decay of the reference signal over time,\textsuperscript{31} our decay showed linear characteristics, which were used for the correction methods.

2.2.3 Region of Interest Definition

To define the regions of interest for analysis, the structural and CEST images were co-registered. An area encompassing the tumour, as visualized on the structural image, was manually delineated on the CEST image. An example is shown in Figure 3, including the corresponding H&E and ISEL stained histology slides. This area (the “mask”) was intentionally drawn conservatively to ensure that the mask remained within the tumour over the entirety of the scan, accounting for small amounts of motion over the length of the scan.
The MTR was then calculated for each voxel within the masks for a given frequency offset using the formula

\[ MTR = 1 - \frac{S}{S_0} \]

where \( S \) is the strength of the MRI signal measured at a given frequency offset of RF saturation and \( S_0 \) is the strength of the MRI signal when no RF saturation is applied.\(^6\) The voxels were then assigned as cell death or tumour based on the MTR. Once the mask was defined, a histogram was created by assigning each voxel into bins by MTR at a given frequency offset. The histogram was fit to a Gaussian distribution to define cutoffs to segment the masks in viable tumour and necrotic/apoptotic tissue.

Using these masks, the cell death index (CDI) was calculated for each tumour by

\[ CDI = \frac{N_{\text{below}}}{N_{\text{total}}} \]

Where \( N_{\text{below}} \) is the number of voxels with MTR below the cutoff (indicating the presence of cell death) and \( N_{\text{total}} \) is the total number of voxels within the mask encompassing the tumour.

The statistical significance between differences in MTR was tested using paired t-test to compare pre- to post-chemotherapy scans and using unpaired t-test to compare viable tumour to cell death.
Figure 3: Representative images of different methods of tumour analysis employed in our study.

A) T2-weighted “structural” MRI image.
B) CEST MRI image divided into pixels for analysis. Overlaid in orange is the mask defining the region of interest for CEST analysis.
C) ISEL stained histology slide: blue indicates viable tumour, purple indicates cell death.
D) H&E stained histology slide.
E) Map of MTR for each mask pixel at 1.8 ppm frequency offset.
All scale bars indicate 1 mm.
2.3 Results

2.3.1 Data Analysis

Fourteen xenografts were scanned. All tumours were scanned before chemotherapy was administered. Three were scanned at 4 h after chemotherapy, four at 8 h, four at 12 h, and three at 24 h. A fourth tumour was scanned in each of the 4 and 24 h groups, but the images were discarded due to extensive motion during the scan. All fourteen xenografts had histology preparation and staining after the post-chemotherapy scan.

Two initial analyses were performed. For the first, three tumours with identifiable necrotic cores were chosen based on visual assessment of the structural T2-weighted images. Masks were then created to estimate the areas of viable tumour and cell death. The mean Z-spectrum of the three viable tumour regions was compared to that of the three cell death regions, as shown in Figure 4A. Although large separations between the spectra were seen at 1.8, 0.6, -0.5, and -3.3 ppm, only the difference at 1.8 ppm was statistically significant in this analysis (p = 0.03).

The second initial analysis examined the CEST spectra of the entire xenograft region, making no attempt to differentiate between viable tumour and cell death regions. For this analysis, masks were created encompassing the entire xenograft (i.e., both areas of viable tumour and regions of cell death) based on visual analysis of the structural T2-weighted images. The mean CEST spectra of all pre-chemotherapy scans were then compared to the post-chemotherapy scans. As seen in Figure 4B, the difference in MTR values between these two groups were much smaller in magnitude than the differences between the areas of viable tumour and cell death compared in Figure 4A. The difference at -3.3 ppm did reach statistical significance (p = 0.035), while differences at other offsets such as 1.8, 0.6 and -0.5 ppm did not (p > 0.05).
Figure 4: CEST spectra.

A) CEST spectra (solid lines) averaged over the regions of viable tumour (blue) and cell death (red) as defined by visual assessment of the T2 structural images with co-registration of the CEST data. Dashed lines indicate standard deviations.

B) CEST spectra (solid lines) averaged over the entire region of interest mask for all pre-chemotherapy scans (green) and post-chemotherapy scans (black). Dashed lines indicate standard deviations.
2.3.2 Defining MTR Characteristics of Viable Tumour and Cell Death

Based on the above results, analysis of CEST characteristics was directed toward the 1.8 and -3.3 ppm frequency offsets. Using the initial masks, which encompassed the entire area of the tumour, including any areas of cell death, the MTR was calculated for each voxel in each scan. At 1.8 ppm, the MTR for all voxels ranged from 0.076 to 0.24. At -3.3 ppm, the MTR ranged from 0.077 to 0.23. Histograms of voxel MTR values are presented in the top row of Figure 5A and 5B.

