STEADY-STATE SUSCEPTIBILITY CONTRAST MRI
OF BREAST CANCER ANGIOGENESIS:
PRECLINICAL VALIDATION AND APPLICATION

by

Eugene Kim

A dissertation submitted to Johns Hopkins University in conformity with the requirements for
the degree of Doctor of Philosophy

Baltimore, Maryland

February, 2014
Abstract

Angiogenesis is essential to the growth and spread of cancer, making it a promising therapeutic target. However, no anti-angiogenic drug is currently approved to treat breast cancer. Furthermore, there are no clinically validated biomarkers of angiogenesis, which would aid the development and optimization of anti-angiogenic therapies. Steady-state susceptibility contrast magnetic resonance imaging (SSC-MRI) is a noninvasive, clinically translatable technique with the potential to fill the need for angiogenesis biomarkers. In this work, these candidate SSC-MRI biomarkers were validated and used to monitor the effects of a novel anti-angiogenic peptide in an orthotopic model of MDA-MB-231 human breast cancer in female athymic mice.

In vivo SSC-MRI was performed on the tumor xenografts to measure biomarkers of fractional blood volume, vessel size index, and vessel density. A multimodal vascular imaging platform was developed to validate the in vivo vascular biomarkers with high-resolution ex vivo micro-computed tomography (μCT). While SSC-MRI biomarkers systematically overestimated μCT-measured vascular morphometrics, there were significant correlations between SSC-MRI and μCT measurements.

An independent longitudinal study was performed to investigate the sensitivity of SSC-MRI biomarkers to the effects of anti-angiogenic peptide therapy. The changes in SSC-MRI biomarker values were significantly different between treated and control groups, reflecting the anti-angiogenic effects of the peptide confirmed by histology. Moreover, SSC-MRI biomarkers detected treatment-induced vascular changes before any detectable reduction in tumor volume or cellularity.

A large-scale, image-based computational platform was developed to investigate the relationship between SSC-MRI biomarkers and the underlying vascular geometry. Geometries
tested ranged from randomly oriented cylinders to μCT-derived tumor vasculature. Similar to the in vivo data, in silico SSC-MRI biomarkers exhibited bias but correlated well with ground-truth vascular parameters. Simulations demonstrated that the performance of the SSC-MRI biomarkers was significantly affected when vascular geometry deviated from the random cylinder model from which these biomarkers were derived.

While SSC-MRI may not be suitable for absolute quantification of tumor vascular morphology, it does provide useful relative measures of vascular volume, size, and density that are sensitive to changes in angiogenic phenotype. Exhaustive characterization of these noninvasive imaging biomarkers demonstrated their clinical potential for evaluating anti-angiogenic drugs and maximizing therapeutic efficacy.

Thesis committee members:

Arvind P. Pathak (advisor, reader)
Zaver M. Bhujwalla
Feilim Mac Gabhann (reader)
Acknowledgements

I dedicate my work to my beloved parents. Words cannot fully convey my gratitude to them, and I likely do not even fully comprehend the depth of their sacrificial love for me. I am also grateful to my older sister, my life’s spare tire, for doing what older sisters do and giving me the occasional kick in the pants when I needed it.

I am very thankful to my advisor, Dr. Arvind Pathak, for his mentorship; for giving me the opportunity, support, and encouragement to get me to this point; for teaching me to be a good scientist while never forgetting the more important things in life – integrity, relationships, service to others, and of course, soccer.

I give special thanks to the other members of my thesis committee, Drs. Zaver Bhujwalla and Feilim Mac Gabhann, for their gracious support and valuable time and insights.

And many thanks to all my past and present colleagues and collaborators: Dr. Jiangyang Zhang for his help with pulse sequence optimization; Dr. Yoshinori Kato for his deft hands for tail vein cannulations; Meiyappan Solaiyappan for sharing his computational expertise in the FPM project; Dr. Douglas Ward for his help with statistical analysis; Karen Hong, Kevin Rhie, and Stacy Gil for their toils at the cryostat and on the bench; Charlesea Plummer for all of her pro bono work and being generally indispensible to the lab; Dr. Aleksander Popel, Dr. Spyros Stamatekos, and Esak Lee for their collaboration on the multiscale and peptide projects; and especially Jana Cebulla for her many research contributions as well as her constant love and support.
All glory and honor and praise be to God, who reveals Himself to us in the impossible intricacy and beauty of His creation.
Table of Contents

List of Tables .................................................................................................................................... viii

List of Figures .................................................................................................................................... viii

1 Background ................................................................................................................................ 1

1.1 Tumor angiogenesis .................................................................................................................. 1

1.1.1 Angiogenesis in breast cancer ............................................................................................ 1

1.1.2 Angiogenesis as a therapeutic target .................................................................................. 2

1.1.3 The need for clinical biomarkers of angiogenesis ............................................................... 3

1.2 Imaging tumor vasculature ....................................................................................................... 4

1.2.1 In vivo steady-state susceptibility contrast MRI ................................................................. 5

1.3 Aims ........................................................................................................................................ 11

2 General methods ..................................................................................................................... 12

2.1 Tumor model ............................................................................................................................ 12

2.2 In vivo MRI protocol .............................................................................................................. 13

2.3 In vivo MRI processing and analysis ....................................................................................... 14

2.4 Statistical analysis .................................................................................................................. 16

3 Multimodal characterization of 3D tumor vascular morphology ............................................. 18

3.1 Introduction ............................................................................................................................. 18

3.1.1 Ex vivo micro-CT angiography ............................................................................................ 18

3.1.2 Ex vivo micro-MRI angiography ......................................................................................... 20

3.2 Methods ................................................................................................................................ 23

3.2.1 Sample preparation for ex vivo angiography ..................................................................... 23

3.2.2 Ex vivo micro-CT ................................................................................................................... 23

3.2.3 Ex vivo micro-MRI ................................................................................................................ 23

3.2.4 Micro-CT and micro-MRI vessel extraction ...................................................................... 24

3.2.5 Co-registration of ex vivo micro-CT to in vivo MRI data .................................................. 28

3.2.6 Computation of vascular morphology .............................................................................. 30

3.3 Results ................................................................................................................................... 32

3.3.1 Micro-CT and micro-MRI vessel segmentation ................................................................ 32

3.3.2 Validation of micro-CT to micro-MRI co-registration ....................................................... 34

3.4 Discussion ............................................................................................................................... 37

4 Validation of in vivo SSC-MRI biomarkers of angiogenesis with ex vivo μCT angiography ...... 39

4.1 Introduction ............................................................................................................................. 39

4.2 Methods ................................................................................................................................ 41

4.2.1 Tumor model and inoculations .......................................................................................... 41

4.2.2 In vivo MRI acquisition and processing ............................................................................. 41

4.2.3 Measurement of USPIO-induced Δχ ................................................................................... 41

4.2.4 Ex vivo image acquisition and processing ......................................................................... 42
4.2.5 Statistical analyses ..................................................................................................... 43
4.3 Results ................................................................................................................................ 45
  4.3.1 Measurement of USPIO-induced Δχ ........................................................................... 45
  4.3.2 Computation of in vivo SSC-MRI biomarkers ............................................................. 45
  4.3.3 Quantitative agreement and correlation between SSC-MRI and μCT ......................... 48
  4.3.4 Predictive value of SSC-MRI biomarkers ................................................................. 51
  4.3.5 Tracking vascular phenotype across tumor stages and sizes ..................................... 52
4.4 Discussion ........................................................................................................................... 54

5 Assessing the effects of anti-angiogenic peptide therapy with in vivo SSC-MRI .............................................................................................................................................. 60
  5.1 Introduction ....................................................................................................................... 60
  5.2 Methods ............................................................................................................................. 63
    5.2.1 Cell culture and inoculation ........................................................................................ 63
    5.2.2 In vivo MRI acquisition ........................................................................................ 63
    5.2.3 Peptide synthesis and treatment ............................................................................... 63
    5.2.4 In vivo MRI image processing ..................................................................................... 64
    5.2.5 Measurement of ferumoxytol-induced Δχ ................................................................. 64
    5.2.6 Histology .................................................................................................................... 65
    5.2.7 Statistical analysis ...................................................................................................... 67
  5.3 Results ................................................................................................................................ 69
    5.3.1 Ferumoxytol-induced Δχ ............................................................................................. 69
    5.3.2 Effect of SP2024 on tumor growth ............................................................................. 69
    5.3.3 Effect of SP2024 on MRI biomarkers .......................................................................... 70
    5.3.4 Histology .................................................................................................................... 76
  5.4 Discussion ........................................................................................................................... 79

6 Large-scale simulations of SSC-MRI using real tumor vasculature .......................... 85
  6.1 Introduction ....................................................................................................................... 85
  6.2 Methods ............................................................................................................................. 89
    6.2.1 Computing magnetic field perturbation maps using the FPM ................................... 89
    6.2.2 Monte Carlo simulation of the MRI signal ................................................................. 93
    6.2.3 Simulations using real tumor vasculature from ex vivo μCT angiography ................. 94
    6.2.4 Simulations using randomly oriented cylinders ......................................................... 95
    6.2.5 Computation of simulated biomarker maps .............................................................. 97
    6.2.6 Statistical analysis ...................................................................................................... 98
  6.3 Results ................................................................................................................................ 101
    6.3.1 Computational performance of large-scale simulations ........................................ 101
    6.3.2 The Good: Fractional blood volume ....................................................................... 101
    6.3.3 The Bad: Vessel size ................................................................................................. 106
    6.3.4 The Ugly: Vessel density ......................................................................................... 110
  6.4 Discussion ........................................................................................................................... 114
List of Tables

Table 4.1 $R^2$ value and percentage of voxels that satisfied goodness-of-fit and $\Delta R_2^*$ calculation criteria.................................................................47

Table 4.2 Spearman correlation coefficients between various quantiles of corresponding $\mu$CT and SSC-MRI parameter distributions .................................................................51

Table 4.3 Median absolute errors from cross-validation analysis .................................................................52

Table 5.1 Changes in tumor parameters and the mutual information between these changes and group membership (control or treated) .................................................................70

Table 6.1 Voxel-wise errors between simulated MRI parameters and their corresponding ground truth values.................................................................................................113

List of Figures

Figure 1.1 Dependence of $\Delta R_2^*$ and $\Delta R_2$ on average cylinder (vessel) radius ........................................7

Figure 1.2 $\Delta R_2^*/\Delta R_2$ increases linearly with average cylinder (vessel) radius ........................................8

Figure 2.1 Histograms of $\Delta R_2^*$ and FBV$_{\mu$CT} illustrating that normality of SSC-MRI and $\mu$CT parameter distributions cannot be generally assumed.................................................................16

Figure 2.2 Tumor-wise scatter plot of median $N_{\text{MRI}}$ vs. $\text{MVD}_{\mu$CT} illustrating that the SSC-MRI and $\mu$CT data do not have bivariate normal distributions ........................................................................17

Figure 3.1 Microfil® as a dual-modality MR and CT contrast agent for $\text{ex vivo}$ angiography ....... 21

Figure 3.2 Flow chart of the $\mu$CT vessel extraction algorithm ........................................................................26

Figure 3.3 Flow chart of the $\mu$MRI vessel extraction algorithm ........................................................................27

Figure 3.4 Vascular morphology and parametric maps derived from high-resolution $\mu$CT .......... 31
Figure 3.5 Illustration of vessel extraction steps for µCT data.................................................. 33
Figure 3.6 Illustration of vessel extraction steps for µMRI data.................................................. 35
Figure 3.7 Validation of vessel extraction and co-registration of µMRI and µCT..................... 36
Figure 4.1 Raw in vivo MR images and corresponding ADC, R₂ *, and R₂ maps.......................... 46
Figure 4.2 Pseudo-color maps of “absolute” SSC-MRI vascular biomarkers............................ 47
Figure 4.3 Pseudo-color maps of “relative” SSC-MRI vascular biomarkers.............................. 48
Figure 4.4 Quantitative error in tumor-wise median FBV,μMRI and VSI,μMRI values............... 49
Figure 4.5 Q-Q plots displaying just the 5th, 25th, 50th, 75th, and 95th quantiles of SSC-MRI parameters vs. their corresponding µCT parameters for every tumor........................................... 50
Figure 4.6 Tumor-wise scatter plots of median µCT and SSC-MRI vascular biomarker values vs. tumor volume................................................................. 53
Figure 5.1 Effect of SP2024 treatment on tumor growth............................................................ 69
Figure 5.2 Raw in vivo MR images and parametric maps of a representative control tumor at days 0 and 14.................................................................................................................. 71
Figure 5.3 Raw in vivo MR images and parametric maps of a representative treated tumor at days 0 and 14.................................................................................................................... 72
Figure 5.4 Plots of changes in median MRI parameter values from day 0 to 14 for individual control (CTRL) and treated (SP2024) tumors............................................................................. 74
Figure 5.5 Box plots of SSC-MRI vascular parameter distributions for individual treated tumors at days 0 and 14.................................................................................................................... 75
Figure 5.6 Histological quantification of viable tumor fraction.................................................... 77
Figure 5.7 Immunofluorescent quantification of tumor vascularization, VEGF, and p-Met........ 78
Figure 6.1 Schematic of the computational pipeline for SSC-MRI simulations.......................... 90
Figure 6.2 Illustration of the overlap-crop method used to expand the FPM to compute large magnetic field perturbation maps...................................................................................... 92
Figure 6.3 Volume renderings of the vascular systems used for SSC-MRI simulations............. 96
Figure 6.4 In silico images and parametric maps generated from the μCT-derived tumor vasculature.

Figure 6.5 Voxel-wise scatter plots of simulated SSC-MRI blood volume biomarkers vs. their ground truth values.

Figure 6.6 Plots of voxel-wise Pearson and Lin’s concordance correlation coefficients between $\text{FBV}_\text{SIM}$ and $\text{FBV}_\text{TRUTH}$ as functions of $\Delta \chi$.

Figure 6.7 Voxel-wise 2D histograms of simulated SSC-MRI biomarkers vs. their ground truth analogs for the μCT tumor data.

Figure 6.8 Voxel-wise scatter plots of simulated SSC-MRI vessel size biomarkers vs. their ground truth values.

Figure 6.9 Plots of voxel-wise Pearson correlation coefficients between $\text{VSI}_\text{SIM}$ and $\langle R \rangle \text{TRUTH}$ as functions of $\Delta \chi$.

Figure 6.10 Plots of voxel-wise errors of $\text{VSI}_\text{SIM}$ with respect to $\text{VSI}_\text{TRUTH}$ as a function of $\langle R \rangle \text{TRUTH}$, $\text{FBV}_\text{TRUTH}$, and $\Delta R^2\text{SIM}$ for the μCT tumor data.

Figure 6.11 Voxel-wise scatter plots of simulated SSC-MRI vessel density biomarkers vs. their ground truth values.

Figure 6.12 Plots of voxel-wise Pearson correlation coefficients between $Q\text{SIM}$ and $N\text{TRUTH}$ and $N\text{SIM}$ and $N\text{TRUTH}$ as functions of $\Delta \chi$. 
1 Background

1.1 Tumor angiogenesis

1.1.1 Angiogenesis in breast cancer

Angiogenesis is the formation of new blood vessels from preexisting vasculature and is critical for tumor growth, invasion, and metastasis. The achievable size of solid tumors is limited without neovascularization to supply the oxygen and nutrients needed for rapid growth. While different tumor types have different vascular phenotypes, there is generally activation of an “angiogenic switch” caused by an imbalance between pro- and anti-angiogenic factors that triggers the formation of new blood vessel and thus enables malignant tumor progression (Baeriswyl and Christofori, 2009; Hanahan and Folkman, 1996). One of the primary pro-angiogenic factors implicated in breast cancer is vascular endothelial growth factor A (VEGF-A), one of the five-member VEGF gene family (Claesson-Welsh and Welsh, 2013; Relf et al., 1997). Increased VEGF expression correlates with decreased response to treatment and shorter survival in breast cancer patients (Schneider and Miller, 2005). Triple-negative breast cancer (TNBC), which accounts for 10-17% of all breast cancers, has been shown to express higher levels of VEGF and is significantly more aggressive and associated with a poorer prognosis than other breast cancer subtypes (Badve et al., 2011; Linderholm et al., 2009; Pal et al., 2011). Chemotherapy is the only systemic treatment option for TNBC because their triple-negative status (lacking estrogen receptor (ER) and progesterone receptor (PR) expression, as well as human epidermal growth factor receptor 2 (HER2) overexpression) makes them unresponsive to currently available targeted therapies. However, several targeted drugs, including anti-angiogenic agents, are being evaluated in TNBC in ongoing clinical trials (Greenberg and Rugo, 2010; Pal et al., 2011).
1.1.2 Angiogenesis as a therapeutic target

The concept of “anti-angiogenesis” as a cancer treatment strategy was first proposed by Judah Folkman in 1971 (Folkman, 1971) and has since led to a large and active field of research in developing anti-angiogenic agents for cancer treatment. Some of these, most prominently the anti-VEGF monoclonal antibody bevacizumab, are clinically approved to treat certain cancers. Other clinically approved anti-angiogenic drugs include sunitinib and sorafenib, which are tyrosine kinase inhibitors (TKI). Despite the promising results from preclinical studies and early clinical trials that led to FDA approval of these drugs, the impact of anti-angiogenic treatment on tumor progression and patient survival is not yet clear and often fails to translate to increased overall patient survival, as discussed in detail in a review by de Oliveira et al. (Bergers and Hanahan, 2008; Leite de Oliveira et al., 2011). Specifically in the case of metastatic breast cancer, recent clinical trials showed that bevacizumab in combination with chemotherapy conferred a small increase in progression free survival compared to chemotherapy alone. However, there was no improvement in overall survival. The FDA recently concluded that the modest gains did not outweigh the increased toxicities and revoked its approval for the breast cancer indication for bevacizumab.

The primary mechanism of action of currently available anti-angiogenic agents is inhibition of the VEGF/VEGFR signaling pathway. Studies have shown that tumors can escape anti-VEGF therapy via adaptive upregulation of alternative angiogenic signaling pathways such as the fibroblast growth factor (FGF) and hepatocyte growth factor (HGF)/c-Met pathways (Bergers and Hanahan, 2008). Furthermore, VEGF inhibition can lead to increased tumor invasiveness and metastatic potential (Ebos and Kerbel, 2011; Paez-Ribes et al., 2009). We still need a better understanding of how anti-angiogenic agents affect cancer cells, the tumor microenvironment, and the patient as a whole. Additional knowledge of the effects of these drugs will help optimize
treatment by identifying targets or combinations of targets that prevent tumors from acquiring
evasive resistance, thus providing sustained treatment efficacy.

1.1.3 The need for clinical biomarkers of angiogenesis

Currently, there are no validated biomarkers of response to anti-angiogenic therapy (Jain et al.,
2009). Tumor volume is a commonly used surrogate marker to determine the efficacy of anti-
angiogenic therapy. However, it may not be a good indicator of angiogenic changes because
inhibition of angiogenesis can occur before detectable decreases in tumor volume (Lindner and
Borden, 1997). Traditionally, breast cancer angiogenesis has been assessed by histological
measurements of microvessel density (MVD) in biopsy samples, which has been associated with
vascular endothelial growth factor (VEGF) expression (Guidi et al., 1997) and tumor
aggressiveness (Weidner, 1995). However, it is not clinically feasible to repeatedly perform
patient biopsies to obtain MVD measurements throughout the course of treatment. In addition,
a single, small biopsy sample may not accurately represent the heterogeneous angiogenic
phenotype of the whole tumor. Thus, there is a need for reliable and repeatedly measureable
biomarkers of angiogenesis to help facilitate: i) development and evaluation of new anti-
angiogenic drugs; ii) identification of patients most likely to benefit from anti-angiogenic
treatment; iii) more effective monitoring of treatment response; iv) prediction of drug
resistance; and consequently v) more adaptable, efficacious, and personalized treatment
planning.
1.2 Imaging tumor vasculature

There are several noninvasive in vivo imaging techniques such as positron emission tomography (PET), ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) that provide information on vascular function and structure while circumventing the limitations of traditional endpoints of anti-angiogenic therapy such as MVD.

Dynamic contrast enhanced (DCE) MRI and CT are currently used for imaging tumor vascular function both clinically and preclinically (Batchelor et al., 2007; Fournier et al., 2010; Kan et al., 2005). The basic principles of these DCE imaging techniques are reviewed by (O’Connor et al., 2011). In brief, the general concept is to intravenously administer a contrast agent bolus and dynamically image the change in signal over time. The signal change can be related to contrast agent concentration based on the physics of the imaging modality, e.g., CT contrast agents increase X-ray attenuation whereas MRI contrast agents modulate the magnetization relaxation rates of hydrogen nuclei (protons) in water molecules in tissue. Next, pharmacokinetic models are used to analyze the concentration-time curve and obtain various parameters related to perfusion, blood flow, blood volume, and vascular permeability. Although these parameters are routinely used to evaluate anti-angiogenic and anti-vascular therapies in preclinical and clinical studies, they are not yet considered clinically validated biomarkers of angiogenesis (Miles et al., 2012; Nielsen et al., 2012b; O’Connor et al., 2012; Sessa et al., 2008).

While functional vascular imaging is a promising and active area of research, the focus of this work is on imaging the structure of breast cancer vasculature. There are several intravital optical microscopy techniques that have been used to image vasculature in preclinical tumor models. These include multi-photon fluorescence microscopy (Tozer et al., 2005) and optical coherence tomography (Vakoc et al., 2012). While these methods have very high spatial resolution, imaging
depth is limited to 1-2 mm, so their clinical application is limited. MRI and CT have no depth limitations but cannot match the resolution of optical imaging techniques. In preclinical applications of 3D MR angiography (MRA) and CT angiography (CTA), vessels as small as 40-60 μm can be identified (Figueiredo et al., 2011). Even in clinical settings, contrast enhanced MRA can be used to detect tumor induced changes in vascularity (van Vliet et al., 2005); it is also feasible to quantify treatment-induced changes in the 3D morphology of blood vessels down to ~0.4 mm in radius (Bullitt et al., 2007). However, microvascular structure cannot be imaged with clinical angiography.