Cutoffs to label tumour and viable tissue based on the MTR were then determined. The bottom row of Figure 5 shows scatter plots of the histogram data at 1.8 ppm (Figure 5A) and -3.3 ppm (Figure 5B) offset with several candidate tumour-cell death cutoffs defined: the mean of the distribution (labelled in purple), 1 standard deviation below the mean (1 SD; orange) and 0.5 standard deviations below the mean (0.5 SD; green).

Figure 6A-C shows an example of the tumour and cell death mask areas using the three different cutoffs (with MTR values measured at 1.8 ppm) compared with the structural T2-weighted MR image and ISEL stained histology slide for the same tumour. Figure 6D shows a T2-weighted structural image is seen with the corresponding tumour mask and ISEL stained histology slide. Visual comparison suggests that the heterogeneously-enhancing core of the tumour on the T2 image correlates with the shape and size of the dark-stained cell death region of the ISEL histology slide. Similarly, the shape and size of the cell death region seen using T2-weighted imaging and ISEL more closely matches the cell death region determined using the 0.5SD cutoff (Figure 6B) than the 1SD cut-off (which tended to underestimate the amount of necrosis; Figure 6A) or the mean value cut-off (which tended to overestimate the amount of necrosis; Figure 6C) in this example.
Figure 5: Histograms of MTR values.

Histograms of MTR for each pixel from all scans combined (pre- and post-chemotherapy). MTR are counted in bins of 0.005, for 40 bins in total ranging from 0.05 to 0.25.

A) Histograms generated at 1.8 ppm.
Top: Bar graph showing histogram data.
Bottom: Scatter plot of same data as top with Gaussian curve fit to data (red). Vertical lines indicate the mean (purple; MTR = 0.14), 0.5 standard deviations below the mean (green; MTR = 0.12) and 1 standard deviation below the mean (yellow; MTR = 0.10).

B) As in (A) but generated at -3.3 ppm.
Top: In the bottom subplot, mean (purple) has MTR = 0.10, 0.5 standard deviations below the mean (green) has MTR = 0.12, and 1 standard deviation below the mean (orange) has MTR = 0.15.
Figure 6: Differences in cell death regions defined at different MTR cutoffs.

Example of definitions of viable tumour (orange) and cell death (yellow) regions using different candidate MTR cutoffs:
A) 1 standard deviation below the mean (MTR = 0.10).
B) 0.5 standard deviations below the mean (MTR = 0.12).
C) Mean (MTR = 0.14).
D) From left to right: the T2-weighted structural image, pixelated CEST image with mask region overlaid in orange, and ISEL stained histology images for reference.
All scale bars indicated 1 mm.
To validate this qualitative observation, the CDI that was measured using the ISEL stained images was compared with the CDI using different MTR cutoffs. For each time point, $\Delta_{CDI}$ was calculated by

$$\Delta_{CDI} = |CDI_{ISEL} - CDI_{MTR}|$$

where $CDI_{ISEL}$ is the mean CDI calculated using ISEL for all post-chemotherapy xenografts at a given time point and $CDI_{MTR}$ is the mean CDI calculated using MTR. The sum of all $\Delta_{CDI}$ calculations ($\Delta_{CDI \ Total}$) were then compared to determined which MTR cutoff most closely agreed with the CDI measured using ISEL (lower $\Delta_{CDI \ Total}$ suggests better agreement). The CDI using the 0.5 SD cutoff for both the 1.8 ppm and -3.3 ppm offset most closely agreed with the CDI measured using ISEL, as shown in Table 1.

Table 1: Cell death index measured by ISEL staining and MTR values.

<table>
<thead>
<tr>
<th>Chemo time</th>
<th>ISEL Histogram mean</th>
<th>1.8 ppm cutoff</th>
<th>-3.3 ppm cutoff</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 SD</td>
<td>1 SD</td>
</tr>
<tr>
<td>4h</td>
<td>0%</td>
<td>32.6%</td>
<td>5.1%</td>
</tr>
<tr>
<td>8h</td>
<td>12.7%</td>
<td>37.7%</td>
<td>10.2%</td>
</tr>
<tr>
<td>12h</td>
<td>20.5%</td>
<td>42.7%</td>
<td>13.6%</td>
</tr>
<tr>
<td>24h</td>
<td>29.5%</td>
<td>47.7%</td>
<td>19.1%</td>
</tr>
<tr>
<td></td>
<td>$\Delta_{CDI \ Total}$</td>
<td>98.2</td>
<td>24.8</td>
</tr>
</tbody>
</table>

Percent values represent the mean CDI of all post-chemotherapy xenografts from a given time point.
2.3.3 Comparison of Viable Tumour to Cell Death

The difference in CEST parameters amongst tumours that had identifiable cell death was then examined. Masks defining regions of viable tumour and cell death were created using the MTR map at 1.8 ppm (Figure 7A) and -3.3 ppm (Figure 7B); the cutoff between tumour and cell death used to define these regions was set at the 0.5 SD cutoff for each offset (MTR = 0.12 at 1.8 ppm, MTR = 0.125 at -3.3 ppm).

The spectra for tumour and cell death regions are shown in Figure 7A using masks generated at 1.8 ppm and in Figure 7B using masks generated at -3.3 ppm. Regardless of which offset was used to define the masks, the maximum separation between the curves outside of the direct effect region was observed at 1.8 and -3.3 ppm. The mean MTR of the masks for each individual xenograft are shown in Figure 7C (using MTR at 1.8 ppm to define the masks) and 7D (using MTR at -3.3 ppm to define the masks). The differences in MTR were statistically significant for all shown cases (p ≤ 0.001).

Figure 8 shows the mean change in measured CDI as a function of time after chemotherapy. Although no differences between experimental times reached statistical significance, a trend is evident with the maximum cytotoxic effect at 8-12 h after chemotherapy administration.
Figure 7: CEST spectra comparison between cell death and viable tumour regions.

A) CEST spectra (solid lines) averaged over the regions of viable tumour (blue) and cell death regions (red) as defined by the MTR for each voxel at 1.8 ppm using MTR = 0.12 (0.5 standard deviations below the mean of the calculated histogram) as the cutoff. Dashed lines indicate standard deviations.

B) Mean MTR of the masks for each individual xenograft used in Section A. The tumour and cell death masks are differentiated using MTR = 0.12. The MTR difference between the masks at the 1.8 ppm and -3.3 ppm cutoffs are both statistically significant using this cutoff (p ≤ 0.001).

C) CEST spectra (solid lines) averaged over the regions of viable tumour (blue) and cell death regions (red) as defined by the MTR for each voxel at -3.3 ppm using MTR = 0.125 (0.5 standard deviations below the mean of the calculated histogram) as the cutoff. Dashed lines indicate standard deviations.

D) Mean MTR of the masks for each individual xenograft used in Section C. The tumour and cell death masks are differentiated using MTR = 0.125. The MTR difference between the masks at the 1.8 ppm and -3.3 ppm cutoffs are both statistically significant using this cutoff (p ≤ 0.001).

T = viable tumour regions; CD = cell death regions
Figure 8: Change in cell death index (CDI) by time after chemotherapy administration

Average change in cell death index from pre- to post-chemotherapy scans as defined at 1.8 ppm frequency offset using the MTR = 0.12 cutoff for viable versus dead tumour. Error bars denote standard error of the mean. The differences between groups did not reach statistical significance.
2.4 Discussion

This study investigated methods for differentiating viable tumour from tumour regions containing cell death using CEST MRI. Statistically significant differences in MTR were identified at 1.8 and -3.3 ppm between regions of viable and dead tissue. An MTR cutoff of 0.12 at 1.8 ppm or 0.125 at -3.3 ppm most closely approximated the cell death pattern shown by histological assessment. Using this cutoff to determine CDI, a maximum increase in cell death was observed between 8-12 h after chemotherapy, after which the CDI diminished. We have here confirmed the previous findings of Desmond et al. (which used a small sample size of MDA tumours) that MTR analysis can differentiate viable tumour from cell death in this cell line.

Previous pre-clinical research has demonstrated that CEST MRI can be used to differentiate between tissue types, including differentiating between muscle and tumour, different tumour cell lines, and between viable tumour and cell death. In a sample of 20 Lewis lung carcinoma (LLC) xenografts and four MDA breast cancer xenografts, Desmond et al. have studied a variety of MRI parameters, including T1 and T2 relaxation; diffusion (ADC); and CEST parameters such as MTR and Lorentzian curve peak amplitudes corresponding to amide, amine, and aliphatic groups within CEST spectra. These MTR analyses were focused on 3.5 ppm to maximize the contribution of amide protons. Those results indicated that differentiation between viable tumour and necrotic tissue for both MDA breast cancer and LLC lung cancer xenografts could be obtained by measuring the amplitude of Lorentzian peaks fitted to the Z-spectrum centered on the resonance frequencies of amide (3.5 ppm), amine (2 ppm) and aliphatic (-3 ppm) protons. MTR at 5 ppm was the only other metric that could statistically significantly differentiate between the two tissue types.