1.2.1 In vivo steady-state susceptibility contrast MRI

Steady-state susceptibility contrast (SSC)-MRI is a contrast enhanced in vivo imaging technique that does not directly visualize the vasculature but provides quantitative information about vascular morphology based on the behavior of the MR signal. Susceptibility contrast is created by an intravascular (super)paramagnetic agent such as ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles. SSC-MRI is an established preclinical imaging method, and the recent development of FDA-approved USPIO agents has enabled its adoption in clinical trials (Fredrickson et al., 2012a; Stabi and Bendz, 2011). The contrast agent creates a difference in magnetic susceptibility (Δχ) between the intra- and extravascular spaces, which results in microscopic local magnetic field perturbations. This causes water protons to experience different magnetic fields, causing them to precess at different Larmor frequencies, leading to loss of phase coherence and ultimately an increase in the rate at which the bulk transverse magnetization of the tissue decays. This MR signal decay is characterized by the transverse relaxation rate constants R_2 (for a spin echo experiment) and R_2* (for a gradient echo experiment).
The steady-state contrast agent-induced changes in these transverse relaxation rates ($\Delta R_2$ and $\Delta R_2^*$) depend on vascular morphology, the diffusion coefficient ($D$) of water, and the characteristic frequency shift created by the intravascular contrast agent. For an ensemble of randomly oriented cylinders, this characteristic frequency shift ($\delta \omega$) is given by (Yablonskiy and Haacke, 1994):

$$\delta \omega = \frac{4}{3} \pi \cdot \gamma \cdot \Delta \chi \cdot B_0$$  \hspace{1cm} [1]

where $\gamma$ is the gyromagnetic ratio of the $H^1$ nucleus and $B_0$ is the main magnetic field strength.

In a seminal paper, Boxerman et al. used ensembles of randomly oriented and randomly distributed magnetized cylinders to model blood vessels and ran Monte Carlo simulations to determine the dependence of $\Delta R_2$ and $\Delta R_2^*$ on various biophysical parameters (Boxerman et al., 1995). They showed that both $\Delta R_2$ and $\Delta R_2^*$ increased linearly with vascular (cylinder) volume fraction (Figure 1.1a). Their simulations also showed that $\Delta R_2^*$ increased with vessel radius $R_V$ and then plateaued for radii greater than capillary size. In contrast, $\Delta R_2$ is a non-monotonic function of vessel size, peaking for vessels around capillary size and then decreasing for larger vessels (Figure 1.1b). The small values of $\Delta R_2^*$ and $\Delta R_2$ for small radii are the result of motional narrowing, which occurs when the characteristic time for a water molecule to diffuse a distance comparable to a vessel’s radius ($\tau_D = R_V^2/D$) is much less than the characteristic time of spin dephasing caused by the local magnetic field inhomogeneities created by that vessel ($1/\delta \omega$). In other words, when $\delta \omega \cdot \tau_D << 1$, the Brownian motion of water molecules is rapid enough to average out the phases of neighboring nuclear spins. The static dephasing regime holds when the opposite is true and $\delta \omega \cdot \tau_D >> 1$, i.e., spin dephasing caused by static local field gradients occurs more rapidly than diffusion can average out spin phases. As $\delta \omega \cdot \tau_D \to \infty$, $\Delta R_2^*$ approaches...
its static dephasing limit; but the 180° pulse used in spin echo (SE) pulse sequences increasingly refocuses the spins, and $\Delta R_2$ approaches zero.

![Figure 1.1 Results of Monte Carlo simulations of SSC-MRI from Boxerman et al. a) both $\Delta R_2^*$ and $\Delta R_2$ increase linearly with cylinder (vascular) volume fraction. b) $\Delta R_2^*$ and $\Delta R_2$ depend differently on cylinder (vessel) radius. $\Delta R_2^*$ increases monotonically and then plateaus for larger vessels, while $\Delta R_2$ peaks around the size of capillaries. Adapted from (Boxerman et al. 1995).](image)

Dennie et al. determined that the differential vessel size dependencies of $\Delta R_2$ and $\Delta R_2^*$ could be exploited to gain information on the average vessel size in an imaging voxel (Dennie et al., 1998). Their Monte Carlo simulations showed that the ratio of $\Delta R_2^*$ to $\Delta R_2$ increases linearly with cylinder radius (Figure 1.2), and in vivo measurements of $\Delta R_2^*/\Delta R_2$ in a rat glioma model were greater in the tumor than in contralateral gray matter, consistent with the larger tumor vessel diameters measured from histology.

Yablonskiy and Haacke developed a theoretical framework to analytically describe gradient echo (GE) signal behavior in the static dephasing regime in various model biological systems, including a vascular network represented by randomly oriented and distributed cylinders (Yablonskiy and...
Haacke, 1994). They determined that for randomly oriented cylinders and for the long time scale ($\delta \omega t \geq 1.5$), the GE signal decays monoexponentially with a relaxation rate constant given by:

$$\Delta R_2^* = \frac{2}{3} FBV \delta \omega$$

where $FBV$ is the fractional blood volume (Eqs. 21-22 in [Yablonskiy and Haacke, 1994]). Thus, blood volume can be indirectly measured by measuring $\Delta R_2^*$ and $\Delta \chi$. Tropres et al. rearranged Eq. 2 for the application of SSC-MRI to measure FBV (Tropres et al., 2001):

$$FBV_{MRI} = \frac{3}{4\pi} \frac{\Delta R_2^*}{\Delta \chi C B_0}$$

where $\Delta \chi C$ is the difference in the susceptibility of blood with and without the presence of contrast agent.

Figure 1.2 Results of Monte Carlo simulations of SSC-MRI from Dennie et al. show that the ratio $R = \Delta R_2^*/\Delta R_2$ increases linearly with average cylinder (vessel) radius. Adapted from (Dennie et al. 1998).
Similarly, Kiselev and Posse extended the analytical model of Yablonskiy and Haacke to describe SE signal behavior near the static dephasing regime, i.e., in the slow diffusion regime where the echo time $TE << R_V^2/D$ (Kiselev and Posse, 1999). They estimated that when $TE >> \delta \omega^{-1}$:

$$R_2 = 0.694 \left( D\delta \omega^2 \right)^{1/3} \text{VSI}^{-2/3}$$  \[4\]

VSI is the vessel size index defined by:

$$\text{VSI}^{-2/3} = \int_0^\infty R_V^{-2/3} f(R_V) \, dR_V$$  \[5\]

where $f(R_V)$ is the volume fraction of vessels with radius $R_V$. Tropres et al. proposed an equation for measuring VSI with SSC-MRI by solving Eq. 2 for $\delta \omega$, substituting the result into Eq. 4, and solving for VSI (Tropres et al., 2001):

$$\text{VSI}_{MRI} = 0.425 \left( \frac{D}{\gamma \Delta \chi B_0} \right)^{1/2} R_{MRI}^{3/2}$$  \[6\]

$$R_{MRI} \equiv \frac{\Delta R_2}{\Delta R_2^*}$$  \[7\]

Jensen and Chandra introduced an SSC-MRI measure of vessel density called $Q$ (Jensen and Chandra, 2000):

$$Q \equiv \frac{\Delta R_2}{(\Delta R_2^*)^{2/3}}$$  \[8\]

$Q$ is related to the histologic vessel density $N$, which they defined as:

$$N = \frac{f}{2\pi(R_V^*)}$$  \[9\]
where $f$ is the area fraction of vessels, and $\langle x \rangle$ is the mean of any distribution $x$. Substituting $\text{FBV}_{\text{MRI}}$ (Eq. 3) for $f$ and $\text{VSI}_{\text{MRI}}^2$ (Eq. 6) for $\langle R_v^2 \rangle$ in Eq. 8 results in $N \sim Q^3 / D$. Here, an SSC-MRI measure of vessel density is defined by assuming equality of the expression:

$$N_{\text{MRI}} = \frac{Q^3}{D}$$

[10]
1.3 Aims

Anti-angiogenic therapy in clinical trials has produced mixed results, but it is still a viable avenue of cancer treatment that is being actively researched. This area of research is particularly relevant to TNBC, for which systemic treatment options are currently lacking. TNBCs are typically highly angiogenic, so anti-angiogenic drugs may help fill this need. Efforts are also underway to develop biomarkers of angiogenesis to aid the development, evaluation, and optimization of novel anti-angiogenic drugs. Current candidate biomarkers include histologic MVD; blood serum markers such as circulating VEGF and endothelial progenitor cells; and various advanced imaging parameters measured by PET, US, CT, and MRI. SSC-MRI is a method used to measure vascular morphological parameters, which are emerging as potential translatable biomarkers of anti-angiogenic treatment response. The goal of this work is to evaluate the utility of SSC-MRI vascular biomarkers in a preclinical model of human TNBC. The following specific aims have been accomplished to address this goal:

1. Develop a method to segment, co-register, and characterize blood vessels from ex vivo 3D micro-MRI (μMRI) and micro-CT (μCT) angiography data for biomedical applications (Chapter 3).

2. Validate in vivo steady-state susceptibility contrast (SSC)-MRI biomarkers of angiogenesis in an orthotopic human breast cancer model (MDA-MB-231) with 3D vascular morphology acquired by ex vivo μCT (Chapter 4).

3. Use these SSC-MRI biomarkers to evaluate the early in vivo effects of a novel anti-angiogenic peptide in MDA-MB-231 breast tumor xenografts (Chapter 5).

4. Incorporate the μCT angiography data from Aim 2 into image-based simulations to elucidate the biophysical factors affecting the accuracy of in vivo SSC-MRI biomarkers of angiogenesis (Chapter 6).
2 General methods

2.1 Tumor model

The tumor model used for this research was an orthotopic MDA-MB-231 human breast cancer model in female athymic NCr-nu/nu mice. The MDA-MB-231 breast adenocarcinoma cell line was established at M. D. Anderson Hospital and Tumor Institute (Houston, TX) from a single pleural effusion from a breast cancer patient (Cailleau et al., 1974). MDA-MB-231 cells are triple-negative; and like many TNBC, they are invasive, metastatic, and express high levels of VEGF (Bhujwalla et al., 2001).

MDA-MB-231 cells were grown in RPMI-1640 cell culture medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). Female athymic NCr-nu/nu mice were anesthetized with ketamine/acepromazine, and $3 \times 10^6$ MDA-MB-231 cells in 50 μL of Hank's balanced salt solution (Sigma-Aldrich) were orthotopically inoculated into the lower thoracic mammary fat pad. All animal studies were performed in accordance with the Johns Hopkins University Animal Care and Use Committee guidelines.
2.2 *In vivo MRI protocol*

*In vivo* MRI experiments were performed on a Bruker Biospin (Billerica, MA) 9.4T small animal MRI system using a custom-built 18-mm diameter radiofrequency (RF) transceiver surface coil. Mice were anesthetized with ketamine/acepromazine or isoflurane, cannulated via the tail vein for contrast agent administration, and secured on a custom cradle with the tumor positioned within the RF coil. A heating blanket was used to maintain the temperature of each mouse at 37°C. Mice were maintained under 1-2% isoflurane administered through a nose cone during imaging. The isoflurane-air mixture was adjusted to maintain a respiration rate of ~50 breaths per minute to reduce motion artifacts. Tumors were imaged using the following MRI sequences:

i) 2D diffusion-weighted imaging (DWI): TE = 26.6 ms, repetition time (TR) = 1000 ms, diffusion gradient amplitude G = 14 G/cm, diffusion gradient duration δ = 5 ms, diffusion gradient separation Δ = 11 ms, number of averages (NA) = 2; one non-diffusion-weighted image and three diffusion-weighted images with a \( b \)-value of 327 s/mm\(^2\) and diffusion-sensitizing gradient orientations along the x-, y-, and z-axes were acquired.

ii) 2D multi-echo GE (MGE): six echoes with 3 ms echo spacing and first TE = 4.2 ms, or six echoes with 5 ms echo spacing and first TE = 5 ms, TR = 800 ms, NA = 4.

iii) 2D multi-echo SE (MSE): six echoes with 8.4 ms echo spacing and first TE = 8.4 ms, or eight echoes with 10 ms echo spacing and first TE = 10 ms, TR = 1500 ms, NA = 2.

For SSC imaging, MGE and MSE images were acquired before and 5 min after tail vein injection of either ferumoxide (Feridex I.V.*®, Bayer Healthcare Pharmaceuticals, Montville, NJ) at a dose of 25 mg Fe/kg or ferumoxytol (Feraheme®*, AMAG Pharmaceuticals, Waltham, MA) at a dose of 5 mg Fe/kg. For all scans, matrix size = 128 × 128, and slice thickness = 1 mm. The number of slices varied to cover the spatial extent of each tumor and ranged from three to eight. Total scan time for each mouse was approximately 1 h.
2.3 *In vivo* MRI processing and analysis

Voxel-wise maps of apparent diffusion coefficients (ADC), $R_2$, and $R_2^*$ were calculated from DWI, MSE, and MGE images, respectively. ADC maps were calculated using DTI-Studio (H. Jiang and S. Mori, Department of Radiology, Johns Hopkins University, Baltimore, MD). The MR signal intensity can be expressed as a function of $b$-value and ADC:

$$S = S_0 \exp (-b \times \text{ADC})$$ \[11\]

where $S$ and $S_0$ are the signal intensities with and without diffusion weighting, respectively. The $b$-value of 327 s/mm² used for the DWI acquisition was calculated from the following equation:

$$b = \gamma^2 G^2 \delta^2 \left( \Delta - \delta / 3 \right).$$ \[12\]

Solving Eq. 11 for ADC for the three DW images with orthogonal diffusion-sensitizing gradients gives the ADC along each gradient direction: $ADC_x$, $ADC_y$, and $ADC_z$. The final ADC map is a map of the mean diffusivity:

$$ADC = (ADC_x + ADC_y + ADC_z) / 3.$$ \[13\]

Using the Analysis of Functional NeuroImages (AFNI) software package (Cox, 1996), voxel-wise pre- and post-contrast $R_2$ and $R_2^*$ maps were calculated using a least-squares fit of the signal intensity vs. TE data to a mono-exponential model with a constant noise term:

$$S(\text{TE}) = S_0 \exp \left( -R_2^* \times \text{TE} \right) + \epsilon.$$ \[14\]

In some voxels, the post-contrast signal had already significantly decayed by the first TE due to high vascularity and thus high contrast agent concentration. This resulted in underestimation of $S_0$ and $R_2^*$. Since technical limitations of the scanner precluded shortening the TE, the post-contrast signal fitting was improved by using the pre-contrast data as follows: theoretically, the
post-contrast $S_0$ should be equal to or greater than the pre-contrast $S_0$ due to $T_1$ shortening effects of the USPIO contrast agent. Therefore, if the initial estimated post-contrast $S_0$ was less than the pre-contrast $S_0$, that pre-contrast $S_0$ value was appended to the post-contrast signal at TE = 0, and the exponential fit recalculated.

All $R_2$ and $R_2^*$ maps were thresholded by the $F$ statistic ($F$-stat) to retain only those voxels for which the full (i.e., exponential) model fit the data significantly better ($p < 0.05$) than the reduced (i.e., constant) model. $\Delta R_2$ and $\Delta R_2^*$ maps were calculated by subtracting the pre-contrast $R_2$ and $R_2^*$ maps from the corresponding post-contrast maps. Voxels exhibiting negative $\Delta R_2$ or $\Delta R_2^*$ were discarded from further analysis. ADC, $\Delta R_2$, and $\Delta R_2^*$ maps were then used to generate $FBV_{MRI}$, $VSI_{MRI}$, $R_{MRI}$, $Q$, and $N_{MRI}$ maps according to Eqs. 3, 6, 7, 8, and 10. In addition to these five parameters, we also considered $\Delta R_2$ as a biomarker of microvascular blood volume, $R_{MRI}^{3/2}$ as a biomarker of vessel size, and $Q^3$ as a biomarker of vessel density. Parameters that are functions of only $\Delta R_2$ and $\Delta R_2^*$ ($\Delta R_2$, $\Delta R_2^*$, $R_{MRI}$, $R_{MRI}^{3/2}$, $Q$, and $Q^3$) were categorized as “relative” biomarkers because they are proportional to measures of vascular morphology. Since $\Delta R_2^*$ is directly proportional to $FBV_{MRI}$ it was not considered as a separate biomarker. In contrast, parameters that incorporated ADC and/or $\Delta \chi_C$ ($FBV_{MRI}$, $VSI_{MRI}$, and $N_{MRI}$) were categorized as “absolute” biomarkers because they are measures of vascular morphology in the appropriate units (e.g., $VSI_{MRI}$ measures vessel radius in $\mu$m).
2.4 Statistical analysis

In generally, neither the μCT nor MRI data were normally distributed. Thus, unless stated otherwise, nonparametric statistics were used since they do not make any assumptions about the shapes of the underlying distributions. Because intratumor distributions were heavily skewed (Figure 2.1), their central tendencies were measured by the median, which are less sensitive to outliers than the mean.

![Histograms of ΔR² and FBVμCT](image)

**Figure 2.1** The highly skewed histograms of ΔR² and FBVμCT from one MDA-MB-231 breast tumor xenograft illustrate that normality of SSC-MRI and μCT parameter distributions cannot be generally assumed.

When comparing two independent groups, the nonparametric Mann-Whitney U test was used instead of the commonly used t test, which assumes that both groups follow normal distributions with equal variances. In contrast, the Mann-Whitney U test makes no assumptions about distribution shape; and instead of testing for a difference in means, it tests for a difference in the medians of the two groups. The Mann-Whitney U test is almost as powerful...
(able to accurately reject the null hypothesis) as the $t$ test when the assumptions of the $t$ test are met; it is more powerful than the $t$ test when these assumptions are not met (Dawson and Trapp, 2004).

Similarly, the Spearman correlation coefficient ($r_s$) was used instead of the more common Pearson product moment correlation coefficient ($r$), which assumes that the two variables in question have a bivariate normal distribution. The Spearman correlation coefficient, essentially $r$ computed for the ranks of the data, is nonparametric and more robust to outliers than the Pearson correlation coefficient. This is illustrated in Figure 2.2.

![Figure 2.2](image)

**Figure 2.2** Tumor-wise scatter plot of median $N_{\text{MRI}}$ vs. $\text{MVD}_{\mu\text{CT}}$. The data do not have a bivariate normal distribution, and the outlier in the upper right results in a very high Pearson correlation ($r = 0.97$). The Spearman correlation coefficient is insensitive to outliers and shows that the correlation of the ranks of the data is relatively lower ($r_s = 0.79$).
3 Multimodal characterization of 3D tumor vascular morphology

3.1 Introduction

Tumor vascular morphology is highly heterogeneous and markedly different from normal vasculature. It is characterized by dilated and tortuous vessels, chaotic branching, and blind ends (Vaupel, 2004). It is also strongly dependent on tumor size, location within the tumor, and local angiogenic activity (Vajkoczy and Menger, 2000). Therefore, the vascular architecture provides insights into the angiogenic phenotype, which traditionally has been characterized using histologic techniques in terms of microvessel density (MVD) (Weidner, 1995). Although optical microscopy is a powerful tool for imaging the microvasculature at submicron resolutions, it offers only limited coverage, and recovering the 3D vascular and tissue morphology after sectioning requires complex reconstruction.

Multiphoton microscopy is a 3D optical imaging technique that has been used to image murine cortical capillaries at submicron resolution (Verant et al., 2007), but the imaging depth is limited to a few hundred microns. A recent study used optical coherence tomography (OCT) to image blood and lymphatic vascular morphology, as well as tissue viability in various tumor models in vivo (Vakoc et al., 2009). OCT offers a wider field of view and greater tissue penetration at the expense of spatial resolution relative to multiphoton microscopy, and does not require the administration of contrast agents. However, the maximum imaging depth is between 1 and 2 mm below the tissue surface; hence, coverage is still limited relative to modalities such as MRI and CT.

3.1.1 Ex vivo micro-CT angiography

Micro-CT (μCT) is a method in which a 3D image is reconstructed from 2D X-ray projections taken at incremental angles around an axis of rotation. For vascular imaging, the blood vessels
are filled with a contrast agent with low viscosity and high radiopacity (i.e., strongly attenuates X-rays), which provides high positive contrast in CT images (Figure 3.1a). A typical vascular casting procedure is described in Verli et al. (Verli et al., 2007); in brief, the animal is anesthetized and then perfused first with a solution such as heparinized saline for exsanguination, followed with a fixative such as formaldehyde, and finally with a vascular filling agent. Microfil® (Flow Tech Inc., Carver, MA), a silicone rubber compound is commonly used for μCT angiography and is also used in this work. Micro-CT is capable of imaging the vasculature of entire tumors at isotropic resolutions of up to ~3 μm (Folarin et al., 2010). This spatial resolution is high enough to image the vascular architecture down to the capillary level, as demonstrated by a study that showed excellent agreement between μCT- and scanning electron microscopy (SEM)-measured cortical vessel diameters (Heinzer et al., 2006). Another advantage of μCT is that tissue maceration is not required, which allows samples to be preserved for further study (e.g., histology). Micro-CT has also been successfully combined with MRI to create a high-resolution, 3D atlas of the neurovasculature in a CBA mouse (Dorr et al., 2007). This multi-modality dataset shows the placement of the major arteries, their branches, and the brain structures they feed. As one of the first 3D atlases of the cerebral vasculature in mice, it is proving useful for comparative studies involving the murine neurovasculature in healthy and disease models.

Recently, a hierarchical imaging method was developed that involved using synchrotron radiation μCT (SRμCT) to image corrosion casts of 3D capillary networks at 1.4 μm resolution in select regions of interest (ROIs) 1mm³ in size (Heinzer et al., 2006). Another study employed SRμCT on 2.5 mm diameter cylindrical samples from 9L tumor-bearing rat brains and obtained high contrast between the vasculature and background tissue at 1.4 μm resolution (Risser et al., 2007). The sub-capillary spatial resolution of their 3D images enabled them to ascertain that
both tumor and normal brain vasculature display fractal organization on small spatial scales. Chien et al. employed SRµCT to profile “microangiogenesis” in a panel of subcutaneous tumor models as well as in an orthotopic pancreatic tumor model (Chien et al., 2011). They could assess small differences between the vasculature of pancreatic tumors derived from control or radiation activated PANC1 tumor cells. Its combination of large volumetric coverage, high spatial resolution, and high contrast makes µCT uniquely suited for imaging tumor vascular structure. Further applications of vascular imaging with µCT can be found in the review by Zagorchev et al. (Zagorchev et al., 2010).

3.1.2 Ex vivo micro-MRI angiography

MR microscopy or micro-MRI (µMRI) is essentially MRI at high resolution, which is enabled by high main magnetic field and gradient field strengths, highly sensitive radiofrequency (RF) coils, and long acquisition times (Glover and Mansfield, 2002). Micro-MRI is commonly defined as MR imaging with resolution higher than 100 µm and is capable of acquiring images with isotropic resolution down to ~20 µm (Johnson et al., 2007). It has previously been applied to phenotyping various transgenic mouse models (Benveniste and Blackband, 2002; Johnson et al., 2002), and to image the vasculature of mouse embryos (Berrios-Otero et al., 2009; Smith et al., 1994) and adult rat brains (Johnson et al., 1997) using gadolinium-doped gelatin as an intravascular contrast agent. We developed an ex vivo µMRI angiography technique that employs the µCT-compatible vascular filling agent Microfil, which acts as a negative MRI contrast agent because it lacks the mobile water protons necessary for producing an MR signal. Thus, a 3D µMRI image can be acquired in which blood vessels appear dark against bright background tissue (Figure 3.1b).
Figure 3.1 Dual-modality *ex vivo* angiography. a) Micro-CT maximum intensity projection and (b) μMRI minimum intensity projection of the same Microfil-perfused mouse brain. Microfil provides positive contrast in CT images and negative contrast in MR images.

We exploited the fact that Microfil provides excellent contrast in both CT and MR images to construct a pipeline for multimodal imaging and characterization of tumor vasculature. *Ex vivo* μCT is used to obtain high-resolution, 3D information on the vascular morphology of whole tumor xenografts, while the dual vascular and soft tissue contrast of *ex vivo* μMRI is exploited to facilitate co-registration between *ex vivo* μCT and *in vivo* MRI data (Cebulla et al., submitted for publication; Kim et al., 2013; Kim et al., 2012). The co-registered μCT vascular morphometric data generated from this aim (Aim 1) was used to help accomplish Aims 2 and 4: it was used as
a reference standard to validate in vivo SSC-MRI vascular biomarker measurements (Chapter 4) and as an input to image-based SSC-MRI simulations (Chapter 6). This chapter describes the details of the ex vivo imaging, vessel extraction, co-registration, and vascular morphometric characterization; and briefly discusses other applications of this vascular imaging platform.
3.2 Methods

3.2.1 Sample preparation for ex vivo angiography

After in vivo MRI (discussed in Chapter 2.2), mice were deeply anesthetized with isoflurane and then sacrificed by perfusion fixation. The chest was opened to expose the heart, and a butterfly needle inserted into the left ventricle. Mice were first perfused with 20 ml of heparinized phosphate-buffered saline (PBS) for exsanguination, and then with 20 ml of formaldehyde fixative, and finally with 20 ml of a 1:2 mixture of Microfil and diluent with 5% v/v curing agent. The jugular veins were cut to allow drainage of the perfusates. The Microfil was allowed to cure at room temperature for 90 minutes before the tumors were excised and immersion fixed in formaldehyde for 48 h at 4°C.

3.2.2 Ex vivo micro-CT

Tumors were sent to Numira Biosciences (Salt Lake City, UT) to be imaged on a high-resolution volumetric μCT scanner (μCT40, ScanCo Medical, Brüttisellen, Switzerland). 3D vasculature data were acquired with the following imaging parameters: 8 μm isotropic resolution, 55 kVp, 144 μA tube current, 300 ms exposure time, 2000 views, and five frames per view.

3.2.3 Ex vivo micro-MRI

Twenty-four hours before μMR imaging, samples were immersed in phosphate-buffered saline doped with 1 mM Gd-DTPA (Omniscan, GE Healthcare, Oslo, Norway) to enhance $T_1$ relaxation and soft tissue contrast. Each sample was imaged in a nuclear magnetic resonance (NMR) tube filled with Fomblin (Solvay Solexis, Milano, Italy), which provides a dark, uniform image background because it does not contribute any proton signal to the MR images. Tumors were imaged on a 400-MHz spectrometer in a 10-mm volume RF coil (Bruker BioSpin Corp., Billerica,
MA, USA) using a 3D MGE sequence with six echoes, echo spacing = 4.8 ms, first TE = 4.9 ms, TR = 150 ms, NA = 14, resolution = 40 μm isotropic, and acquisition time ~11-18 h.

### 3.2.4 Micro-CT and micro-MRI vessel extraction

A multi-step vessel extraction algorithm was developed in ImageJ (Rasband, W.S., National Institutes of Health, Bethesda, MD, USA, [http://rsbweb.nih.gov/ij/](http://rsbweb.nih.gov/ij/)). Flow charts of the μCT and μMRI vessel extraction algorithms are presented in Figure 3.2 and Figure 3.3, respectively.

First, for both modalities, a tubeness filter described by Sato et al. (Sato et al., 1998) was applied to enhance the contrast between the vasculature and surrounding tissue. The filter determines how tube-like structures are based on the eigenvalues of the Hessian matrix at each voxel. The Hessian of a function \( f(x,y,z) \) is a matrix of its second-order partial derivatives:

\[
\mathbf{H}(f(x,y,z)) = \begin{bmatrix}
    \frac{\partial^2 f}{\partial x^2} & \frac{\partial^2 f}{\partial x \partial y} & \frac{\partial^2 f}{\partial x \partial z} \\
    \frac{\partial^2 f}{\partial y \partial x} & \frac{\partial^2 f}{\partial y^2} & \frac{\partial^2 f}{\partial y \partial z} \\
    \frac{\partial^2 f}{\partial z \partial x} & \frac{\partial^2 f}{\partial z \partial y} & \frac{\partial^2 f}{\partial z^2}
\end{bmatrix}
\]  \[15\]

The filter convolves the image \( I(x,y,z) \) with a Gaussian kernel \( G(x,y,z;\sigma) \) and computes the Hessian of the result:

\[
\mathbf{H}(G(x,y,z;\sigma) * I(x,y,z))
\]  \[16\]

Then, a tubeness metric is calculated from the Hessian eigenvalues \( \lambda_1, \lambda_2, \lambda_3 \) given by Eq. 16 in (Sato et al., 1998):

\[
\lambda_{123}(x,y,z) = \begin{cases}
    \left|\lambda_3\right| \left(\frac{\lambda_2}{\lambda_3}\right)^{Y_{23}} \left(1 + \frac{\lambda_1}{\left|\lambda_2\right|}\right)^{Y_{12}}, & \lambda_3 < \lambda_2 < \lambda_1 \leq 0 \\
    \left|\lambda_3\right| \left(\frac{\lambda_2}{\lambda_3}\right)^{Y_{23}} \left(1 - \alpha \frac{\lambda_1}{\left|\lambda_2\right|}\right)^{Y_{12}}, & \lambda_3 < \lambda_2 < 0 < \lambda_1 < \frac{\left|\lambda_3\right|}{\alpha} \\
    0, & \text{otherwise}
\end{cases}
\]  \[17\]
For a bright cylindrical object against a dark background, none of the three Hessian eigenvalues will be positive. At the center of the cylinder, the eigenvector corresponding to $\lambda_1$ will point along the cylinder axis, resulting in $\lambda_1 = 0$. The two orthogonal eigenvectors will lie in the cross-sectional plane, and their corresponding eigenvalues $\lambda_2$ and $\lambda_3$ will be negative. Thus, this metric was designed to have large values when $\lambda_1 \approx 0$ and $\lambda_2, \lambda_3 << 0$. Because the tubeness filter expects bright vessels, the $\mu$MRI images were first inverted. The filter response can be tuned by varying the parameters $\gamma_{12}, \gamma_{23}$, and $\alpha$. For our data, we confirmed Sato’s observation that the filter performance is fairly independent of $\gamma_{12}$ and $\gamma_{23}$ for values between 0.5 and 1. Thus, the suggested parameter values of $\gamma_{12} = \gamma_{23} = 1$ and $\alpha = 0.25$ were used for all of our application.

The standard deviation $\sigma$ of $G$ can be varied to sensitize the tubeness filter to vessels of different sizes. To extract vessels of all sizes, the filter was applied multiple times using different values of $\sigma$, and the outputs integrated using a maximum operator to obtain a multiscale tubeness metric:

$$T(x,y,z) = \max_\sigma \lambda_{123}(x,y,z; \sigma)$$  \hspace{1cm} [18]

The filter was applied with $\sigma = 0.8, 1, 1.2, $ and $1.4$ for the $\mu$MRI data and $\sigma = 1$ and $1.5$ for the $\mu$CT data. Then, for the $\mu$CT data, $T(x,yz)$ was smoothed using a 3D mean filter (6-connected neighborhood kernel). The coarser $\mu$MRI resolution precluded smoothing of $T(x,yz)$ for the $\mu$MRI data. This result was manually thresholded to extract an initial binary vasculature. Large values of $\sigma$ led to dilation of smaller vessels, so the tubeness filter could not be used to extract vessels with diameters larger than approximately four voxels. The high contrast between large vessels and surrounding tissue allowed extraction of these vessels by applying a threshold to the original image. These were then combined with the initial binary vasculature.
Figure 3.2 Flow chart of the μCT vessel extraction algorithm. Images represented by the blue boxes labeled (a-d) are shown in the corresponding panels of Figure 3.5.

The presence of hypointense tumor tissue in the μMRI images necessitated additional steps to extract vessels in these regions where the contrast between vessels and surrounding tissue was low. A conservative threshold was applied to $T(x,y,z)$ to extract the initial binary vasculature, ensuring that no noise was included. However, this resulted in exclusion of vessels in low contrast regions. So a less conservative threshold was applied to $T(x,y,z)$ to produce a second binary image that included vessels in the low contrast regions but also non-vessel tissue structures with intermediate image intensities. MIPAV (Medical Image Processing, Analysis, and Visualization, National Institutes of Health, Bethesda, MD) was used to perform image intensity-
based K-means clustering to segment the raw μMRI data into three to five classes. The classes corresponding to these intermediate intensities were used to mask out the noise in the second binary image. The class corresponding to the lowest intensities accounted for the large vessels that could not be properly extracted by the tubeness filter. This class was combined with the initial binary vasculature and the masked second binary image.

**Figure 3.3** Flow chart of the μMRI vessel extraction algorithm. Images represented by the blue boxes labeled (a-h) are shown in the corresponding panels of **Figure 3.6**.
For both the μCT and μMRI data, 3D morphological closing was performed on the combined extracted vessels using a six-connected neighborhood structuring element to fill small holes or gaps in the vasculature. Finally, 3D regions smaller than 27 voxels (μCT) or 6 voxels (μMRI) in volume determined by a 26-connected neighborhood were considered noise and removed.

The quality of the vessel extraction was qualitatively validated by optical microscopy. Due to positive contrast of the Microfil in μCT images, superficial tumor blood vessels were visible and could be extracted. The same vessels were then imaged by bright-field microscopy and visually compared. Superficial vessels were not visible in the μMRI data since both vessels and background appear dark, so intratumoral vessels had to be visually compared with optical microscopy images. To do this, a 1-mm-thick tumor section was optically cleared by immersion in a mixture of water and glycerin; the glycerin concentration was increased every 24 hours from 50% (v/v) to 75%, 85% and finally 100% glycerin. A bright-field image of the tumor section was then acquired at 2× magnification.

3.2.5 Co-registration of \textit{ex vivo} micro-CT to \textit{in vivo} MRI data

Immediately after excising the tumors, cotton strings soaked in Gd-DTPA were attached along the anteroposterior axis of each tumor. The string was visible in both μCT and μMRI images and served as a fiducial marker to aid co-registration. Co-registration of \textit{ex vivo} μCT to \textit{in vivo} MRI was accomplished in two steps using Amira® (FEI Visualization Sciences Group, Burlington, MA): first, the \textit{ex vivo} μMRI was co-registered to the \textit{in vivo} MRI; second, the μCT was co-registered to the μMRI.

In the first step, the string fiducial was used to correctly orient the raw μMRI image relative to the \textit{in vivo} MRI data. Manual alignment of the two datasets was aided by the similar MR-based soft tissue contrast. A thorough comparison of the tumor shapes between both datasets was
then used to define the final rigid-body transformation for the alignment of the μMRI tumor volume to the in vivo MRI volume. The transformation was executing using Lanczos interpolation, during which the original voxel size was preserved. The same transformation was used to co-register the extracted μMRI vasculature and the manually drawn tumor mask to the in vivo MRI data. The tumor mask was downsampled to the in vivo MRI spatial resolution for ROI analysis of μCT vascular morphometrics.

To co-register the μCT data to the transformed μMRI data, the string fiducial was again used to perform an initial manual alignment. The alignment was refined using landmark-based rigid-body registration. Between five and ten landmark pairs were manually placed on corresponding vessel bifurcations in the raw μCT and μMRI images. Automatic registration was performed by minimizing the total distance between landmark pairs. The extracted μCT vasculature was transformed with the same parameters as those obtained from the registration of the raw μCT data to the μMRI data. This resulted in co-registration of the ex vivo μCT vasculature to the in vivo MRI data.

The co-registration of extracted μMRI and μCT vasculature was validated visually by overlaying the extracted tumor vasculature obtained from both modalities. In addition, we compared the co-registered blood volume maps from the two modalities. Fractional blood volume (FBV) maps for μMRI and μCT data were computed by superimposing a spatial grid of 1 mm isotropic voxels on each co-registered dataset and computing the fractional occupancy of vessels within each grid voxel. Linear regression between the μMRI and μCT FBV was used to determine the quality of both co-registration and vessel segmentation.
### 3.2.6 Computation of vascular morphology

The spatial graph of the co-registered 3D binary vascular network was reconstructed using the Amira skeletonization option. This involved three steps: first, a distance transform was applied to calculate the Euclidean distance from each voxel in the vasculature to the nearest background voxel. Second, the vasculature was skeletonized using an iterative thinning algorithm. Finally, the skeleton was traced to reconstruct the vascular network represented as a spatial graph. The information contained in the spatial graph includes the Cartesian coordinates of all the points of the vascular skeleton, the vessel radius at each of these points, and the length of each vessel segment. A volume rendering of the spatial graph of a breast tumor xenograft section is shown in **Figure 3.4a**. This spatial graph information was used to compute ex vivo μCT vascular morphometric maps analogous to the in vivo SSC-MRI vascular biomarker maps (**Chapter 2.3**). Custom MATLAB (Mathworks, Inc., Natick, MA) functions were written to take the spatial graph data and calculate the average vessel radius \( \langle R \rangle_{\mu CT} \) and the number of vessel segments \( \text{MVD}_{\mu CT} \) in each voxel of the co-registered in vivo MRI image to generate vessel size and density maps (**Figure 3.4c,d**). Another MATLAB function was written to compute μCT FBV (FBV\(_{\mu CT}\)) maps directly from the binary vasculature by calculating the fractional occupancy of vessels in each co-registered in vivo MRI voxel (**Figure 3.4b**).
Figure 3.4 Vascular morphology and parametric maps derived from high-resolution μCT. a) Volume rendering of the extracted vascular spatial graph from a 1 mm thick section of a PIW 5 breast tumor xenograft. Vessels are color-coded by radius. b) FBV_μCT, (c) ⟨R⟩_μCT, and (d) MVD_μCT maps computed from (a).
3.3 Results

3.3.1 Micro-CT and micro-MRI vessel segmentation

The results of the µCT vessel extraction are demonstrated in Figure 3.5. A slice of the raw µCT data is displayed in **Figure 3.5a** with the radio-opaque Microfil causing the blood vessels to appear bright. **Figure 3.5b** shows the result of combining the outputs of the tubeness filter with $\sigma = 1.0$ and 1.5, wherein the most tube-like structures appear brightest. The multiscale tubeness result was subjected to a 3D mean filter and manually thresholded to obtain an initial binary vasculature (red, **Figure 3.5c**). A few remaining holes in large caliber tumor blood vessels had to be filled by applying a conservative threshold on the raw µCT data (green, **Figure 3.5c**) and combining the result with the initial binary vasculature. The final extracted tumor blood vessels after 3D morphological closing and small region removal is displayed in **Figure 3.5d**.

**Figure 3.6** illustrates the outputs of the steps necessary to reliably extract the breast tumor vasculature from the 3D µMRI data. **Figure 3.6a** shows the raw µMRI data in which the Microfil-containing blood vessels appear dark. **Figure 3.6b** shows the output of the multiscale tubeness filter. The result of the K-means clustering of the raw µMRI data is presented in **Figure 3.6c**. **Figure 3.6d** demonstrates that a tubeness filter alone was not sufficient for extracting all the blood vessels because blood vessels of large caliber or in regions with low contrast-to-noise ratio were not extracted by this method. **Figure 3.6e** shows how the combination of K-means clustering and tubeness filtering permitted extraction of blood vessels with low contrast relative to surrounding tumor tissue. **Figure 3.6f** illustrates how the K-means clustering successfully segmented hypointense tumor blood vessels larger than ~80 μm in radius from the raw µMRI data. In **Figure 3.6g**, the results of these three approaches are overlaid, indicating that each step detects a different subset of vessels. **Figure 3.6h** illustrates the final extracted 3D tumor vasculature.
Figure 3.5 Illustration of vessel extraction steps for µCT data. a) Slice of raw µCT data. Blood vessels appear bright due to presence of radio-opaque Microfil. (b) Result of the multiscale tubeness filter using $\sigma = 1.0$ and 1.5. c) Result of the multiscale tubeness filter after applying a 3D mean filter and manual threshold (red), and conservatively thresholded raw data (green). d) Final extracted blood vessels after 3D morphological closing and removal of isolated 3D region of less than 27 voxels are shown in red.

We qualitatively validated the extraction of tumor blood vessels from µCT data by directly comparing the resulting vasculature with optical microscopy. Figure 3.7a shows a surface rendering of superficial vessels extracted from a µCT image. These vessels were also visible in a 4× bright-field microscopy image (arrows), which was overlaid on a fluorescent image showing
constitutive DsRed fluorescent protein expression in the MDA-MB-231 cells (Figure 3.7b). Vessels smaller than ~8µm were only visible in the bright-field image and not in the µCT. A similar comparison was carried out for the µMRI-derived tumor vasculature. Figure 3.7c shows binary vessels extracted from µMRI data and Figure 3.7d shows a bright-field microscopy image of the same tumor region. Vessels that are visible in both images are indicated by red arrows.

3.3.2 Validation of micro-CT to micro-MRI co-registration

The co-registered and extracted µMRI and µCT vessels of a 1-mm section of a tumor were overlaid as shown in Figure 3.7e. This overlay illustrates the high degree of overlap of the extracted tumor vasculature from the two datasets (white arrows) and is indicative of good inter-modality co-registration. This was quantified by linear regression of voxel-wise scatter plots of µMRI- and µCT-derived FBV maps (Figure 3.7f). The R² values for the linear regression ranged between 0.60 and 0.87 with a mean R² of 0.72 (n=8 tumors). Besides the spatial agreement, we observed that partial volume effects resulted in dilation of the µMRI-derived blood vessels compared to both optical microscopy and µCT-derived vessels (Figure 3.7c-e). In addition, small vessels (< 20 µm radius) were visible in the µCT data (red arrows, Figure 3.7e) but not the µMRI data.
Figure 3.6 Illustration of vessel extraction steps for µMRI data. a) Slice of raw GE µMRI data. Blood vessels appear dark due to presence of waterless Microfil. b) Result of the multiscale tubeness filter using $\sigma = 0.8, 1.0, 1.2, 1.4$. c) Result of applying K-means clustering with five classes to (a). The blue arrow indicates class 1, the red arrows denote classes 3 and 4. d) Result (green) of thresholding (b), overlaid on the raw µMRI data. e) Result (red) of applying a less conservative threshold to (b), followed by masking out regions corresponding to classes 3 and 4 in (c). f) Class 1 in (c), shown in blue. g) Overlay of (d), (e) and (f). h) Final extracted blood vessels after 3D morphological closing and removal of regions of less than 6 connected voxels.
Figure 3.7 Validation of vessel extraction and co-registration of µMRI and µCT. a) Surface rendering of vessels extracted from µCT data. b) Fluorescence image of the breast tumor xenograft (constitutive DsRed expression) overlaid with a bright-field image of the Microfilled vasculature (4×). c) Blood vessels extracted from µMRI data. d) A bright-field image of the same tumor region in (c). Vessels visible in both (c) and (d) are indicated by red arrows. (e) Overlay of the extracted blood vessels from µMRI (green) and µCT (yellow) data of a breast tumor xenograft after co-registration. White arrows in the zoomed inset indicate vessels that are visible in both µMRI and µCT data, and red arrows indicate vessels only visible in the µCT. f) Voxel-wise scatter plot of the µMRI-measured FBV versus the µCT-measured FBV for a representative breast tumor xenograft with the linear regression line shown in red.
3.4 Discussion

Quantifying angiogenesis-induced changes in tumor vasculature from medical images is often challenging due to limited contrast-to-noise ratio, image artifacts and spatial resolution limitations. Additionally, each imaging modality has its own unique biophysical contrast mechanism, strengths, and weaknesses when it comes to imaging the tumor vasculature (Pathak et al., 2008a). Therefore, in this work we exploited the unique advantages of ex vivo μMRI and μCT to develop an integrated platform for characterizing angiogenesis at multiple spatial scales in a human breast cancer model.