Zhou et al. compared APT imaging with anatomical (T1 and T2-weighted) and DW-MRI after treating human GBM xenografts with radiotherapy. Changes in APT signal were observed at 3 and 6 days after treatment, while the other techniques showed no change at these times points. As well, APT was able to differentiate between radiation necrosis and both glioma and gliosarcoma xenografts; neither gadolinium-enhanced T1 nor T2-weighted imaging could differentiate glioma from radiation necrosis while gliosarcoma could only be differentiated by
T2-weighted MRI. This data suggests that CEST-based imaging may be better than other MRI techniques at differentiating cell death from viable tumour.

A clinical study by Mehrabian et al.\textsuperscript{53} of tumour progression versus radiation-induced cell death following stereotactic radiosurgery for brain metastases showed that maximum MTR difference between cell death and progressive tumour has been found in the amide and aliphatic regions of the CEST spectra, corresponding to 3.5 and -3.5 ppm, respectively. The -3.5 ppm offset used by Mehrabian et al. is similar to the -3.3 ppm offset with maximum separation between the pre- and post-chemotherapy spectra in this study. This finding may reflect the importance of the NOE, hypothesized to be the contrast mechanism of aliphatic groups in amino acid side chains. In the work presented here, comparing MTR for viable tumour and cell death at -3.3 ppm also showed a statistically significant difference.

Schmitt et al.\textsuperscript{53} reported on a small cohort of six women with breast cancer imaged with CEST MRI. Their CEST technique used saturation RF energy between 1.2 – 1.8 ppm. In the 3 analyzable patients in the cohort, high CEST signal correlated well with tumour identified using DCE-MRI and CEST signal values were higher in tumour than in surrounding fibroglandular tissue. In the work presented here, similar findings were demonstrated, with significantly higher MTR values (i.e. higher CEST signal) measured for viable tumour at 1.8 ppm compared with cell death regions. These findings suggest that CEST around the 1.8 ppm frequency offset is of particular interest in detecting viable breast cancer.

Imaging methods other than CEST MRI can detect cell death \textit{in vivo}, albeit at later stages of advanced necrosis. When these methods have been applied at varying times after treatment, a trend is evident whereby the cell-death inducing effect of the treatment increases to a point after which it begins to decrease. Tadayyon et al.\textsuperscript{21} used high (20 MHz) and low frequency (7 MHz) QUS to study cell death in MDA-MB-231 xenografts using the same chemotherapy regimen used in the work here. Histological analysis showed an increase in CDI up to 24 h after chemotherapy, with the CDI at 48 h lower than at 24 h, although still statistically significantly increased over baseline. A similar pattern was demonstrated for the change in average acoustic concentration (ΔAAC), which was highest at 24 h after chemotherapy followed by a reduction at 48 h. A separate study\textsuperscript{23} which treated HTB-67 melanoma xenografts with photodynamic therapy
(PDT) and used high frequency (26 MHz), QUS showed similar patterns in the parameters of mid-band fit and spectral slope, which have been correlated with cell death.\textsuperscript{76,77} The peak effect was observed between 12-20 h after treatment, followed by a decline. In the work described here, the CDI calculated using the 0.5 SD cutoff to define necrotic tissue demonstrated a similar trend. The largest average increase in CDI was seen 8-12 h after chemotherapy, with the increase reduced after 24 h. However, the differences between the time points did not reach statistical significance.

The time required to set up and conduct each scan was approximately 3 hours. As this experiment was primarily intended to demonstrate proof-of-concept, preparing and scanning a large number of tumours (e.g. 5 or more) per time point would take an unnecessarily large amount of time and resources, such as machine time and animal specimens. Therefore, 3-4 specimens per post-chemotherapy time period was deemed a reasonable compromise between experimental expediency and sample size.

During the scan time, some tumour movement could have been experienced such as due to slow drifts in the equipment position or deflation of pads and pillows used to set up the mouse on the scanner. Image registration was employed in the fitting algorithms to minimize the effects of such motion. Registration is more accurate when multiple slices are acquired (allowing 3D registration). In this work, however, only single slices were acquired in Z-spectra, limiting registration to in-plane.