Due to its high spatial resolution and wide spatial coverage, μCT can be regarded as the ‘gold standard’ for 3D blood vessel visualization in large or whole tissue specimens. We confirmed with optical microscopy that we were able to accurately extract the 3D tumor vasculature from μCT images. One limitation of our vessel extraction procedure was that it involved the manual determination of segmentation thresholds. Although this made our procedure operator dependent, it was necessitated by differing image contrast profiles from tumor to tumor. Thus, manual thresholding resulted in higher-fidelity vessel extraction than automatic thresholding alone. Others have also demonstrated the utility of μCT for quantifying tumor angiogenesis down to the capillary level (Lang et al., 2012) as well as for validating tumor vascular response to antiangiogenic therapy (Sampath et al., 2013; Savai et al., 2009; Shojaei et al., 2007; Ungersma et al., 2010). In addition, we have used our μCT vascular spatial graph data in image-based computational modeling of blood flow to investigate the hemodynamics of whole-tumor vascular networks for the first time (Stamatelos et al., in press).

We employed μMRI to facilitate co-registration between macroscopic in vivo MRI (~100 μm) and microscopic μCT (8 μm) vascular data. The intermediate (~40 μm) spatial resolution of μMRI in
conjunction with its excellent soft tissue contrast facilitates co-registration and discrimination of tumor tissue from normal tissue for ROI analyses. The spatial resolution of μMRI is limited by the MRI scanner hardware specifications such as the main magnetic field and gradient strengths, and also by the image acquisition time. While the limited resolution does not permit imaging of microvessels and produces partial volume effects that overestimate vascular volume and vessel size, we have shown here that μMRI-derived vessels are sufficiently distinguishable to enable multiscale imaging of the tumor vasculature. We have also shown previously that μMRI can be used to quantify and distinguish the vascular phenotype of 9L brain tumor xenografts from contralateral brain as well as between brain tumors at different stages (Kim et al., 2011). An advantage of μMRI is its ability to provide complementary, co-registered physiological information by exploiting various endogenous contrast mechanisms. For example, we have combined our μMRI angiography technique with diffusion tensor imaging to simultaneously visualize brain tumor angiogenesis and invasion (Pathak et al., 2011).

The multimodal vascular imaging method presented here is relevant to a variety of tumor-related applications and may be a useful tool for studying other pathologies with characteristic abnormal vasculature, including stroke and Alzheimer’s disease. The following chapters will expound on the utility of high-resolution μCT angiography data for validating SSC-MRI vascular biomarkers. Chapter 4 describes the use of μCT vascular morphometric maps as the ground truth to validate in vivo SSC-MRI vascular biomarker measurements in MDA-MB-231 breast tumor xenografts. Chapter 6 details the integration of μCT-derived high-resolution 3D tumor vasculature into a new image-based computational modeling platform for SSC-MRI simulations. These simulations were used to investigate the effect of various biophysical factors on the accuracy of in silico SSC-MRI vascular biomarkers.
4 Validation of in vivo SSC-MRI biomarkers of angiogenesis with ex vivo μCT angiography

4.1 Introduction

SSC-MRI is a noninvasive, clinically translatable technique that provides quantitative information about the underlying vasculature such as blood volume, average blood vessel size, and blood vessel density (Fredrickson et al., 2012a; Jensen and Chandra, 2000; Tropres et al., 2001). Each of these MRI-derived parameters has been employed as a biomarker for monitoring the effects of anti-angiogenic therapies on various preclinical and clinical tumors (Batchelor et al., 2007; Farrar et al., 2011; Howe et al., 2008). SSC-MRI biomarkers have been predominantly measured in brain tumor studies, but there are few such studies of human breast cancer. Ferretti et al. showed that treatment with patupilone, a microtubule stabilizer, significantly decreased FBV and VSI in a rat mammary carcinoma model (Ferretti et al., 2005). A recent study by Bauerle et al. used SSC-MRI to measure blood volume and VSI in a mouse model of breast cancer bone metastasis and found that these parameters changed significantly after anti-angiogenic therapy compared to untreated controls (Bauerle et al., 2010).

Recent studies have looked to quantitatively validate these MRI biomarkers with histology and other imaging modalities. For example, Farrar et al. compared the ratios $R_{\text{MRI}}$ and $R_{\text{MRI}}^{3/2}$ calculated from SSC-MRI to vessel radii measured using intravital optical microscopy and histology in a U87 brain tumor mouse xenograft model. They found that $R_{\text{MRI}}$ was in better agreement with the optical imaging methods than $R_{\text{MRI}}^{3/2}$ (Farrar et al., 2010).

Ungersma et al. performed SSC-MRI to measure $FBV_{\text{MRI}}$, $VSI_{\text{MRI}}$, and $N_{\text{MRI}}$ in colorectal tumor xenografts implanted in the hind limb of mice and compared them with equivalent parameters
calculated from histology and high-resolution, *ex vivo* micro-computed tomography (μCT). They observed a significant correlation between mean VSI\textsubscript{MRI} and μCT-derived mean vessel radius, mean VSI and histological mean vessel radius, mean N\textsubscript{MRI} and histological microvessel density, but not between mean FBV\textsubscript{MRI} and μCT vascular volume fraction (Ungersma et al., 2010).

However, the relative utilities of all SSC-MRI vascular parameters for assessing angiogenesis in a human breast cancer model have not been systematically compared. Here, we used SSC-MRI to simultaneously measure FBV\textsubscript{MRI}, ΔR\textsubscript{2}, VSI\textsubscript{MRI}, R\textsubscript{MRI}, Q, and N\textsubscript{MRI} in orthotopic MDA-MB-231 human breast tumor xenografts and assessed the ability of each MRI biomarker to predict its 3D vascular correlate derived from high-resolution, *ex vivo* μCT. We also examined the sensitivity of each angiogenesis biomarker to changes in vascular phenotype as a function of tumor size. The goal of this study was to systematically establish which MRI biomarkers are relevant for evaluating breast cancer angiogenesis *in vivo*.
4.2 Methods

4.2.1 Tumor model and inoculations

Eleven female athymic NCr-nu/nu mice were anesthetized with a cocktail of ketamine/acepromazine, and $3 \times 10^6$ MDA-MB-231 cells inoculated into the lower left thoracic mammary fat pad of each mouse. Further details are provided in Chapter 2.1.

4.2.2 In vivo MRI acquisition and processing

In vivo MRI experiments were performed as described in Chapter 2.2. Five mice were imaged at PIW 3 with tumor volume $= 99 \pm 27 \text{ mm}^3$ (mean $\pm$ SD), and six mice imaged at PIW 5 with tumor volume $= 292 \pm 131 \text{ mm}^3$. All tumors were imaged using the following sequences and parameters: (i) diffusion-weighted (DW) SE; (ii) 2D MGE, TE = 4.2 ms, six echoes with 3 ms echo spacing, repetition time $= 800 \text{ ms}$; (iii) 2D MSE, TE = 8.4 ms, six echoes with 8.4 ms echo spacing, repetition time $= 1500 \text{ ms}$. For SSC imaging, MGE and MSE images were acquired before and 5 min after injection of Feridex (Bayer Healthcare Pharmaceuticals, Montville, NJ) at a dose of 25 mg Fe/kg bodyweight. For all scans, field of view (FOV) = 14 mm $\times$ 14 mm, in-plane resolution = 109 $\mu$m, and slice thickness = 1 mm. See Chapter 2.2 for more details on the in vivo MRI protocol.

Voxel-wise maps of ADC, $\Delta R_2$, and $\Delta R_2^*$ were calculated from DWI, MSE, and MGE images, respectively, as described in Chapter 2.3. These were used to compute the SSC-MRI vascular biomarkers defined by Eqs. 3, 6, 7, 8, and 10 introduced in Chapter 1.2.1. The value of $\Delta \chi_c$ used in Eqs. 3 and 6 was calculated using the method described below.

4.2.3 Measurement of USPIO-induced $\Delta \chi$

The susceptibility difference between blood with and without contrast agent ($\Delta \chi_c$) was measured using the same MRI scanner and RF coil used for the in vivo MRI experiments. A
phantom was constructed consisting of two sealed pipette tips filled with heparinized mouse blood from the same freshly drawn sample. Feridex was added to one pipette tip at a concentration estimated from the average dose of 25 mg Fe/kg administered in vivo. Assuming a total mouse blood volume of 72 L/kg bodyweight (Diehl et al., 2001), the blood iron concentration is 

$$\frac{(25 \text{ mg Fe/kg bodyweight})}{(72 \text{ mL blood/kg bodyweight})} = 0.35 \text{ mg Fe/mL blood.}$$

The pipette tips were placed parallel to and maximally distant from each other inside a plastic vial (13 mm diameter) filled with water. The phantom was placed in the RF coil with the tubes perpendicular to the main magnetic field, and the resonant frequencies of pre- and post-contrast blood were measured. \( \Delta \chi \) (cgs units) was calculated from the expression for the Lorentz-corrected field shift inside an infinite cylinder oriented perpendicular to \( B_0 \) (Weisskoff and Kiihne, 1992):

$$\Delta \chi = \frac{3}{2\pi} \frac{\gamma_{\text{post-contrast}} - \gamma_{\text{pre-contrast}}}{B_0}. \quad [19]$$

4.2.4 Ex vivo image acquisition and processing

Following in vivo MRI, mice were sacrificed via intracardial perfusion with PBS, zinc formalin (Richard-Allan Scientific, Kalamazoo, MI), and Microfil as described in Chapter 3.2.1. After allowing the Microfil to cure, the tumors were excised and immersion fixed in zinc formalin for 48 hours. The tumors were scanned with \( \mu \)MRI and then sent to Numira Biosciences for \( \mu \)CT imaging. Details of ex vivo imaging are provided in Chapter 3.2.2 and Chapter 3.2.3. The tumor vessels were extracted from the ex vivo angiography data (Chapter 3.2.4), the \( \mu \)CT vessels were co-registered to the in vivo MRI data via co-registration to the \( \mu \)MRI (Chapter 3.2.5), and finally the co-registered \( \mu \)CT vessels were used to compute ground truth vascular morphometric maps analogous to the in vivo SSC-MRI vascular biomarker maps (Chapter 3.2.6). Some \( \mu \)CT images exhibited small (1-2 voxels), bright, spherical inclusions. These were later determined to be from
the gadolinium-based contrast agent used for the μMRI scans and precipitation of the zinc formalin fixative, which is commonly used for its superior preservation of cytoarchitecture and immunoreactivity compared to conventional formaldehyde fixatives (Dapson, 1993). These inclusions could not be excluded by the vessel extraction algorithm. Therefore, voxels of the μCT morphometric maps where \( \langle R \rangle_{\mu CT} > 8 \, \mu m \) were excluded from all subsequent analyses.

4.2.5 Statistical analyses

Statistical analyses were performed using MATLAB and NCSS statistical analysis and graphics software.

4.2.5.1 Quantitative agreement and correlation between SSC-MRI and μCT

The median value of each MRI and μCT parameter was calculated for each tumor. The percent error of median FBV\textsubscript{MRI} and VSI\textsubscript{MRI} values were computed relative to their corresponding μCT parameters. These were the only two MRI-μCT parameter pairs that were measured in identical units (fractional blood volume in % and average vessel radius in \( \mu m \)) and could therefore be compared directly. In addition to tumor medians, the 5\textsuperscript{th}, 25\textsuperscript{th}, 75\textsuperscript{th}, and 95\textsuperscript{th} quantiles of each parameter were calculated for each tumor. The Spearman correlation coefficient \( (r_s) \) between each quantile of each MRI parameter and its μCT analog was computed, and a Student’s t-test performed to determine whether \( r_s \) was significantly different from zero (\( \alpha = 0.05 \)). Quantile-quantile (Q-Q) plots of corresponding MRI and μCT parameter pairs were used to examine the similarity between their distributions. Due to a few extreme outliers in the MRI data and for the sake of clarity, only the 5\textsuperscript{th}, 25\textsuperscript{th}, 75\textsuperscript{th}, and 95\textsuperscript{th} quantiles of each tumor were included in the Q-Q plots.
4.2.5.2  Predictive value of SSC-MRI biomarkers

The predictive value of each MRI biomarker was evaluated using leave-one-out cross-validation (LOOCV) analysis. First, the measurements from one tumor was set aside as validation data, and the median values of the other tumors were used to perform linear regression to model the relationship between each *in vivo* MRI parameter (i.e., the predictor variable) and its corresponding μCT analog (i.e., the response variable). The left-out MRI data point was used to test the linear model, and the error of the predicted μCT median value with respect to the observed μCT value was calculated. This procedure was repeated $n$ (number of tumors) times to use the data from every tumor as the validation data. The median absolute error (MAE) of each SSC-MRI biomarker was computed to measure its relative predictive value. To determine whether predictive accuracy differed significantly between “relative” and “absolute” biomarkers of the same vascular parameter, a two-tailed Mann–Whitney U test ($\alpha = 0.05$) was performed between their MAEs.

4.2.5.3  Tracking vascular phenotype across tumor stages and sizes

Finally, to assess the utility of the MRI biomarkers to track changes in breast tumor angiogenesis with progression, $r_s$ between each vascular parameter and tumor volume was computed. In addition, a two-tailed Mann–Whitney U test ($\alpha = 0.05$) was performed to determine whether these parameters were significantly different between PIW 3 and PIW 5 tumors.
4.3 Results

4.3.1 Measurement of USPIO-induced $\Delta \chi$

A frequency shift of -93.904 Hz was measured between the blood and blood+Feridex peaks, which corresponded to $\Delta \chi_c = 0.112$ ppm (cgs units). This value was used to calculate FBV$_{\text{MRI}}$ and VSI$_{\text{MRI}}$ defined by Eqs. 3 and 6.

4.3.2 Computation of in vivo SSC-MRI biomarkers

Parametric maps were computed for each tumor. One PIW 5 tumor exhibited motion in the slice select direction between post-contrast GE and SE scans, preventing accurate calculation of $\Delta R_2^*$. Thus, only FBV$_{\text{MRI}}$ was computed for that tumor. Figure 4.1a–c shows representative non-DW and DW images and the corresponding ADC map. Figure 4.1d–f shows the pre-contrast GE image, corresponding $R_2^*$ map, and a 3 × 3 voxel matrix illustrating the quality of the exponential curve fitting of the signal intensity vs. TE data. Figure 4.1g-i, j-l and m-o show analogous data for post-contrast GE, pre-contrast SE, and post-contrast SE images, respectively. Table 4.1 summarizes the percentage of voxels that satisfied the nonlinear regression F-stat threshold, the mean $R_2$ of the regression models for those voxels, and the percentage of total voxels that had non-negative $\Delta R_2^{(*)}$. Figure 4.2 and Figure 4.3 show maps of the “absolute” and “relative” in vivo MRI biomarkers from representative PIW 3 and PIW 5 tumors.
Figure 4.1 a) Non-DW image, b) DW image, and c) corresponding masked ADC map of a PIW 5 tumor slice. d) Raw in vivo pre-contrast GE image (TE=5 ms), e) corresponding R2* map, and f) exponential fits for the ROI indicated by the green square super-imposed on the raw image in (d). g-i) Corresponding post-contrast GE data analogous to panels (d-f). j) Raw in vivo pre-contrast SE (TE = 8.4 ms) image, k) corresponding R2 map, and l) exponential fits for the ROI indicated by the green square superimposed on the raw image in (j). m-o) Corresponding post-contrast SE data analogous to panels (j-l).
Table 4.1 $R^2$ value and percentage of voxels that satisfied goodness-of-fit and $\Delta R_2^{(*)}$ calculation criteria

<table>
<thead>
<tr>
<th>Image</th>
<th>$R^2$</th>
<th>F-stat p &lt; 0.05 [mean ± SD]</th>
<th>Parameter</th>
<th>$\Delta R_2^{(*)} &gt; 0$ [mean ± SD]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-contrast SE</td>
<td>0.987 ± 0.009</td>
<td>[99.7 ± 0.5]%</td>
<td>$\Delta R_2$</td>
<td>[76.1 ± 10.5]%</td>
</tr>
<tr>
<td>Post-contrast SE</td>
<td>0.990 ± 0.005</td>
<td>[99.9 ± 0.1]%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-contrast GE</td>
<td>0.983 ± 0.004</td>
<td>[97.9 ± 1.8]%</td>
<td>$\Delta R_2^{*}$</td>
<td>[78.6 ± 8.1]%</td>
</tr>
<tr>
<td>Post-contrast GE</td>
<td>0.995 ± 0.001</td>
<td>[99.9 ± 0.1]%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.2 Pseudo-color maps of “absolute” SSC-MRI vascular biomarkers from (a–c) a PIW 3 tumor and (d–f) a PIW 5 tumor, respectively, overlaid on a pre-contrast SE image slice.
Figure 4.3 Pseudo-color maps of “relative” SSC-MRI vascular biomarkers from (a–c) a PIW 3 tumor and (d–f) a PIW 5 tumor, respectively, overlaid on a pre-contrast SE image slice.

4.3.3 Quantitative agreement and correlation between SSC-MRI and μCT

Plots of median FBV\(_{\text{MRI}}\) and FBV\(_{\text{μCT}}\) vs. the corresponding FBV\(_{\text{μCT}}\) rank (Figure 4.4a) and of median VSI\(_{\text{MRI}}\) and VSI\(_{\text{μCT}}\) vs. the corresponding VSI\(_{\text{μCT}}\) rank (Figure 4.4b) show that in vivo MRI overestimated FBV and VSI with respect to μCT. There was no significant correlation between median FBV\(_{\text{μCT}}\) and the FBV\(_{\text{MRI}}\) error (\(r_s = -0.43, p = 0.19\)), while the VSI\(_{\text{MRI}}\) error increased with increasing \(\langle R\rangle_{\text{μCT}}\) (\(r_s = 0.73, p = 0.02\)). The average error of median MRI measurements with respect to μCT was (28.75 ± 27.73)% (mean ± SD) for FBV and (171.99 ± 146.04)% for VSI (Figure
The agreement between other MRI-μCT parameter pairs could not be directly assessed because they did not have the same units.

**Figure 4.4** MRI and μCT measurements of median tumor FBV (a) and VSI (b) plotted against the ranks of the μCT measurements. c) Dot plots of the percent error in MRI measurements of median tumor FBV and VSI with respect to μCT-measured values. Dotted lines indicate the average percent error for each parameter.

The Q-Q plots of FBV$_{MRI}$ vs. FBV$_{μCT}$ (**Figure 4.5a**) and ΔR$_2$ vs. FBV$_{μCT}$ (**Figure 4.5b**) were strongly linear, which suggests that the shapes of the FBV$_{MRI}$ and ΔR$_2$ distributions are similar to the shapes of the FBV$_{μCT}$ distributions. The slope of the FBV$_{MRI}$ vs. FBV$_{μCT}$ Q-Q plot is greater than 1, indicating that there was a discrepancy in the scales of the distributions. The Q-Q plots of VSI$_{MRI}$ vs. ⟨R⟩$_{μCT}$ were supralinear (**Figure 4.5c**), indicating that the VSI$_{MRI}$ distributions were much more positively skewed than the ⟨R⟩$_{μCT}$ distributions. In comparison, the shapes of the relative...
biomarker $R_{\text{MRI}}$ distributions were more similar to those of $\langle R \rangle_{\mu\text{CT}}$ as illustrated by the more linear Q-Q plots (Figure 4.5d). The same trends were seen for the vessel density parameters – the Q-Q plots of the relative biomarker $Q_{\text{MRI}}$ vs. MVD$_{\mu\text{CT}}$ (Figure 4.5f) were more linear than those of the absolute biomarker $N_{\text{MRI}}$ vs. MVD$_{\mu\text{CT}}$ (Figure 4.5e), indicating that the shapes of the $Q_{\text{MRI}}$ distributions were more similar to the shapes of the corresponding MVD$_{\mu\text{CT}}$ distributions.

Figure 4.5 Q-Q plots displaying just the 5th, 25th, 50th, 75th, and 95th quantiles of SSC-MRI parameters vs. their corresponding $\mu\text{CT}$ parameters for every tumor. Top row: absolute SSC-MRI parameters, bottom row: relative SSC-MRI parameters. The dashed lines represent the line of equality ($y = x$).
Table 4.2 lists the Spearman correlation coefficients between various quantiles of each SSC-MRI biomarker and the corresponding μCT parameter quantiles. In general, $r_S$ was greatest between MRI- and μCT-measured medians compared to the correlations between other quantiles. With the exception of $\Delta R_2$, there was a significant correlation between all median SSC-MRI biomarker values and their μCT analogs.

Table 4.2  Spearman correlation coefficients between various quantiles of corresponding μCT and SSC-MRI parameter distributions

<table>
<thead>
<tr>
<th>Ex vivo μCT parameter</th>
<th>In vivo SSC-MRI parameter</th>
<th>Quantile</th>
<th>5th</th>
<th>25th</th>
<th>50th</th>
<th>75th</th>
<th>95th</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBV$_{\text{μCT}}$</td>
<td>$\Delta R_2$</td>
<td></td>
<td>0.53</td>
<td>0.53</td>
<td>0.62</td>
<td>0.62</td>
<td>0.56</td>
</tr>
<tr>
<td>FBV$_{\text{MRI}}$</td>
<td></td>
<td></td>
<td>0.61*</td>
<td>0.77*</td>
<td>0.90*</td>
<td>0.93*</td>
<td>0.60</td>
</tr>
<tr>
<td>$\langle R \rangle_{\text{μCT}}$</td>
<td>R</td>
<td></td>
<td>0.62</td>
<td>0.68*</td>
<td>0.76*</td>
<td>0.42</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>VSI$_{\text{MRI}}$</td>
<td></td>
<td>0.56</td>
<td>0.67*</td>
<td>0.73*</td>
<td>0.50</td>
<td>0.19</td>
</tr>
<tr>
<td>MVD$_{\text{μCT}}$</td>
<td>Q</td>
<td></td>
<td>0.43</td>
<td>0.40</td>
<td>0.78*</td>
<td>0.44</td>
<td>0.66*</td>
</tr>
<tr>
<td></td>
<td>N$_{\text{MRI}}$</td>
<td></td>
<td>0.28</td>
<td>0.40</td>
<td>0.79*</td>
<td>0.72*</td>
<td>0.73*</td>
</tr>
</tbody>
</table>

* $p < 0.05$

4.3.4 Predictive value of SSC-MRI biomarkers

Table 4.3 lists the MAE values from the LOOCV analysis, which measure the ability of the tumor-wise median value of each in vivo MRI biomarker to predict the median value of its μCT analog. The MAEs computed from the LOOCV analysis were not significantly different between the blood volume biomarkers (FBV$_{\text{MRI}}$ and $\Delta R_2$), or between the vessel size biomarkers ($R_{\text{MRI}}$, $R_{\text{MRI}}^{3/2}$, and VSI$_{\text{MRI}}$). However, the relative vessel density biomarker Q had a significantly greater MAE than the absolute vessel density biomarker N$_{\text{MRI}}$. 

51
### Table 4.3 Median absolute errors from cross-validation analysis

<table>
<thead>
<tr>
<th>Ex vivo μCT parameter</th>
<th>In vivo SSC-MRI parameter</th>
<th>LOOCV MAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBV&lt;sub&gt;μCT&lt;/sub&gt; (%)</td>
<td>ΔR&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>FBV&lt;sub&gt;MRI&lt;/sub&gt;</td>
<td>0.47</td>
</tr>
<tr>
<td>(R)&lt;sub&gt;μCT&lt;/sub&gt; (μm)</td>
<td>R&lt;sub&gt;MRI&lt;/sub&gt;</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>R&lt;sub&gt;MRI&lt;/sub&gt;&lt;sup&gt;3/2&lt;/sup&gt;</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>VSI&lt;sub&gt;MRI&lt;/sub&gt;</td>
<td>0.12</td>
</tr>
<tr>
<td>MVD&lt;sub&gt;μCT&lt;/sub&gt; (mm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>Q</td>
<td>75.0</td>
</tr>
<tr>
<td></td>
<td>Q&lt;sup&gt;3&lt;/sup&gt;</td>
<td>30.6</td>
</tr>
<tr>
<td></td>
<td>N&lt;sub&gt;MRI&lt;/sub&gt;</td>
<td>13.3*</td>
</tr>
</tbody>
</table>

* MAE of N is significantly less (<p < 0.05) than MAE of Q

#### 4.3.5 Tracking vascular phenotype across tumor stages and sizes

Median FBV<sub>μCT</sub>, FBV<sub>MRI</sub>, and ΔR<sub>2</sub> were all significantly greater for PIW 3 tumors than PIW 5 tumors (Figure 4.6a–c). Similarly, there were significant negative Spearman rank correlations between tumor volume and all three blood volume parameters. The decrease in median ΔR<sub>2</sub> with tumor size indicated that larger tumors contained a smaller fraction of microvessels than smaller tumors. This was also reflected in the increased blood vessel size in PIW 5 tumors indicated by median (R)<sub>μCT</sub>, VSI<sub>MRI</sub>, and R<sub>MRI</sub>; however, none of these parameters were significantly different between PIW 3 and PIW 5 tumors (Figure 4.6d–f), and none correlated significantly with tumor volume. PIW3 tumors exhibited higher vessel density compared to PIW 5 tumors. Median MVD<sub>μCT</sub> and N<sub>MRI</sub> significantly differed between groups and correlated with tumor volume but median Q did not (Figure 4.6g–i).
Figure 4.6 Tumor-wise scatter plots of median μCT and SSC-MRI vascular biomarker values vs. tumor volume. Top row: blood volume parameters, middle row: vessel size parameters, bottom row: vessel density parameters. * Significant difference between PIW 3 and PIW 5 tumors ($p < 0.05$). † Spearman correlation ($r_S$) between vascular parameter and tumor volume is significantly different from zero ($p < 0.05$).
4.4 Discussion

The sensitivity of SSC-MRI to the underlying vasculature enables one to generate parametric maps of *in vivo* tumor angiogenesis. These *in vivo* MRI biomarkers include tumor blood volume, average blood vessel size, and blood vessel density and can be broadly grouped into two classes: (i) “relative” biomarkers or those that are proportional to a vascular parameter, e.g. $R_{\text{MRI}}$ is proportional to average vessel size; (ii) “absolute” biomarkers or those that are direct measures of a vascular parameter, e.g. $V_{\text{MRI}}$ is a measure of average blood vessel radius in microns. However, it is not known which of these MRI biomarkers are the most relevant to the assessment of breast cancer angiogenesis *in vivo*. Therefore, in this study we systematically validated each *in vivo* SSC-MRI biomarker of angiogenesis against its 3D vascular analog derived from high-resolution μCT.

Micro-CT has the unique ability to image the intact 3D vasculature of the whole tumor at very high spatial resolution (8 μm isotropic) over a spatial scale spanning three orders of magnitude (μm to mm). Additionally, whole tumor 3D μCT circumvents sampling errors typically associated with “hot-spot” analysis commonly employed when computing vascular morphology from immunostained tumor sections (Hlatky et al., 2002). This is especially relevant when trying to characterize spatially heterogeneous tumor vasculature. Furthermore, for μCT imaging, the vessel lumina are filled with Microfil, allowing for more accurate measurements of vascular morphology. In contrast, immunohistochemical stains typically label the endothelial cell wall or vascular basement membrane, which makes it challenging to accurately quantify the 3D morphology of the tumor vasculature. Furthermore, the challenge of assessing vascular morphometrics from immunostained tissue sections is compounded by tissue sectioning artifacts and the need for stereologic corrections (Pathak et al., 2001). Therefore, while capillaries < 4 μm in radius could not be fully resolved at the resolution employed in this study,
μCT has several advantages as a viable technique for determining the 3D morphology of the tumor vasculature. Finally, it is worth noting that neither μCT nor histology necessarily images only the vessels that were perfused during in vivo SSC-MRI, as tumor blood flow can be both spatially and temporally heterogeneous (Vaupel and Hockel, 2000). This caveat must be considered when making comparisons between ex vivo and in vivo data and may explain some of the differences between MRI and μCT parameters reported here. Intermittent tumor perfusion, along with other factors such as noise and tissue deformation, also makes voxel-wise comparison of MRI and μCT parameters challenging. Thus, tumor-wise analyses were conducted in this study.

The median FBV_{μCT} measured in this study was found to be in good agreement with histologic measurements in MDA-MB-231 tumors reported by a previous study (Garvin et al., 2006) despite the fact that the μCT system could not resolve microvessels < 4 μm in radius. The results of this study show that FBV_{MRI} generally overestimated the blood volume measured by μCT, which was also observed by Ungersma et al. (Ungersma et al., 2010). Similarly, VSI_{MRI} significantly overestimated \langle R \rangle_{μCT}. Previous studies employing various tumor models have also reported that VSI_{MRI} overestimated vessel radius measurements from high-resolution ex vivo techniques such as immunohistochemistry (Bauerle et al., 2010; Lemasson et al., 2013; Persigehl et al., 2013; Tropres et al., 2004b; Ungersma et al., 2010; Zwick et al., 2009) and two-photon laser scanning microscopy (Douma et al., 2010). Monte Carlo simulations have demonstrated that VSI_{MRI} increasingly overestimates the true vessel radius with decreasing Δχ_{C} (Tropres et al., 2001). This could be explained by a shift in peak sensitivity of ΔR_{2} toward larger vessels (Boxerman et al., 1995) or by an increase in the lower limit of the range of vessel sizes for which VSI_{MRI} is valid (Tropres et al., 2001). However, considering the high contrast agent dose employed in this study, simulations have shown (Boxerman et al., 1995; Pathak et al., 2008b;
Tropres et al., 2001; Weisskoff et al., 1994) that the contribution of these effects on the VSIMRI overestimation reported here may be relatively small. The MRI overestimation of blood volume and vessel size may be attributable to the discarding of voxels exhibiting negative $\Delta R_2^*$ in regions of low vascularization or poor perfusion, as well as to the characteristically irregular tumor vascular architecture, which significantly deviates from the infinite cylinder model used to approximate vessel geometry in deriving Eqs. 2-10 (Yablonskiy and Haacke, 1994). Moreover, the accuracy of $FBV_{MRI}$ and $VSI_{MRI}$ are also sensitive to measurements of $\Delta \chi_C$ and ADC, which are necessary for their computation and may introduce additional potential sources of error. The systematic overestimation of $FBV_{MRI}$ and $VSI_{MRI}$ diminishes their utility as “absolute” biomarkers. However, as stated previously, the tumor-wise medians of all in vivo biomarkers except $\Delta R_2$ exhibited significant correlations with their $\mu$CT analogs, indicating that they are suitable for relative and comparative measurements.

It is not surprising that $\Delta R_2$ did not significantly correlate with $FBV_{\mu CT}$ because $FBV_{MRI}$ and $FBV_{\mu CT}$ are measures of total blood volume while $\Delta R_2$ is primarily sensitive to the microvascular blood volume. Typically, the $\Delta R_2$ vs. vessel caliber curve exhibits peak sensitivity for capillary-sized vessels (Figure 1.1b) but depends on the magnetic field strength, contrast agent dose, and TEs employed (Boxerman et al., 1995). The significant decrease in $\Delta R_2$ with tumor progression suggests that changes in angiogenic phenotype between PIW 3 and PIW 5 tumors primarily involved the microvasculature, which is consistent with our understanding of angiogenic sprouting (Hillen and Griffioen, 2007).

Previous studies have observed that the simple ratio $R_{MRI}$ was in better agreement with histological measurements than the parameter $R_{MRI}^{3/2}$ (Farrar et al., 2010). In this study, LOOCV analysis demonstrated that there was no significant difference between $R_{MRI}$, $R_{MRI}^{3/2}$, and
VSI\textsubscript{MRI} in terms of their accuracy as predictors of \(\langle R \rangle\)\textsubscript{\(\mu\textsc{CT}\)}. In conjunction with the systematic overestimation of \(\langle R \rangle\)\textsubscript{\(\mu\textsc{CT}\)} discussed above, these data indicate that the “absolute” biomarker VSI\textsubscript{MRI} did not provide an advantage over the “relative” biomarkers R\textsubscript{MRI} and R\textsubscript{MRI} \(3/2\). The same was true of the blood volume biomarkers, i.e. FBV\textsubscript{MRI} overestimated FBV\textsubscript{\(\mu\textsc{CT}\)}, and its ability to predict FBV\textsubscript{\(\mu\textsc{CT}\)} was not significantly different from that of \(\Delta R_2\) as determined by LOOCV. Therefore, the “absolute” biomarker FBV\textsubscript{MRI} did not provide any advantage over the “relative” biomarkers \(\Delta R_2^*\) and \(\Delta R_2\). While the absolute agreement between MRI and \(\mu\textsc{CT}\)-measured vessel density parameters could not be assessed because the former measured area density and the latter volume density, LOOCV analysis indicated that the “absolute” biomarker N\textsubscript{MRI} did not provide an advantage over the “relative” biomarker Q\textsuperscript{3} but was a more accurate predictor of \(\mu\textsc{CT}\) vessel density than Q. However, inspection of Q-Q plots revealed that the intratumoral distributions of the relative biomarkers Q and R are more similar in shape to the distributions of their respective \(\mu\textsc{CT}\) analogs compared to those of the absolute biomarkers VSI\textsubscript{MRI} and N\textsubscript{MRI}. This suggests that the exponents of 3/2 and 3 in the theoretical expressions for VSI\textsubscript{MRI} (Eq. 6) and N\textsubscript{MRI} (Eq. 10), respectively, may be too large in practice.

Collectively, our results suggest that making additional \(\Delta \chi_c\) and ADC measurements to compute the “absolute” biomarkers did not confer a significant advantage over the “relative” biomarkers when assessing angiogenesis for this breast tumor model. These “relative” biomarkers are easy to compute; correlate well with \(\mu\textsc{CT}\)-measured vascular morphology; are sensitive to changes in the tumor vasculature with tumor progression; and as demonstrated by other studies, are also well suited to monitor the effects of anti-angiogenic therapies (Batchelor et al., 2007; Bauerle et al., 2010; Farrar et al., 2011; Howe et al., 2008; Ungersma et al., 2010). In addition, the “relative” parameters may be more clinically relevant than the “absolute” parameters because the theoretical assumptions on which the latter are based can be violated at lower, clinically
relevant contrast agent doses (Jensen and Chandra, 2000; Tropres et al., 2004a). This is especially pertinent in light of a recent preliminary study demonstrating the feasibility of applying SSC-MRI to cancer patients (Fredrickson et al., 2012a). Thus, a standalone SSC-MRI protocol that allows for the calculation of $\Delta R_2$ and $\Delta R_2^*$ appears to be a viable option for assessing angiogenesis-induced changes in vascular morphology in breast tumor models with the potential for clinical translation.

On the other hand, diffusion-weighted imaging may be beneficial, independent of computing the angiogenic biomarkers presented here, as ADC has been shown to be a potent oncologic biomarker in its own right (Galons et al., 1999; Moffat et al., 2005). However, the intra- and inter-tumor variations in ADC make it necessary to obtain a spatial map of the ADC for each tumor and perform voxel-wise calculations to accurately compute $\text{VSI}_{\text{MRI}}$ or $\text{N}_{\text{MRI}}$ and fully characterize the vascular heterogeneity within each tumor. Similarly, to obtain accurate measurements of $\text{FBV}_{\text{MRI}}$ and $\text{VSI}_{\text{MRI}}$, individual $\Delta \chi_C$ measurements would have to be made as it depends on factors such as the type of contrast agent, dose, and magnetic field strength.

**Chapter 6** details the integration of high-resolution μCT angiography data in a computational model of susceptibility-based contrast (Pathak et al., 2008b) to better understand the interplay between the tumor vasculature and these biophysical factors, as well as their effect on SSC-MRI biomarker accuracy.

In conclusion, SSC-MRI allows for the measurement of several *in vivo* biomarkers of tumor angiogenesis. In this study, we systematically validated these biomarkers in an orthotopic MDA-MB-231 human breast cancer model against their 3D vascular correlates obtained from high-resolution *ex vivo* μCT. While the “absolute” parameters $\text{FBV}_{\text{MRI}}$ and $\text{VSI}_{\text{MRI}}$ are not accurate quantitative readouts (i.e., they overestimate their μCT correlates), the “relative” parameters
are still promising candidates for noninvasive, *in vivo* biomarkers of breast cancer angiogenesis. The lack of FDA approved anti-angiogenic therapies for breast cancer makes these findings especially relevant because such biomarkers will facilitate the development and *in vivo* monitoring of such therapies. **Chapter 5** presents a study wherein we demonstrate the utility of SSC-MRI in evaluating the *in vivo* effects of a novel biomimetic anti-angiogenic peptide in MDA-MB-231 tumor xenografts.
5 Assessing the effects of anti-angiogenic peptide therapy with in vivo SSC-MRI

5.1 Introduction

Angiogenesis is a hallmark of cancer and is essential to the progression and metastasis of most solid tumors (Hanahan and Weinberg, 2011). As such, it is a prominent focus of cancer research and a promising therapeutic target. Although several anti-angiogenic drugs have been approved by the US Food and Drug Administration to treat certain cancers, none are currently approved for treating breast cancer. Therefore, there is a need for new targeted therapies, particularly for triple negative breast cancers (TNBC), an aggressive subtype of breast cancer with poor prognosis. TNBC is defined by a lack of estrogen receptor and progesterone receptor expression and human epidermal growth factor receptor 2 overexpression, making them unresponsive to current receptor targeted therapies (Greenberg and Rugo, 2010). Since TNBC tends to be highly vascularized and express elevated levels of vascular endothelial growth factor (VEGF) (Linderholm et al., 2009; Nalwoga et al., 2011), anti-angiogenic agents have the potential to improve TNBC patient outcomes.

Recently, peptides have emerged as promising therapeutic agents because they exhibit advantages over large proteins or small molecule therapeutics, such as low molecular weights below the immunogenic threshold; superior synthetic fidelity since peptide synthesis is based on amino acids, which are standardized building blocks; and high diversity since single amino acid substitutions can be made to engineer peptides with high specificity to various target molecules. We have identified more than 100 anti-angiogenic peptides using a bioinformatics-based methodology (Karagiannis and Popel, 2008). Among them, peptides derived from collagen type IV (SP2000, seq:LRRFSTMPFMCNINVCNF) potently inhibited angiogenesis in vivo and in vitro.
To increase their bioavailability, the peptides can be encapsulated in polymeric carriers designed to release them gradually, peptide bonds can be replaced with non-peptidic constraints, or natural amino acids can be substituted with more stable, synthetic analogs (Pirogova et al., 2011; Rosca et al., 2011a). For example, we developed a biomimetic peptide (SP2012, seq:LRRFSTMPPFMF(Abu)NINNV(Abu)NF), based on the SP2000 peptide that was more translational by replacing two cysteines with 2-aminobutyric acid (Abu), a synthetic cysteine analog. We found that SP2012 interacts with integrin beta 1 and interferes with the vascular endothelial growth factor receptor 2 (VEGFR2) signaling pathway in endothelial cells (Koskimaki et al., 2013). Most recently, we replaced Abu and asparagine (N) with isoleucine (I) and aspartic acid (D), respectively (SP2024, seq:LRRFSTMPPFMFIDIDDVINF) to achieve greater activity and stability compared to earlier iterations.

Change in tumor volume is often used as a biomarker of therapeutic efficacy. However, since tumor volume changes can lag behind vascular changes caused by anti-angiogenic therapies (Lindner and Borden, 1997), there is a crucial need for translational in vivo biomarkers of early anti-angiogenic response to assess the efficacy and optimize the administration of new anti-angiogenic therapies (Sessa et al., 2008). SSC-MRI has demonstrated the potential to provide such biomarkers and has been used to monitor tumor response to anti-angiogenic treatments in various preclinical cancer models (Bauerle et al., 2010; Farrar et al., 2011; Nielsen et al., 2012a; Ullrich et al., 2011; Ullersma et al., 2010; Walker-Samuel et al., 2011; Zwick et al., 2009). The potential for clinical translation of SSC-MRI has been enhanced by the availability of ferumoxytol (Feraheme®, AMAG Pharmaceuticals, Inc., Lexington, MA), an i.v. drug indicated for the treatment of iron deficiency anemia in adult patients with chronic kidney disease, that can also be used as a superparamagnetic MRI contrast agent. Recently, a Phase 1 clinical trial was conducted to evaluate the feasibility of translating SSC-MRI to cancer patients (Fredrickson et
al., 2012b). Several other Phase 1 and Phase 2 clinical trials have been conducted or are currently underway to evaluate the safety and efficacy of ferumoxytol as a clinical MRI contrast agent (Stabi and Bendz, 2011).

There were two primary aims of this study. The first aim was to demonstrate the utility of SSC-MRI for evaluating the early effects of SP2024 peptide treatment on tumor blood volume, vessel size, and vessel density in orthotopic human TNBC xenografts in mice. In addition, diffusion weighted imaging (DWI) was used to measure the in vivo apparent diffusion coefficients (ADC), a promising biomarker of treatment-induced changes in tumor cellularity (Thoeny and Ross, 2010). However, ADC is not directly related to vascularity. Like tumor volume, changes in tumor cellularity may occur later than changes in the vasculature following anti-angiogenic therapy. The second aim of this study was to test the hypothesis that the vasculature-specific SSC-MRI biomarkers are more responsive than tumor size or ADC to the early effects of our anti-angiogenic peptide.
5.2 Methods

5.2.1 Cell culture and inoculation

3×10⁶ MDA-MB-231 human breast cancer cells in 50 μL of Hank’s balanced salt solution (Sigma-Aldrich) were orthotopically inoculated into the lower right thoracic mammary fat pad of 10 female athymic NCr-\textit{nu/nu} mice. Further details are given in Chapter 2.1.

5.2.2 \textit{In vivo} MRI acquisition

When tumor xenografts had grown to approx. 70 mm³, they were imaged \textit{in vivo} on a Bruker Biospin (Billerica, MA) 9.4T small animal MRI system using an 18-mm diameter radiofrequency transceiver surface coil the following sequences: (i) two-dimensional (2D) DWI; (ii) 2D MGE, TE = 5 ms, six echoes with 5 ms echo spacing, TR = 800 ms. (iii) 2D MSE, TE = 10 ms, eight echoes with 10 ms echo spacing, TR = 1500 ms. MGE and MSE images were acquired before and five minutes after i.v. injection of ferumoxytol at a dose of 5 mg Fe/kg. For all imaging sequences, field of view (FOV) = 16 mm × 16 mm, in-plane resolution = 125 μm, and slice thickness = 1 mm. Further details of the imaging protocol and sequences can be found in Chapter 2.2.

5.2.3 Peptide synthesis and treatment

The SP2024 peptide was produced by a commercial manufacturer (New England Peptide, Gardner, MA) using a solid-phase synthesis technique. High-performance liquid chromatography and mass spectrometry analyses of the peptide were provided by the manufacturer to demonstrate greater than 95% purity. The peptide was stored at -80°C and thawed at room temperature just before use. For preparation of peptide stock solutions, DMSO (Sigma Aldrich, St. Louis, MO) was used as a solvent at a concentration of 5% (vol/vol) in PBS. Prior to imaging, the mice were randomized into two groups of five. Starting on the day of imaging (day 0) after imaging was completed, one group received daily i.p. injections of 100 μl of SP2024 solution at
a dose of 10 mg/kg, while the other group received daily i.p. injections of 100 μl of 5% DMSO in PBS to control for solvent effects. Tumor volumes were approximated daily using the formula: \( V = 0.52ab^2 \), where \( a \) is the long axis and \( b \) the short axis of the tumor, measured by calipers. After 14 days of treatment, the tumors were imaged again (day 14) using the same in vivo MRI protocol as for day 0.