Resource management and patient comfort considerations make long scans untenable in human trials. Reducing the number of frequency offsets used in clinical trials, for example by obtaining data from several offsets around 1.8 ppm while minimizing the data taken in other offset regions, would permit the use of shorter scans, consequently reducing scan costs and improving patient satisfaction by not requiring long periods of cooperation lying in an MRI scanner. Measurements at fewer offsets may also allow for longer RF saturation times given the availability of multiple RF amplifiers, which generally have limited duty cycles, on a clinical scanner. This data can be used to guide decisions to optimize scan protocols for future planned clinical trials.
Detection of cell death *in vivo* provides a promising avenue for early response assessment and prediction for patients undergoing neoadjuvant chemotherapy for locally advanced breast cancer. This data further supports the ability of CEST to detect cell death in breast cancer. Differences in MTR measurements at 1.8 ppm should be a point of interest in studies attempting to translate CEST MRI analysis into clinical practice and may be investigated alone or in combination with previously studied metrics such as Lorentzian peak amplitude to develop prediction algorithm based on multiple CEST parameters. Further study, in both animal models and humans, can combine CEST MRI with other validated imaging modalities to further refine detection methods to detect cell death and improve predictive models for response and clinical outcomes.

2.5 Conclusions

Analysis of magnetization transfer ratio using CEST MRI can differentiate between viable tumour and cell death in MDA-231 xenografts. Maximal tumour response to chemotherapy is seen at 8-12 h after administration.
Chapter 3
Summary and Future Work

3.1 Summary

This thesis investigated the use of CEST MRI to distinguish between areas of viable tumour and cell death *in vivo* in breast cancer xenografts. The first chapter described previous clinical efforts to develop imaging-based methods to monitor the response of cancer to therapy and to predict ultimate response early in the treatment course. It also described the downsides presented by previously studied methods including questions regarding sensitivity and specificity of signal changes after cytotoxic chemotherapy, the need for injected contrast agents, cost, and patient inconvenience in integrating new modalities into existing clinical pathways. This chapter also reviewed the physics of CEST MRI contrast and the characteristics that make it a very promising modality for treatment response monitoring.

Chapter 2 described experimental efforts to characterize the *in vivo* CEST parameters. Tumours were scanned before administration of chemotherapy and then again after a series of different intervals after chemotherapy administration (4, 8, 12, and 24 h). Histological specimens were obtained from each tumour and stained to differentiate areas of viable tumour and cell death. Visual comparison between these stained histological specimens with high resolution MRI provided a method to distinguish between viable tumour and cell death on the images. Registration of the CEST images with the high-resolution images allowed the delineation of areas of cell death and viable tumour on the CEST image.

Preliminary comparison of the Z-spectra of viable tumour areas with cell death regions suggested that the 1.8 ppm frequency offset showed maximum separation between the two regions compared to all other frequencies. A second frequency of interest, -3.3 ppm, was found by comparing the mean Z-spectrum of the entire pre-chemotherapy xenografts to the mean Z-spectrum of the entire post-chemotherapy xenografts. Histograms of the MTR values for each voxel at these two frequencies were generated. Segmentation of the tumours was then performed
using a variety of threshold values to differentiate cell death and viable tumour based on these histograms. Using the 1.8 ppm offset, the threshold MTR value of 0.12 was found to provide the most accurate definition of these regions (using ISEL staining as the gold standard), while a threshold value of 0.125 provided the most accurate definition using the MTR measurements from the -3.3 ppm offset; both these cutoffs represented the value 0.5 standard deviations below the mean of the calculated histogram for that offset. Regions defined using this threshold showed significant differences in MTR values between the two regions.

Finally, establishing a threshold MTR value to define areas of cell death allowed the mean change in CDI between the pre-chemotherapy and post-chemotherapy scans to be measured as a function of interval between chemotherapy administration and post-chemotherapy scan time. An increase in CDI to 8-12 h followed by a decrease at 24 h was measured, although the changes did not meet statistical significance, likely due to the relatively small numbers of tumours studied. This time course is similar to that measured after treatment by other methods.78,79

3.2 Future Work

The results described here represent a basis for detection of cell death both before treatment and after administration of chemotherapy (e.g., apoptosis and/or necrosis). Future work expanding on these findings should focus on three avenues of inquiry: 1) validating the results, 2) translating CEST protocols for response detection and monitoring for human breast cancer, and 3) further expansion to other cancer types.