5.2.4 In vivo MRI image processing

Manually drawn masks were used to exclude voxels containing non-tumor tissue from all subsequent analyses. Voxel-wise maps of ADC, \( \Delta R_2 \), and \( \Delta R_2^* \) were calculated from DWI, MSE, and MGE images, respectively, as described in Chapter 2.3. These were used to compute the SSC-MRI vascular biomarkers defined by Eqs. 3, 6, 7, 8, and 10 introduced in Chapter 1.2.1. The value of \( \Delta \chi \) in Eqs. 3 and 6 was calculated using the method described below. ADC and pre-contrast \( T_2 \) were considered as biomarkers of tumor cellularity (Jakobsen et al., 1995; Thoeny and Ross, 2010).

5.2.5 Measurement of ferumoxytol-induced \( \Delta \chi \)

The difference in magnetic susceptibility induced by ferumoxytol (\( \Delta \chi_c \)) was measured using the same MRI scanner and radiofrequency coil used for the in vivo experiments. A tube filled with saline was placed in the coil perpendicular to the main magnetic field (\( B_0 \)). The resonant frequency was measured inside a 4 mm isotropic voxel placed in the center of the tube. This was repeated for a tube filled with ferumoxytol diluted with saline to the concentration used for the in vivo scans. \( \Delta \chi_c \) was calculated using Eq. 19 for the Lorentz-corrected frequency shift inside a cylindrical, magnetized object oriented perpendicular to \( B_0 \).
5.2.6 Histology

Following the \textit{in vivo} MRI experiments, mice were sacrificed by cervical dislocation. The tumors were immediately excised and immersion fixed in freshly prepared 2\% formaldehyde at 4°C for 48 hours. The samples were then transferred to 30\% sucrose + 0.02\% sodium azide and stored at 4°C for two weeks. The fixed, cryoprotected tumor samples were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc., Torrance, CA) and frozen in liquid nitrogen. Serial 12-μm thick sections were cut at -20°C and mounted for H&E and immunofluorescence staining of laminin (an endothelial basement membrane protein), VEGF, and phosphorylated Met receptor tyrosine kinase (p-Met). Met is a receptor tyrosine kinase that is activated through phosphorylation by binding of its natural ligand, hepatocyte growth factor (HGF). Met in turn activates multiple signal transducers such as phospholipase C-γ (PLC-γ), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and focal adhesion kinase (FAK) (Christensen et al. Cancer Lett 2005). These downstream Met signaling pathways are important for angiogenesis and cell migration, proliferation, and survival.

5.2.6.1 H&E staining and quantification

A Nikon DXM1200 microscope was used to acquire color images of H&E-stained breast tumor sections at 2× magnification. Several images needed to be taken to cover each whole section. ImageJ (National Institutes of Health, Bethesda, MD) was used to apply background subtraction and to stitch together the different frames taken for each tumor section to create a single image of the whole section. Color H&E images were converted to grayscale images. The darker regions, indicative of abundant hematoxylin staining, were considered viable tumor regions; lighter regions stained only with eosin were considered non-viable. The grayscale images were binarized using the default ImageJ automatic threshold algorithm to quantify the viable tumor fraction of each tumor section.
5.2.6.2 Laminin staining and quantification

After blocking with 90% Carbo-Free blocking solution (Vector Laboratories, Inc., Burlingame, CA) and 10% goat serum for 20 minutes at room temperature and washing in PBST (PBS + 0.3% Triton) for 5 min, sections were incubated with rabbit anti-laminin antibodies (1:500) overnight at 4°C. After washing in PBST for 5 min, sections were incubated with Alexa Fluor® 546-conjugated goat anti-rabbit antibodies (1:1000, Life Technologies, Carlsbad, CA) for 2.5 h at room temperature. Sections were then washed in PBST for 5 min, treated with DAPI (1:10,000, Hoffmann-La Roche Ltd., Basel, CH) for 5 min, and washed again in PBST for 5 min. Coverslips were mounted using Faramount aqueous mounting medium (Dako North America, Inc., Carpinteria, CA).

Fluorescent images were obtained using a Nikon Eclipse E400 microscope (Nikon Instruments Inc., Melville, NY). For each group, laminin-positive area fractions were quantified in 24 random fields (10× magnification) acquired from three control and four treated different tumors. Using ImageJ, a median filter (2 pixel radius) and background subtraction (50 pixel radius rolling ball) were applied before binarizing the images (manual threshold) and measuring the positive area fractions.

5.2.6.3 VEGF and p-Met staining and quantification

After blocking with 5% donkey or goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBST for 1 h at room temperature, tumor sections were incubated with rabbit anti-p-Met antibodies (1:50, Cell Signaling Technology, Inc., Danvers, MA) or goat anti-VEGF165 antibodies (1:100, R&D Systems, Minneapolis, MN) overnight at 4°C. After 3 rinses with PBST, sections were incubated with Cy3-conjugated goat anti-rabbit antibodies or TRITC-conjugated donkey anti-goat antibodies (1:500, Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room
temperature. After 3 rinses, sections were treated with DAPI for 5 min at room temperature, followed by another rinse with PBST. Coverslips were mounted with the ProLong Gold anti-fade reagent (Life Technologies) and incubated overnight in darkness at room temperature.

Fluorescent images were obtained using an LSM-510 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, DE). VEGF165 and p-Met-positive signals were binarized using the default ImageJ automatic threshold algorithm, and the positive area fractions were measured in 24 random fields (10× magnification) for each marker and for each group. Fields were acquired from three control tumors and three treated tumors.

5.2.7 Statistical analysis

The two-tailed Mann-Whitney U test ($\alpha = 0.05$) was used to determine: (1) whether there was a significant difference in tumor volume and growth between control and treatment groups; (2) whether the changes in the MRI parameter medians from day 0 to day 14 were significantly different between control and treatment groups; (3) whether median MRI biomarker values changed significantly on an individual tumor basis; and (4) whether histologically measured viable tumor fraction, laminin area fraction, and VEGF and p-Met levels were significantly different between control and treatment groups.

A mutual information metric was used to quantify the utility of each measured parameter (tumor volume, pre-contrast $T_2$, ADC, FBV, $\Delta R_2$, VSI, $R$, $Q$, and $N$) as a biomarker of SP2024 treatment response. First, the change in the median value of each parameter $\Delta X$ for each tumor was binarized by applying a threshold $t$:

$$\Delta X_t = \begin{cases} 1, & \Delta X > t \\ 0, & \Delta X \leq t \end{cases}$$

[20]
Then the mutual information $I(\Delta X_t, G)$ between these binary parameter changes $\Delta X_t$ and group membership ($\in \{\text{control, treatment}\}$) was calculated:

$$I(\Delta X_t, G) = H(G) - H(G|\Delta X_t) \quad [21]$$

The marginal entropy $H(G) = 1$ because the control and treatment groups were of equal size. The conditional entropy $H(G|\Delta X_t)$ was computed using the following equation:

$$H(G|\Delta X_t) = -\sum_{x\in\Delta X_t} p(x) \sum_{g\in G} p(g|x) \log_2 p(g|x) \quad [22]$$

where $p(x)$ is the probability that $\Delta X_t = x$ and $p(g|x)$ is the conditional probability that $G = g$ given $\Delta X_t = x$. For each parameter, the maximum mutual information as a function of the threshold ($I_{\text{max}}$) as well as the mutual information given a threshold of zero ($I_{t=0}$) were calculated.
5.3 Results

5.3.1 Ferumoxytol-induced $\Delta \chi$

The resonance frequency was 3.91 Hz in saline and -74.34 Hz in the ferumoxytol solution with an iron concentration equivalent to the in vivo dose of 5 mg/kg. This corresponded to a $\Delta \chi_c$ of 0.09 ppm (cgm units) as calculated by [19].

5.3.2 Effect of SP2024 on tumor growth

There was no difference in tumor volume at day 0 between treatment (68.2 ± 8.02 mm$^3$, median ± median absolute deviation) and control (67.8 ± 11.0 mm$^3$) groups. While the treated tumors grew more slowly compared to the control tumors (Figure 5.1a), the tumor sizes at day 14 (98.6 ± 17.9 mm$^3$ for treated and 185.0 ± 98.8 mm$^3$ for control) were still not significantly different between the two groups ($p = 0.1$). There was also no significant difference in the change in tumor volume from day 0 to 14 ($p = 0.06$) (Table 5.1, Figure 5.1b).

![Figure 5.1](image)

**Figure 5.1** a) Tumor growth curves showing the median tumor volumes of control (CTRL) and treated (SP2024) groups. Error bars indicate the median absolute deviation. b) Plot of changes in tumor volume from day 0 to 14 for individual tumors. Horizontal lines indicate the group medians.
Table 5.1 Changes in tumor parameters and the mutual information between these changes and group membership (control or treated)

<table>
<thead>
<tr>
<th>Tumor Parameter</th>
<th>Δ[Median]</th>
<th>CTRL</th>
<th>SP2024</th>
<th>p</th>
<th>$I_{max}$</th>
<th>$I_{t=0}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor volume (mm$^3$)</td>
<td></td>
<td>117.2 ± 87.82</td>
<td>38.41 ± 13.04</td>
<td>0.06</td>
<td>0.610</td>
<td>0</td>
</tr>
<tr>
<td>$T_2$ (ms$^3$)</td>
<td></td>
<td>4.672 ± 2.496</td>
<td>0.888 ± 1.981</td>
<td>0.10</td>
<td>0.396</td>
<td>0.108</td>
</tr>
<tr>
<td>ADC ($\times 10^{-5}$ mm$^2$/s)</td>
<td></td>
<td>5.830 ± 15.47</td>
<td>6.369 ± 16.76</td>
<td>1</td>
<td>0.108</td>
<td>0</td>
</tr>
<tr>
<td>FBV (%)</td>
<td></td>
<td>1.167 ± 1.081</td>
<td>-0.978 ± 0.416</td>
<td>&lt;0.01</td>
<td>1</td>
<td>0.610</td>
</tr>
<tr>
<td>$\Delta R_2$ (s$^{-1}$)</td>
<td></td>
<td>-0.182 ± 0.222</td>
<td>0.673 ± 0.264</td>
<td>0.22</td>
<td>0.396</td>
<td>0.125</td>
</tr>
<tr>
<td>$\Delta R_2^{*}/\Delta R_2$</td>
<td></td>
<td>8.598 ± 1.498</td>
<td>-7.556 ± 5.022</td>
<td>&lt;0.01</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>VSI (μm)</td>
<td></td>
<td>18.13 ± 7.133</td>
<td>-25.75 ± 10.70</td>
<td>&lt;0.01</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Q (s$^{-1/3}$)</td>
<td></td>
<td>-0.159 ± 0.015</td>
<td>0.122 ± 0.052</td>
<td>&lt;0.01</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N (mm$^2$)</td>
<td></td>
<td>-70.13 ± 50.17</td>
<td>37.37 ± 3.252</td>
<td>0.02</td>
<td>0.610</td>
<td>0.610</td>
</tr>
</tbody>
</table>

5.3.3 Effect of SP2024 on MRI biomarkers

Figure 5.2 shows MRI parametric maps from a representative control tumor at day 0 and day 14. The first row shows pre-contrast MSE (TE = 40 ms) and MGE (TE = 5 ms) images. Below, the parametric maps are overlaid on their respective SE images. Figure 5.3 shows corresponding images for a representative treated tumor. Voxels in the maps that had negative $\Delta R_2^{*}$ or that did not meet the F-statistic criterion were not displayed.
Figure 5.2 Raw images and parametric maps of a representative control tumor at days 0 and 14. Top row: Raw SE (TE=40 ms) and GE (TE=5 ms). Second row: T2 and ADC maps. Third row: ΔR$_2$ (microvascular volume) and FBV (total blood volume) maps. Fourth row: R$_{MRI}$ and VSI$_{MRI}$ maps (vessel radius). Bottom row: Q and N$_{MRI}$ maps (vessel density). Parametric maps are overlaid on their respective SE images. Voxels with negative ΔR$_2$(*) or that did not satisfy the F-stat criterion are not displayed.
Figure 5.3 Raw images and parametric maps of a representative treated tumor at days 0 and 14. 

Top row: Raw SE (TE=40 ms) and GE (TE=5 ms). Second row: T2 and ADC maps. Third row: $\Delta R_2$ (microvascular volume) and FBV (total blood volume) maps. Fourth row: $R_{\text{MRI}}$ and $V_{\text{SI}_{\text{MRI}}}$ maps (vessel radius). Bottom row: Q and $N_{\text{MRI}}$ maps (vessel density). Parametric maps are overlaid on their respective SE images. Voxels with negative $\Delta R_2^*$ or that did not satisfy the F-stat criterion are not displayed.
The median pre-contrast $T_2$ increased from day 0 to day 14 in all control tumors and all but one treated tumor; there was no significant difference in the change in median $T_2$ between groups (Figure 5.4a). There was also no significant difference in the change in median ADC between control and treatment groups, with positive and negative changes observed in both groups (Figure 5.4b). With the exception of $\Delta R_2$, all of the changes in the vascular biomarkers were significantly different between control and treatment groups (Figure 5.4c-h). In general, median $FBV_{\text{MRI}}$, $R_{\text{MRI}}$, and $VSI_{\text{MRI}}$ increased while $\Delta R_2$, $Q$, and $N_{\text{MRI}}$ decreased in the control tumors. In contrast, median $FBV_{\text{MRI}}$, $R_{\text{MRI}}$, and $VSI_{\text{MRI}}$ decreased while $\Delta R_2$, $Q$, and $N_{\text{MRI}}$ increased in the treated tumors. Table 5.1 lists the changes in median parameter values for each group and the $p$-values from the two-tailed Mann-Whitney $U$ tests to compare the changes between control and treatment groups.

Figure 5.5 shows the quartile values of the vascular biomarker maps at day 0 and 14 for each treated tumor (M1-M5). Median $FBV_{\text{MRI}}$ significantly decreased during the treatment period in all tumors except M3 (Figure 5.5a). Median $\Delta R_2$ changed significantly in all five tumors but increased in three while decreasing in two (Figure 5.5b). M1 showed no change in median $VSI_{\text{MRI}}$ or $N_{\text{MRI}}$, but the other four tumors showed significant decreases in $VSI_{\text{MRI}}$ and increases in $N_{\text{MRI}}$ (Figure 5.5c,e). Median $R_{\text{MRI}}$ significantly decreased and median $Q$ significantly increased in all tumors (Figure 5.5d,f). The opposite was seen in control tumors: median $FBV_{\text{MRI}}$ did not change significantly in two and increased in three; median $\Delta R_2$ did not change in one, increased in one, and decreased in three; median $VSI_{\text{MRI}}$ and $R_{\text{MRI}}$ increased in all five; median $N_{\text{MRI}}$ and $Q$ decreased in all five (data not shown).
Figure 5.4 Plots of changes in median MRI parameter values from day 0 to 14 for individual control (CTRL) and treated (SP2024) tumors. Horizontal lines indicate group medians. There was no significant difference between control and treatment groups in the changes in $T_2$ (a), and ADC (b). With the exception of $\Delta R_2$ (c), there was a significant difference in the changes in all of the vascular parameters (d-h). * $p < 0.05$, two-tailed Mann-Whitney U test.
Figure 5.5 Box plots (excluding whiskers) of SSC-MRI vascular parameter distributions for individual treated tumors (M1–M5) at days 0 and 14. In general, total blood volume (a) and vessel size (c, d) decreased while vessel density increased (e, f) from day 0 to 14. $\Delta R_2$ changed significantly in all tumors but not all in one direction (b). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-tailed Mann-Whitney $U$ test.
The ability of each parameter to predict whether a mouse received treatment was measured by the mutual information $I(\Delta X_t, G)$ between group membership and the change in that parameter. $I(\Delta X_t, G) = 1$ indicates that knowing the change in the median value of a particular parameter in a tumor from day 0 to 14 removes all uncertainty of that tumor’s group membership, i.e., there is no overlap between control and treatment groups. Comparing values of $I_{max}$ suggests that all of the vascular parameters except $\Delta R_2$ were as good as or better than any of the non-vascular parameters at predicting group membership (Table 5.1). In fact, there was no overlap between the control and treated tumors in the change in median FBV$_{MRI}$, R$_{MRI}$, VSI$_{MRI}$, or $Q$ ($I_{max} = 1$). The values of $I_{t=0}$ indicate that, if it were only known whether a parameter increased or decreased, all of the vascular parameters would be better predictors of group membership than any of the non-vascular parameters.

5.3.4 Histology

Figure 5.6 shows raw images of H&E sections from representative control and treated tumors (Figure 5.6a,b) and the corresponding binary images in which black areas represent viable tumor (Figure 5.6d,e). There was no significant difference in the viable tumor fraction calculated from H&E images between control (43.5 ± 4.4%, median ± median absolute deviation) and treated tumors (46.6 ± 0.5%) (Figure 5.6e). In contrast, significantly less laminin was observed in treated tumors (1.90 ± 0.93% fractional area) than in control tumors (5.61 ± 2.73%) (Figure 5.7a-c), which is indicative of the anti-angiogenic activity of the SP2024 peptide. VEGF expression was also lower in the treated tumors (0.44 ± 0.26 normalized to the median VEGF-positive area in control tumors), but the difference was not significant ($p = 0.22$) (Figure 5.7d-f). Treated tumors did, however, show significantly lower levels of p-Met (0.24 ± 0.24 normalized to control, $p = 0.02$) (Figure 5.7g-i).
Figure 5.6 a, b) Images of H&E sections from representative control (a) and SP2024-treated (b) tumors. H&E images were binarized based on grayscale image intensity (c, d) with black pixels representing viable tumor. e) Dot plot of the viable tumor fractions of control and treated tumors, quantified from the binarized H&E images. Horizontal lines indicate the group medians.
Figure 5.7  a, b) 20x fluorescent images of representative laminin-stained sections from control (a) and SP2024-treated (b) tumors. c) Bar plot of the tumor-wise average laminin-positive area fractions for control and treated tumors. d, e) Representative 10x fluorescent images of VEGF165-stained sections from control (d) and treated (e) tumors. f) Bar plot of the tumor-wise average VEGF165-positive area fractions for control and treated tumors. g, h) Representative 10x fluorescent images of p-Met-stained sections from control (d) and treated (e) tumors. f) Bar plot of the tumor-wise average p-Met-positive area fractions for control and treated tumors. Error bars in bar plots indicate median absolute deviations.
5.4 Discussion

There were two primary goals of this study: (i) to use in vivo MRI biomarkers to assess the ‘early’ anti-angiogenic effects of a novel biomimetic peptide in an orthotopic human breast cancer model; and (ii) to compare vasculature-specific SSC-MRI biomarkers to non-vascular biomarkers such as tumor volume and MRI-measured ADC and $T_2$ in terms of their responsiveness to anti-angiogenic therapy. Treatment with the SP2024 peptide induced strong anti-angiogenic effects in MDA-MB-231 human breast cancer xenografts in mice, as demonstrated by the significant changes in SSC-MRI biomarkers of blood volume, vessel size, and vessel density. In contrast, changes in ADC and $T_2$ were not significantly different between the treatment and control groups. While SP2024 treatment reduced tumor growth rates, the changes in tumor volume during the two-week treatment period were not significantly different between the experimental groups. Collectively, these results indicate that the vascular SSC-MRI biomarkers were more sensitive for detecting the early anti-angiogenic effects of SP2024 peptide than tumor volume, ADC, or $T_2$.

While anti-angiogenic therapy does have anti-tumoral effects, vascular changes have been shown to arise before significant inhibition of tumor growth (Hoff et al., 2012; Lindner and Borden, 1997; Sahani et al., 2013). Changes in ADC have also been shown to be predictive of response to anti-angiogenic therapy (Nowosielski et al., 2010) and to precede changes in tumor size (Sahani et al., 2013). But at the early tumor stages employed in this study, control and treated tumors could not be distinguished by changes in ADC. A recent study showed that SP2024 induced apoptosis in MDA-MB-231 xenografts as measured by caspase-3 activity (Rosca et al., 2011b). Decreased cell density due to apoptosis may have contributed to the increased median ADC in three of the five treated tumors. However, median ADC also increased in three of the five control tumors, possibly due to increased edema or necrosis. Median $T_2$ increased in all
control tumors and all but one treated tumor, which also suggests an increase in extracellular volume (Jakobsen et al., 1995), but there was no significant difference between groups. Consistent with the ADC and T2 data, quantitative H&E staining did not show any significant difference in viable tumor fraction between treated and control groups.

An investigation of the effects of SP2024 using dynamic contrast enhanced MRI measurements of vascular volume and permeability in MDA-MB-231 xenografts showed no significant differences between control and treatment groups after 10 days of treatment (Rosca et al., 2011b). There was also no significant difference between the mean control and treated mean tumor volumes after 27 days of treatment. Based on these results, two weeks were determined to be a sufficient time for assessing any measurable effects of SP2024 on the tumor vasculature without significantly inhibiting tumor growth. This permitted us to determine if SSC-MRI vascular biomarkers enable earlier detection of anti-angiogenic efficacy. A clinical biomarker should not only be sensitive to early therapeutic changes, but also be noninvasive and capable of repeated measurements for longitudinal patient monitoring. Such a biomarker would enable more flexible and adaptive study designs and treatment strategies. In this regard, MRI is superior to conventional biopsy and histological evaluations; but its repeatability is limited by cost, MRI scanner availability and, for contrast enhanced methods, the biological and plasma half-lives of the contrast agent.

In this study, control tumors underwent vascular changes opposite to those in treated tumors. However, it is possible that tumor vasculature may naturally evolve in a way that obscures anti-angiogenic treatment effects, as was the case for tumor cellularity discussed above. For example, we previously observed lower fractional blood volumes in MDA-MB-231 tumors at post-inoculation week (PIW) 5 than in tumors at PIW 3 (Kim et al., 2013). The presence of
necrosis in the larger tumors suggests that the decreased fractional blood volume was caused by
tumor growth outstripping the available blood supply. Since anti-angiogenic therapy is designed
to decrease tumor blood volume, treatment was initiated when the tumors were smaller than
the PIW 3 tumors from our previous study to ensure that the blood volume did not also
decrease in the control tumors during the two-week treatment period.

The increased $\text{FBV}_{\text{MRI}}$ and $\text{VSI}_{\text{MRI}}$ in control tumors seen here are consistent with the elevated
blood volume and dilated vessels that result from dysregulated angiogenesis driven by VEGF
signaling in tumors (Carmeliet, 2005). Treated tumors demonstrated a direct reversal of these
trends, with decreased $\text{FBV}_{\text{MRI}}$ and $\text{VSI}_{\text{MRI}}$ after SP2024 treatment. The SP2024-induced decrease
in blood volume was corroborated by the significantly reduced presence of laminin staining in
immunofluorescence images of treated tumors compared to control tumors. However, it should
be borne in mind that laminin is a component of the vascular basement membrane, and thus
blood vessel lumens were not stained by this method (illustrated by Figure 5.7a,b), making
direct quantification of blood volume and vessel radii and density challenging. This issue is
common to most histological methods of vascular labeling, although it can be mitigated by
cutting thicker sections or employing stereologic techniques (Pathak et al., 2001). An alternative
for preclinical studies would be to perfuse the animal with a vascular filling agent such as
Microfil®.

Although it might be expected that anti-angiogenic therapy would cause vessel density to
decrease, the SSC-MRI vessel density parameters increased in the SP2024 treated tumors and
decreased in the control tumors. We have previously shown using high-resolution μCT that
vessel density decreases in MDA-MB-231 tumors with tumor progression and that $N_{\text{MRI}}$ and $Q$
correlated with μCT-measured vessel density (Kim et al., 2013). It is possible that SP2024
treatment altered this natural evolution of the vascular phenotype. Another possibility lies in
the fact that N is not an independent parameter but a function of FBV_{MRI} and VSIMRI – Eq. 10 is
derived directly from substituting FBV_{MRI} for f and VSIMRI for \langle R^2 \rangle in Eq. 9 (Jensen and Chandra,
2000). Thus, any errors in FBV_{MRI} and VSIMRI will propagate to N_{MRI}. Even if FBV_{MRI} and VSIMRI
correctly reflected the decreased blood volume and vessel caliber in treated tumors, errors in
the magnitudes of these changes could have caused a spurious increase in N_{MRI}.

The mutual information between group membership and change in median parameter value
was used here to quantify the difference in how control and treated tumors changed from day 0
to day 14. This metric can be used to compare the relative utilities of various biomarkers. For
example, there was less distinction between control and treatment groups based on the change
in median \Delta R_2 compared to the other SSC-MRI biomarkers (Table 5.1). This may be explained by
the opposing effects of blood volume and vessel radius on \Delta R_2, which increases with increasing
blood volume but decreases with increasing vessel radius (after some peak radius that typically
corresponds to capillary-sized vessels) (Boxerman et al., 1995). In general, \Delta R_2 decreased in
control tumors and VSIMRI increased. This is consistent with a decrease in microvascular volume
fraction that was likely due to vessel dilation. The one control tumor in which median \Delta R_2
increased also saw the largest increase in median FBV_{MRI}, which suggests that the increase in
total blood volume offset any decrease in microvascular volume. The opposite trend was seen in
the treated tumors – VSIMRI decreased in all treated tumors and \Delta R_2 increased in three. This
indicates that SP2024 therapy resulted in reduction of average vessel size and a concomitant
increase in the number of small vessels to which \Delta R_2 is most sensitive, thereby increasing \Delta R_2.
However, the median \Delta R_2 decreased in two treated tumors, which may be attributed to the
countervailing effect of decreased total blood volume, which was observed in all treated
tumors. Overall, our results indicate that knowing both \Delta R_2^* and \Delta R_2 is more informative for
monitoring anti-angiogenic therapy than knowing either parameter separately. And as mentioned previously, the vascular SSC-MRI biomarkers were able to distinguish treated tumors from control tumors better than tumor volume, $T_2$, or ADC.

Histology showed that VEGF-A expression was reduced by treatment but not to an extent that was statistically significant. This may have been a product of the sparse random sampling that is a drawback of histologic analysis in general. On the other hand, SP2024 may inhibit angiogenesis via alternative pathways independent of VEGF. For example, SP2024 has been shown to inhibit $\beta_1$ integrin interactions and phosphorylation of VEGFR2 as well as downstream signal transducers PLC-$\gamma$ and FAK in endothelial cells (Rosca et al., 2011b). Here, we showed that SP2024 also inhibits phosphorylation of Met. Munshi et al. showed that treatment with a selective Met inhibitor significantly inhibited in vitro proliferation and in vivo tumor xenograft growth in several human cancer cell lines including MDA-MB-231 (Munshi et al., 2010). Met has several of the same downstream targets as VEGFR2, including PLC-$\gamma$, MAPK, FAK, and PI3K (Claesson-Welsh and Welsh, 2013). Studies have shown that Met signaling is a possible alternative pathway via which tumors can evade anti-VEGF therapy (di Tomaso et al., 2011; Shojaei et al., 2010). Shojaei et al. showed that tumors resistant to sunitinib, a VEGFR inhibitor, expressed higher levels of HGF than sunitinib-sensitive tumors and that the combination of sunitinib and a selective Met inhibitor had an additive effect in reducing the growth of resistant tumors compared to sunitinib monotherapy (Shojaei et al., 2010). Yakes et al. showed that cabozantinib, a dual Met and VEGFR2 inhibitor, had strong anti-tumoral and anti-vascular effects in MDA-MB-231 xenografts and did not affect lung metastatic burden, whereas treatment with sunitinib increased tumor burden in the lungs (Yakes et al., 2011). Their data indicates that tumor cells are capable of evading VEGF-based anti-angiogenic therapies by switching to a more invasive and metastatic phenotype (Ebos and Kerbel, 2011; Paez-Ribes et al., 2009). Therefore,
drugs (including SP2024) that target multiple pathways involved in angiogenesis as well as in the spread of cancer cells themselves could circumvent the problems of acquired resistance and increased invasiveness that can result from prolonged inhibition of the VEGF pathway. Future work includes assessing the effects of SP2024 on breast cancer metastasis and its robustness against acquired resistance.

In conclusion, we have shown that the SP2024 peptide is a promising new therapeutic for TNBC; it has a multi-modal mechanism of action that has the potential to overcome some of the limitations of conventional VEGF-targeted anti-angiogenics. We also showed that SSC-MRI biomarkers are highly sensitive to changes in tumor vasculature and more suitable for detecting early response to anti-angiogenic therapy compared to conventional metrics such as tumor volume, $T_2$, and ADC.
Large-scale simulations of SSC-MRI using real tumor vasculature

6.1 Introduction

Abnormal vascular structure and function are associated with a variety of diseases from stroke to cancer. Susceptibility contrast MRI is commonly used to characterize these vascular abnormalities and assess response to therapy, e.g. monitor the effects of anti-angiogenic drugs in tumors. There are dynamic and steady-state approaches of susceptibility contrast MRI; the former involves imaging with high temporal resolution to track the first pass changes in MR contrast agent concentration by measuring $\Delta R_2(t)$ and $\Delta R_2^*(t)$ following bolus administration, while the latter measures $\Delta R_2$ and $\Delta R_2^*$ at the steady-state concentration of an MR contrast agent confined to the intravascular space. Dynamic susceptibility contrast (DSC)-MRI is widely used in the clinic to measure cerebral perfusion and blood volume (Ostergaard, 2005). In comparison, steady-state susceptibility contrast (SSC)-MRI allows for higher signal-to-noise ratio (SNR), is simpler to implement, and does not require measurement of an arterial input function; but it does require measurement of $\Delta \chi$ and, until recently, was limited to preclinical studies. However, the recent FDA approval of ferumoxytol (Feraheme®, AMAG), a USPIO with a long circulation half-life, to treat iron deficiency anemia in patients with chronic kidney disease has led to its off-label use as a contrast agent for SSC-MRI in humans (Christen et al., 2012a; Fredrickson et al., 2012b; Landry et al., 2005). This development has opened the possibility for SSC-MRI to fill a need for clinical biomarkers of angiogenesis, particularly in the area of anti-angiogenic cancer therapy (Sessa et al., 2008).
SSC-MRI biomarkers of fractional blood volume (FBV), vessel size index (VSI), and vessel density (N) are derived from analytical models of susceptibility contrast that make various biophysical assumptions (Kiselev and Posse, 1999; Yablonskiy and Haacke, 1994) that include:

- a vascular network approximated by randomly distributed (position, orientation, radius) infinite cylinders
  - cylinder radius << voxel size, cylinder length and curvature
  - cylinder length >> distance between cylinders
- number of cylinders >> 1
- small blood volume fraction, ignore MR signal inside cylinders
- static dephasing regime (GE signal) or slow diffusion regime (SE signal)
- TE >> 1/δω
- *two homogeneous compartments: intravascular space and extravascular space
- *uniform nuclear spin (water $^1$H nuclei) distribution
- *restricted, isotropic water diffusion

The last three assumptions marked by asterisks are also made by the computational model presented here.

The static dephasing regime holds when the following inequality is satisfied (Yablonskiy and Haacke):

$$\frac{1}{FBV \cdot \delta \omega} \ll \frac{(RV)^2}{6D}$$  \[23\]

where FBV is the fractional blood volume, $\delta \omega$ is defined by Eq. 1, RV is the average vessel radius, and $D$ is the diffusion coefficient of water. Since static dephasing is refocused in an SE experiment, the static dephasing condition is relaxed when modeling the SE signal in order to
consider the effects of diffusion. The slow diffusion regime holds when $\sqrt{D \Delta t} \ll R_V$ (Kiselev and Posse).

Recent studies have reported that the SSC-MRI biomarkers correlate well with corresponding measurements made using high resolution ex vivo techniques such as histology and micro-CT ($\mu$CT), but FBV and VSI exhibit systematic overestimation (Kim et al., 2013; Ungerma et al., 2010; Valable et al., 2008), suggesting that these model assumptions and restrictions may not be appropriate in vivo.

Numerical simulations have also demonstrated that while there is a relationship between $\Delta R_2$ and $\Delta R_2^*$ and vascular morphology (Boxerman et al., 1995), there is a bias in these theoretically derived SSC-MRI biomarkers (Kiselev et al., 2005; Tropres et al., 2001). Most of these simulations used randomly oriented infinite cylinders to model vascular networks because there is an analytical solution to the magnetic field perturbations induced by an ensemble of magnetized cylinders. However, the geometry of characteristically tortuous tumor vessels can deviate significantly from that of simple cylinders. This discrepancy necessitates the incorporation of a more realistic model of the tumor vasculature in numerical simulations exploring the relationship between $\Delta R_2$ and $\Delta R_2^*$ and vascular morphology. This in turn requires specialized numerical methods capable of computing the magnetic field perturbations arising from arbitrary spatial distributions of magnetic susceptibility gradients. These numerical approaches can be broadly classified as iterative finite difference or finite element methods (Bhagwandien et al., 1994; Li et al., 1996) or Fourier-based approaches (Jenkinson et al., 2004; Marques and Bowtell, 2005; Salomir et al., 2003). One such Fourier-based approach called the finite perturber method (FPM) was specifically developed by us to compute the local magnetic field shifts induced by real microvascular structures for SSC-MRI simulations (Pathak et al.,
2008b). Christen et al. also used a Fourier-based approach to investigate the effects of vessel geometry on SSC-MRI-derived FBV measurements, and they found that overestimation of the actual blood volume was greater for murine cortical vessels acquired by two-photon microscopy than for randomly oriented cylinders (Christen et al., 2012b).

SSC-MRI is emerging as a potential candidate to provide clinically relevant imaging biomarkers of angiogenesis capable of facilitating the development of new anti-angiogenic drugs, monitoring of treatment efficacy, and planning of personalized cancer therapy. In this study, we expanded the FPM to enable simulations and validation of SSC-MRI on the scale of whole murine organs or tumors (~100 mm³). We combined ex vivo μCT angiography data and numerical simulations to investigate the effects of various biophysical factors and imaging parameters on SSC-MRI biomarker accuracy in several synthetic and real vascular systems.
6.2 Methods

A schematic of the computational methodology of this study is presented in Figure 6.1. The FPM was used to compute maps of magnetic field perturbations (ΔB) created by various 3D vascular systems. Three categories of vessel geometries were considered: (i) single-voxel randomly oriented cylinders, (ii) multi-voxel randomly oriented cylinders, and (iii) μCT-derived tumor vasculature. Next, the vascular systems and their ΔB maps were incorporated into Monte Carlo simulations of proton diffusion to generate \( \Delta R_2 \) and \( \Delta R_2^* \) maps, from which SSC-MRI vascular biomarkers were calculated using Eqs. 3, 6, 7, 8, and 10. These simulated MRI biomarker maps were compared to ground truth vascular morphometric maps computed directly from the underlying 3D vascular structures. Details of the methods are given below.

6.2.1 Computing magnetic field perturbation maps using the FPM

The FPM approximates the local magnetic field inhomogeneities induced by differences in extra- and intravascular magnetic susceptibilities by modeling the vasculature as a collection of discrete, finite perturbers. The finite perturber geometry is modeled as a cube to ensure complete space filling. The finite perturber field shift (FPF) is that of a sphere, which has a simple analytical expression, scaled by the volumetric ratio of the cubic finite perturber and its inscribed sphere (Pathak et al., 2008b):

\[
\Delta B_{\text{sphere}}(x, y, z) = \frac{4\pi a^3}{r^3} \left( 3 \cos^2 \theta - 1 \right) B_0 \tag{24}
\]

\[
\text{FPF}(x, y, z) = \Delta B_{\text{cube}}(x, y, z) = \frac{6}{\pi} \Delta B_{\text{sphere}}(x, y, z) \tag{25}
\]

where \( \Delta \chi \) is the susceptibility difference in cgs units between the inside and outside of the sphere, \( a \) is the radius of the sphere, \( r \) is the magnitude of the position vector \( \mathbf{p} = [x, y, z] \), and \( \theta \) is the angle between \( \mathbf{p} \) and \( B_0 \). The magnetic field perturbations created by the vasculature is
estimated as the sum of each individual FPF, which is equivalent to the convolution of the vasculature with a single FPF:

$$\Delta B_{\text{vasc}}(x, y, z) = \sum_{\xi=-\infty}^{\infty} \sum_{\eta=-\infty}^{\infty} \sum_{\zeta=-\infty}^{\infty} V(\xi, \eta, \zeta) \text{FPF}(x - \xi, y - \eta, z - \zeta)$$

where $V$ is a binary representation of the underlying vasculature. The circular convolution theorem is exploited to compute this convolution using the fast Fourier transform (FFT).

**Figure 6.1** Schematic of the computational pipeline. White boxes = inputs, gray boxes = outputs, black boxes = computational modules. $\Delta \chi$ is the difference in magnetic susceptibility between the intra- and extravascular compartments, $\Delta B$ is the magnetic field perturbation created by the magnetized 3D vasculature estimated by the FPM, and $D$ is the diffusion coefficient used to simulate random walks of protons through $\Delta B(x, y, z)$. Transverse relaxation rates $\Delta R_2$ and $\Delta R_2^*$ are computed from the simulated spin and gradient echo signals, respectively, and used to generate maps of SSC-MRI biomarkers of angiogenesis. Corresponding ground truth maps are computed directly from the underlying 3D vascular structure.
A limitation of circular convolution is that it results in wrap-around artifacts in the field perturbation map. This is illustrated by a simple case of a single cylinder oriented oblique to the main magnetic field (Figure 6.2a). A method similar to the overlap-save method in signal processing was developed to remove these wrap-around artifacts. In this new “overlap-crop” method, the FPF is first zero-padded to double its original size in all three dimensions, e.g. if the FPF is calculated on an N×N×N grid, N/2 zeros are added to every side to produce a 2N×2N×2N matrix (Figure 6.2b). Due to computational limitations, calculating the magnetic field perturbation map in smaller overlapping blocks is necessary for large datasets. The magnetic field perturbation map is calculated using FFT convolution in overlapping 2N×2N×2N blocks with an overlap width of N (dashed lines, Figure 6.2c). This allows us to crop the outer region of each field block affected by the circular convolution-induced wrap-around, which extends N/2 voxels from each face of the block because of the FPF zero-padding. The remaining central N×N×N field blocks are non-overlapping and immediately adjacent (solid lines, Figure 6.2c), resulting in a whole vascular field map free of wrap-around artifact. Figure 6.2d-f illustrates the need for this correction. Figure 6.2d shows the block of μCT vasculature outlined by the dashed cyan line in Figure 6.2c, and Figure 6.2e shows the field perturbation map obtained by convolving the FPF with the vasculature using FFTs. Figure 6.2f shows the difference between this field with the wrap-around artifact still present (Figure 6.2e) and the corresponding wrap-around-free field calculated using the overlap-crop method. The wrap-around artifact is clearly visible around the edges of the block but does not affect the central N×N×N region delineated by the solid cyan box.
Figure 6.2 a) Slice through the magnetic field perturbation ($\Delta B$) created by a magnetized cylinder calculated using the finite perturber method (FPM). This illustrates the wrap-around artifacts caused by using FFTs to compute the convolution of the object with the finite perturber field (FPF). To remove these artifacts, the FPF is zero-padded (b), and the $\Delta B$ map computed in overlapping blocks as demarcated by dotted lines in (c). The blocks are cropped to keep only the central regions (solid lines), which are free of wrap-around artifacts. These are stitched together to produce the final $\Delta B$ map. d) A block of tumor vasculature that was segmented from high-resolution $\mu$CT images and e) the corresponding $\Delta B$ map with wrap-around artifacts, most visible in the upper corners of the image. f) The difference image of the $\Delta B$ map stitched together by the overlap-crop method and that of the single block in (e), demonstrating wrap-around artifacts outside but not inside the central N×N region (cyan box).
6.2.2 Monte Carlo simulation of the MRI signal

As described in (Pathak et al., 2008b), a Monte Carlo method was used to simulate proton diffusion and estimate the SSC-MRI signal produced by the field perturbation maps computed with the FPM. Initially, protons were placed randomly in simulation space, and their positions \((x,y,z)\) were updated to new positions \((x',y',z')\) at each simulation time step \(\Delta t\):

\[
\begin{align*}
  x' &= x + \Delta x \\
  y' &= y + \Delta y \\
  z' &= z + \Delta z
\end{align*}
\]  

where \(\Delta x\), \(\Delta y\), and \(\Delta z\) are independent random samples from the normal distribution with mean \(\mu = 0\) and standard deviation \(\sigma = \sqrt{2D\Delta t}\). The diffusion coefficient \(D\) was set to a constant value.

For all cases, diffusion was restricted, i.e., protons were not allowed to cross vessel walls.

The phase of the \(k\)th proton at time \(t\) is given by:

\[
\varphi_k(t) = \varphi_k(t - \Delta t) + \gamma \Delta B_{vsc}(p_k(t)) \Delta t
\]  

with all protons having a phase of zero at \(t = 0\). To calculate the SE signal, the phases of all the protons were negated at \(t = nT_{ESE}/2\), where \(T_{ESE}\) is the SE echo time and \(n = 1,3,5,...,2NE-1\) (\(NE = \) number of echoes). At each \(T_{ESE}\) and \(T_{EG}\) (GE echo time), the MRI signal in voxel \(m\) was computed using the following equation:

\[
S_m(TE) = \sum_{n \in \text{protons in voxel } m} \exp(i \varphi_n(TE))
\]

For all simulations, the spatial resolution of simulation space was 1 \(\mu m\), \(D = 1 \times 10^{-5} \text{ cm}^2/\text{s}\), and time step \(\Delta t = 0.1 \text{ ms}\). For each simulation run, \(NE = 20\), with \(T_{EG} = 5 \text{ ms}\) and \(T_{ESE} = 25 \text{ ms}\).
Simulations were repeated using various values of $\Delta \chi$ corresponding to different contrast agent concentrations. It is important to note that the $\Delta \chi$ values reported here correspond to a field strength of 9.4T. USPIOs have been shown to reach their saturation magnetization at low magnetic fields (~1T). At field strengths greater than the saturation field, the product $\Delta \chi B_0$ is constant for a given dose, and thus $\Delta \chi$ decreases with increasing $B_0$.

FPM-based computation of $\Delta B$ maps, Monte Carlo simulations, and image reconstruction were performed in MATLAB on a workstation with dual 3.3 GHz quad-core processors, 128 GB RAM, and a 512 GB solid-state drive. Monte Carlo simulations and image reconstruction were performed using six parallel workers.

6.2.3 Simulations using real tumor vasculature from ex vivo μCT angiography

An orthotopic MDA-MB-231 human breast cancer xenograft was grown in the mammary fat pad of a female athymic NCr-nu/nu mouse for five weeks (tumor volume $= 250$ mm$^3$) (Kim et al., 2013). The mouse was then sacrificed by perfusion fixation followed by perfusion with a radiopaque vascular filling agent (Microfil, Flow Tech Inc.) according to a procedure described by us previously (Kim et al., 2011). The tumor was then excised and sent to Numira Biosciences (Salt Lake City, UT) for μCT imaging using a ScanCo Medical μCT 40 desktop system with the following imaging parameters: 8 μm isotropic resolution, 55 kVp, 144 μA, 300 ms exposure time, 2000 views and 5 frames per view. The vasculature was segmented from the μCT data using an algorithm involving a Hessian-based filter and various morphological operations (Kim et al., 2013; Sato et al., 1998). A volume rendering of a 1-mm thick slice of the segmented tumor vasculature color coded by vessel radius is shown in Figure 6.3a.