The first step is to validate the results in a larger sample of xenograft tumours. The study described in this thesis was intended as a proof-of-concept, and so a balance was sought between developing a robust sample size and experimental expediency given the difficulties of growing tumours and long scan times required. The results obtained were promising, as seen in the large separation in MTR values at the 1.8 ppm offset between tumour and necrosis regions were shown and the ability of MTR mapping at this offset to approximate regions of cell death determined histologically, changes in CDI measured at each time point after chemotherapy
administration did not reach statistical significance. Continuing this experimentation with additional xenografts to increase the sample size would add confidence in the results.

These findings may also inform the development of clinical protocols for response detection and monitoring in human patients with locally advanced breast cancer undergoing pre-operative chemotherapy. One challenge to translation of CEST MRI from preclinical to clinical studies is the long scan time required. In the work presented here, a single CEST scan with RF pulses across the entire frequency spectrum takes ~35 min with additional time required for structural, inversion recovery, B₀ mapping, and other ancillary scans. A clinical scan requiring a similar length of time would present a significant burden on patients’ time. It would also tax existing MRI scanners and staff which are often stretched to accommodate all patients requiring imaging services or would introduce significant capital and operating costs to obtain and run additional MRI scanners. A reduction in scan time would greatly aid uptake of CEST into the clinic.

One way to reduce scan time is to refine the understanding of differences in CEST parameters so as to use only those RF frequencies which provide maximum contrast to answer a given clinical question. The experiments described in this thesis suggest that the region around 1.8 ppm provides the maximum MTR difference between cell death and viable tumour. Therefore, future CEST MRI protocols could focus on RF frequencies around this offset, which would dramatically reduce scan time.

Finally, the experimental methods and experience developed through this experiment can be used to expand CEST MRI for use among other cancer types. Prostate cancer, for example, often presents clinical dilemmas in determining optimal treatment for patients. Most localized prostate cancer is amenable both to surgical resection of the prostate or to radiotherapy, either via external beam radiotherapy or brachytherapy insertion of radioactive sources. However, no directly comparative randomized controlled trials have been performed. One way to guide appropriate treatment would be to determine the sensitivity of a given patient’s tumour to radiation therapy; patients with more radiosensitive tumours could be offered radiotherapy-based treatments while patients with radiation resistant tumours could be steered toward surgery. Because rapidly dividing tissues are both more susceptible to radiotherapy and more metabolically active than more slowly dividing tissue, using CEST MRI to measure metabolic
activity could also serve as a marker for radiation sensitivity. Preclinical work on this topic is currently ongoing within the Stanisz laboratory in the University of Toronto Department of Medical Biophysics.

Further development of CEST MRI and refinement of the ability to detect cell death early in a treatment course and predict ultimate treatment response will provide valuable information for clinicians to optimize treatment protocols (and make appropriate real-time changes if initial decisions prove suboptimal), maximize cure and control rates and avoid unnecessary toxicity.

The promise of CEST MRI for detecting changes in tissue microenvironment, such as after cytotoxic therapy, without attendant risks of other modalities makes it a very promising modality for a range of clinical applications, including treatment response monitoring and prediction. Although there is much work remaining to refine these protocols and applications, the work presented here provides another proof-of-principle for the use of CEST in detecting cytotoxicity which can eventually lead to more involved treatment monitoring algorithms.

### 3.3 Conclusions

This thesis has demonstrated that CEST MRI can be used to differentiate cell death from viable tumour in an *in vivo* breast cancer model. It determined the RF saturation frequency (1.8 ppm) which provides the maximum contrast between these two regions, which should provide a basis for future work to refine the frequency regions scanned in CEST analyses (thus shortening scan time) and to translate this preclinical work into early phase clinical trials. A threshold MTR value which can distinguish between these two regions was also determined and the measured shows a characteristic trend in that the maximum increase in cell death was seen at 8-12 h after chemotherapy while less increase in cell death was measured 24 h after chemotherapy.

The results presented here should serve as an important basis for translation of the CEST MRI technique to clinical trials. Investigations of CEST parameters and early detection of cell death and treatment response can use the RF frequencies and threshold values identified as providing insight into cell death to define their protocols and to reduce scan time by eliminating the need to
scan the entire frequency spectrum. Such protocols will hopefully allow for early detection of cell death and early prediction of ultimate treatment response, allowing for better personalization of cancer treatment and improving patient outcomes while reducing side effects.
References