The magnetic field perturbation map corresponding to the 1-mm slice was computed using a 256×256×256-voxel FPF kernel zero-padded to 512×512×512. The tumor vasculature data was
resampled without interpolation from an isotropic voxel size of 8 μm to 1 μm. Then, owing to its large size (9152×10176×1536 voxels), the resampled image was divided into six overlapping sections, and the field map and Monte Carlo simulations computed separately for each section. The field map was computed with \( B_0 \) oriented along the positive \( y \)-axis (Figure 6.4a). For the simulations, initial proton positions were restricted to within the manually drawn tumor mask and at least half of the overlap width (400 μm) from the edges of each section, eliminating artificial boundaries to diffusion while ensuring uniform distribution of protons throughout the whole slice. These large-scale simulations consisted of a total of approximately \( 96 \times 10^6 \) protons.

The proton position and phase data from the six sections were pooled before generating multi-echo GE and SE images with voxel size of 250×250×250 μm or 125×125×1000 μm using Eq. 29 (Figure 6.4b,c).

### 6.2.4 Simulations using randomly oriented cylinders

Synthetic “vascular” datasets with matrix size 512×512×512 voxels were generated in MATLAB. Each dataset contained randomly distributed, randomly oriented cylinders with average radius \( \langle R \rangle \) varying from 2 to 32 μm and occupying volume fractions \( \langle V_i \rangle \) ranging from 2% to 50%. This was achieved as follows: the radius of each cylinder was determined by sampling from the Poisson distribution with mean parameter \( \lambda = \langle R \rangle \). Because the number of cylinders decreases with increasing radius given a certain fractional volume, a variable number of datasets were created for each \( \langle (R), V_i \rangle \) pair such that the aggregate number of cylinders was at least 1000 to ensure adequate averaging of cylinder orientations. Magnetic field perturbation maps were computed using a 256×256×256 FPF kernel zero-padded to 512×512×512; as described in the previous section, only the central 256×256×256 portions of the field maps were used for the simulations. All simulation runs consisted of \( 2 \times 10^4 \) randomly placed protons. Each 256×256×256 dataset was treated as a single MRI voxel, resulting in one GE and one SE signal time course.
Figure 6.3  a) Volume rendering of the tumor vasculature segmented from high-resolution μCT images and used in SSC-MRI simulations. Vessels are color coded by radius. b) Histogram of mean radii of vessel branches; $V_f$ is the vascular volume fraction and MVD the number of vessel branches per mm$^3$ in the tumor slice. c) Volume rendering of the large multi-voxel random cylinders data set. The radius of each cylinder was determined by sampling from the tumor vessel radius distribution (b). d) The resultant cylinder radius histogram, $V_f$, and number of cylinders.

$V_f = 1.36\%$  \hspace{1cm} MVD = 115.0 mm$^3$

$V_f = 1.21\%$  \hspace{1cm} 180 cylinders
For multi-voxel simulations, a large synthetic “vasculature” dataset with matrix size 2560×2560×1536 was generated containing randomly distributed and randomly oriented cylinders (Figure 6.3c). The radius of each cylinder was determined by sampling from the empirical distribution function defined by the μCT-derived tumor vessel radius histogram (Figure 6.3b), resulting in a similar cylinder radius histogram (Figure 6.3d). The magnetic field perturbation maps were computed using a 256×256×256 FP F kernel zero-padded to 512×512×512, resulting in a final field map matrix size of 2304×2304×1280. The simulations consisted of 8×10⁶ randomly placed protons restricted to the central 2048×2048×1024 of the magnetic field space. Finally, GE and SE images with voxel size of 250×250×250 μm or 125×125×1000 μm were generated.

6.2.5 Computation of simulated biomarker maps

For all three vessel geometry cases (single-voxel random cylinders (RC), multi-voxel RC, and μCT-derived tumor vasculature), ΔR₂, and ΔR₂* were computed voxel by voxel in AFNI (Cox, 1996) by fitting Sₘ(TE) to a mono-exponential model (Eq. 14). For the lowest Δχ value, the first TEs of the GE and SE signals were ignored during fitting to exclude the non-monoexponential decay of the MR signal at short time scales (Kiselev and Posse, 1999; Yablonskiy and Haacke, 1994). Simulated SSC-MRI biomarker maps were computed using Eqs. 3, 6, 7, 8, and 10. For clarity, these simulated MRI parameters will be denoted with a “SIM” subscript (e.g. FBVₘᵦ). Corresponding ground truth maps were computed directly from the multi-voxel RC or μCT tumor vessel data using the method described in Chapter 3.2.6. In brief, cylinder/vessel centerline coordinates and their corresponding radii were computed using the skeletonization routine in Amira®. This data was then imported into MATLAB, and customized scripts written to generate vascular parametric maps using the following equations:
FBV_{\text{TRUTH}} = \frac{V_{\text{vessel}}}{V_{\text{voxel}}} \quad [30]

\langle R \rangle_{\text{TRUTH}} = \frac{1}{N} \sum_{i=1}^{N} R_i \quad [31]

VSI_{\text{TRUTH}} = \left( \frac{\langle R^2 \rangle}{\langle R^4/3 \rangle} \right)^{3/2} \quad [32]

N_{\text{TRUTH}} = \frac{\text{FBV}_{\text{TRUTH}}}{2\pi \langle R^2 \rangle} \quad [33]

where \(V_{\text{vessel}}\) is the volume of cylinders/vessels in a voxel, \(V_{\text{voxel}}\) is the volume of a voxel, \(R_i\) is the cylinder/vessel radius at point \(i\), \(\langle R \rangle_{\text{TRUTH}}\) is the mean cylinder/vessel radius in a voxel, and \(\langle x \rangle\) represents the average of some random variable \(x\) in a voxel. Eqs. 32 and 33 were previously presented in (Jensen and Chandra, 2000) and (Wu et al., 2004). These maps were computed on the same spatial grids as the simulated biomarker maps to allow for voxel-wise comparisons. The ground truth and simulated SSC-MRI biomarker maps for the tumor data are shown in Figure 6.4.

For the single-voxel RC data, skeletonization was not performed due to the large number of individual datasets. Instead, the average cylinder radius was calculated by weighting the radius of each cylinder by its length, and \(N_{\text{TRUTH}}\) was simply the number of cylinders in each voxel.

6.2.6 Statistical analysis

The Pearson correlation coefficient \(r\) was computed for each corresponding pair of simulated MRI and ground truth vascular parameters to assess the linear dependence of the MRI parameters on the underlying vascular structure. Lin’s concordance correlation coefficient \(\rho_C\) (Lin, 1989) and the voxel-wise errors were computed for appropriate parameter pairs (i.e., \(\text{FBV}_{\text{SIM}}\) vs. \(\text{FBV}_{\text{TRUTH}}\), \(\text{VSI}_{\text{SIM}}\) vs. \(\text{VSI}_{\text{TRUTH}}\), \(\text{N}_{\text{SIM}}\) vs. \(\text{N}_{\text{TRUTH}}\)) to assess the quantitative accuracy of the simulated MRI parameters. Two-tailed Wilcoxon signed rank tests and Mann Whitney-U tests
were performed to determine whether the errors between simulated and ground truth parameter values were significant and whether the errors varied as a function of vessel and voxel geometries, respectively (α = 0.01). To allow for better comparison between the three vessel geometries (single-voxel RC, multi-voxel RC, and μCT tumor vessels), only voxels with ground truth parameter values within the range of values for all three cases were used to calculate statistics. As an exception, voxels with \( \text{FBV}_{\text{TRUTH}} \leq 10\% \) were used for computation of \( r \) and \( \rho_c \) for the single-voxel RC data owing to its discretized nature (voxels had \( \text{FBV}_{\text{TRUTH}} = 2\%, 5\%, \) or \( 10\% \)).
Figure 6.4 *In silico* images and parametric maps generated from the μCT-derived tumor vasculature. a) The ΔB map computed using the FPM. b) Simulated gradient echo (GE, TE = 25 ms) and c) spin echo (SE, TE = 100 ms) images reconstructed from Monte Carlo simulations with in-plane resolution = 125 μm and slice thickness = 1000 μm. Overlaid on the simulated SE image are parametric maps of d) ground truth FBV, e) simulated FBV, f) simulated ΔR₂, g) ground truth VSI, h) simulated VSI, i) simulated R, j) ground truth N, k) simulated N, and l) simulated Q. For ground truth maps (d, g, j), voxels that do not contain any vessels are transparent. For simulated maps, voxels for which the exponential fit did not meet the $F$-statistic criterion ($p < 0.05$) are transparent.
6.3 Results

6.3.1 Computational performance of large-scale simulations

FPM-based computation of the magnetic field perturbation map for the multi-voxel RC data took 0.46 h. Monte Carlo simulations of proton diffusion involving ~8 million protons took 5.33 h. Reconstruction of MGE and MSE images (8×8×4 and 16×16×1 matrix dimensions) took a total of 0.23 h.

Computation of the six overlapping blocks of the magnetic field perturbation map for the μCT tumor vessel data took 8.65 h. Monte Carlo simulations involving ~96 million protons took 41.88 h. Reconstruction of MGE and MSE images (64×64×4 and 128×128×1 matrix dimensions) took a total of 12.55 h.

6.3.2 The Good: Fractional blood volume

Figure 6.5a shows voxel-wise scatter plots of \( \text{FBV}_{\text{SIM}} \) vs. \( \text{FBV}_{\text{TRUTH}} \). The single-voxel RC plots only include datasets for which the mean of the Poisson sampling distribution used to determine cylinder radii was 8 μm. The multi-voxel RC and μCT tumor plots are for images with isotropic 250 μm voxel dimensions.

Similar trends were observed for all three vessel geometry cases. The MRI parameter \( \text{FBV}_{\text{SIM}} \) increased linearly with the actual fractional blood volume for small \( \text{FBV}_{\text{TRUTH}} \) (< ~5%). This is best illustrated by the multi-voxel RC data, which had a maximum \( \text{FBV}_{\text{TRUTH}} \) of 5%. The strong linear relationship between \( \text{FBV}_{\text{SIM}} \) and \( \text{FBV}_{\text{TRUTH}} \) is reflected in the voxel-wise Pearson correlation coefficients \( (r) \), which are plotted against \( \Delta \chi \) in Figure 6.6a. The correlation was greatest for the single-voxel RC data, with \( r \) ranging from 0.96 at \( \Delta \chi = 0.05 \) ppm to 0.92 at 0.2 ppm. The correlation was weakest but still strong for the μCT tumor vessel data, with \( r \) between 0.87 and 0.89 for the image with isotropic 250 μm resolution and between 0.77 and 0.87 for the image...
with 125×125×1000 μm resolution. For both multi-voxel RC and tumor vessel data, the correlations between \( \text{FBV}_{\text{SIM}} \) and \( \text{FBV}_{\text{TRUTH}} \) were lower for anisotropic voxels than for isotropic voxels of the same volume. In fact, voxel size had a greater impact on \( r \) than vessel geometry – the correlation for tumor vessels with isotropic voxels was stronger than for multi-voxel RC with anisotropic voxels.

Despite the strong linear relationship, \( \text{FBV}_{\text{SIM}} \) generally overestimated \( \text{FBV}_{\text{TRUTH}} \), especially at lower \( \Delta \chi \) values. This overestimation at small \( \Delta \chi \) was primarily caused by a proportional bias (i.e., an error in scale), as shown in Figure 6.5a with the aid of the dashed lines which represent the line of equality \( (y = x) \). As \( \Delta \chi \) increased, the slope of the data decreased, bringing \( \text{FBV}_{\text{SIM}} \) in closer agreement with \( \text{FBV}_{\text{TRUTH}} \). Interestingly, \( \text{FBV}_{\text{SIM}} \) for single-voxel RC underestimated \( \text{FBV}_{\text{TRUTH}} \) at 0.15 ppm and 0.2 ppm. In contrast, although the proportional bias diminished with increasing \( \Delta \chi \) for the multi-voxel RC and tumor vessel \( \text{FBV}_{\text{SIM}} \), a fixed overestimating bias remained. The median error of both multi-voxel RC and tumor vessel \( \text{FBV}_{\text{SIM}} \) decreased with increasing \( \Delta \chi \) but remained significantly greater than zero for all values of \( \Delta \chi \) (Table 6.1).

The bias in \( \text{FBV}_{\text{SIM}} \) is reflected in the concordance correlation coefficients \( (\rho_c) \) plotted against \( \Delta \chi \) in Figure 6.6b. \( \rho_c \) was greatest for single-voxel RC at all values of \( \Delta \chi \). \( \rho_c \) was greater for multi-voxel RC than the μCT tumor vessels for both voxel dimensions, except at the lowest \( \Delta \chi \). As with \( r \), the anisotropic voxel size had a more deleterious effect on \( \rho_c \) than did the irregular morphology of the tumor vasculature.
Figure 6.5 Voxel-wise scatter plots of (a) $\text{FBV}_{\text{SIM}}$ vs. $\text{FBV}_{\text{TRUTH}}$ and (b) $\Delta R_2_{\text{SIM}}$ vs. $\text{FBV}_{\text{TRUTH}}$ for the single-voxel random cylinders (RC) (top row), multi-voxel RC (middle row), and μCT tumor vessels (bottom row) at various values of $\Delta \chi$, corresponding to different contrast agent doses. For multi-voxel RC and tumor vessels, the data from isotropic voxel (250 μm) images are shown. The dashed lines represent the line of equality ($y = x$).
Figure 6.6 Plots of voxel-wise (b) Pearson and (c) Lin’s concordance correlation coefficients between FBV\textsubscript{SIM} and FBV\textsubscript{TRUTH} as functions of \(\Delta \chi\).

Voxel size also had a greater impact on the tumor vessel simulations than on the multi-voxel RC simulations. For all \(\Delta \chi\) values, the median FBV\textsubscript{SIM} error was significantly greater for anisotropic voxels than for isotropic voxels for the tumor data but not for the RC data. The median errors were significantly different between the multi-voxel RC and tumor data for the anisotropic voxels for all \(\Delta \chi\) except 0.05 ppm; conversely, there was no significant difference in median FBV\textsubscript{SIM} error between the two geometries for the isotropic voxels at any \(\Delta \chi\) except 0.02 ppm.

While median FBV\textsubscript{SIM} error decreased and concordance increased with increasing \(\Delta \chi\), the linear relationship between FBV\textsubscript{SIM} and FBV\textsubscript{TRUTH} weakened, seen qualitatively in the scatter plots (Figure 6.5a) and quantified by the Pearson correlation coefficient (Figure 6.6a). This decrease in linearity can be explained by the increased variance and sublinear relationship between FBV\textsubscript{SIM} and FBV\textsubscript{TRUTH} at large values of FBV\textsubscript{TRUTH}. This plateau effect was more evident at higher \(\Delta \chi\), seen in both the single-voxel RC and \(\mu\)CT tumor data. However, FBV\textsubscript{TRUTH} < 2\% in 85\% (88\%) of isotropic (anisotropic) voxels in the tumor data, meaning a large majority of the data fell within the linear regime. This is illustrated by the 2D histogram of FBV\textsubscript{SIM} vs. FBV\textsubscript{TRUTH} (Figure 6.7a).
Figure 6.7 Voxel-wise 2D histograms of simulated SSC-MRI biomarkers vs. their ground truth analogs for the μCT tumor data (isotropic voxels, $\Delta \chi = 0.1$ ppm). a) $FBV_{\text{SIM}}$ vs. $FBV_{\text{TRUTH}}$. (b) $R_{\text{SIM}}$ vs. $\langle R \rangle_{\text{TRUTH}}$. (c) $Q_{\text{SIM}}$ vs. $N_{\text{TRUTH}}$. 
The voxel-wise scatter plots of $\Delta R_{2, \text{SIM}}$ vs. $FBV_{\text{TRUTH}}$ exhibit even greater plateauing at large $FBV_{\text{TRUTH}}$ (Figure 6.5b). While $\Delta R_{2, \text{SIM}}$ generally increased with $FBV_{\text{TRUTH}}$, it was not as linear of a function of blood volume as $FBV_{\text{SIM}}$ was. Interestingly, the linearity increased with $\Delta \chi$ for the single-voxel RC data, whereas the other two datasets became more nonlinear with increasing $\Delta \chi$.

### 6.3.3 The Bad: Vessel size

**Figure 6.8a** shows voxel-wise scatter plots of $VSI_{\text{SIM}}$ vs. $\langle R \rangle_{\text{TRUTH}}$. The single-voxel RC plots only include datasets for which $FBV_{\text{TRUTH}} = 5\%$. The multi-voxel RC and $\mu$CT tumor plots are for images with isotropic 250 $\mu$m voxel dimensions. For the single-voxel RC data, there was a good linear correlation between $VSI_{\text{SIM}}$ and $\langle R \rangle_{\text{TRUTH}}$ with $r$ ranging from 0.71 to 0.84. Similar to FBV, the variance in $VSI_{\text{SIM}}$ increased with increasing $\langle R \rangle_{\text{TRUTH}}$. The correlation was lower for the multi-voxel RC data, with $r$ ranging from 0.18 to 0.46 for the isotropic voxels and from 0.22 to 0.33 for the anisotropic voxels. For the $\mu$CT tumor data, the correlation was the lowest and even negative for the higher $\Delta \chi$ values (Figure 6.9a).

The lack of correlation for the tumor vessel data resulted from $VSI_{\text{SIM}}$ greatly overestimating small $\langle R \rangle_{\text{TRUTH}}$ values. This is demonstrated qualitatively in Figure 6.4g-i, where the brightest regions of the $VSI_{\text{SIM}}$ and $R_{\text{SIM}}$ maps correspond to the darkest regions of the $VSI_{\text{TRUTH}}$ map. In fact, there was no dependence of $VSI_{\text{SIM}}$ on the true vessel size for these voxels, which are displayed in light gray in the last row of scatter plots of Figure 6.8a. **Figure 6.10** shows scatter plots of the voxel-wise differences between $VSI_{\text{SIM}}$ and $VSI_{\text{TRUTH}}$ plotted against $\langle R \rangle_{\text{TRUTH}}$, $MVD_{\text{TRUTH}}$, $FBV_{\text{TRUTH}}$, and $\Delta R_{2, \text{SIM}}$. The error was smallest for large values of all four of these parameters, but there was a distinct ‘L’ shape to the distribution for the latter three parameters (especially $FBV_{\text{TRUTH}}$ and $\Delta R_{2, \text{SIM}}$) that was not apparent for $\langle R \rangle_{\text{TRUTH}}$.  

106
Figure 6.8 Voxel-wise scatter plots of (a) VS_{SIM} vs. \langle R \rangle_{\text{TRUTH}} and (b) R_{SIM} vs. \langle R \rangle_{\text{TRUTH}} for the single-voxel random cylinders (RC) (top row), multi-voxel RC (middle row), and μCT tumor vessels (bottom row) at various values of \( \Delta \chi \), corresponding to different contrast agent doses. For multi-voxel RC and tumor vessels, the data from isotropic voxel (250 μm) images are shown. The dashed lines represent the line of equality (y = x). For the tumor vessel plots, voxels that did not pass the MVD_{\text{TRUTH}}, FBV_{\text{TRUTH}}, and \Delta R^2_{SIM} thresholds are represented by light gray points.
Figure 6.9 Plots of voxel-wise Pearson correlation coefficients between VSI_{SIM} and \langle R \rangle_{TRUTH} as functions of \Delta \chi. For the tumor vessel data, r was calculated using either (a) all voxels or (b) only the voxels represented by dark gray points in Figure 6.8a.

Figure 6.10 Plots of voxel-wise errors of VSI_{SIM} with respect to VSI_{TRUTH} as a function of a) \langle R \rangle_{TRUTH}, b) FBV_{TRUTH}, and c) \Delta R_{2SIM} computed from the isotropic-voxel \mu CT tumor vessel data for \Delta \chi = 0.1 ppm. These plots were used to determine thresholds (vertical dashed lines) to mask out voxels for which VSI_{SIM} values were invalid (light gray voxels in Figure 6.8).
There were distinguishable threshold values of $MVD_{\text{TRUTH}}$, $FBV_{\text{TRUTH}}$, and $\Delta R^2_{\text{SIM}}$ above which the VS$\text{ISIM}$ error was relatively small and constant. The dark gray points in the last row of scatter plots of Fig. 6a represent voxels in which at least one of these three parameters exceeded their respective thresholds of 1000 mm$^{-3}$, 4%, and 2 s$^{-1}$, which were determined by visual inspection of the plots in Figure 6.10 (vertical dotted lines). Figure 6.9b is the same as Figure 6.9a except that the correlation coefficients for the $\mu$CT tumor data were computed using only the dark gray data points that passed the thresholds. These new $r$ values ranged from 0.22 to 0.64 for the isotropic voxels and from 0.11 to 0.29 for the anisotropic voxels, which are comparable to $r$ for the multi-voxel RC data.

While $FBV_{\text{SIM}}$ estimated the actual fractional blood volume with reasonable accuracy, VS$\text{ISIM}$ systematically overestimated the true average vessel radius by a large margin (Table 6.1). Consequently, $\rho_c$ between VS$\text{ISIM}$ and $\langle R \rangle_{\text{TRUTH}}$ was very low (< 0.1) for all cases. Like FB$\text{VSIM}$, VS$\text{ISIM}$ had a proportional bias that decreased with increasing $\Delta \chi$. However, linearity also decreased with increasing $\Delta \chi$, especially for the tumor vessels.

Voxel size did not have as great of an effect on vessel size correlation or accuracy as it did on blood volume. There were no significant differences between voxel sizes in median VS$\text{ISIM}$ errors for the multi-voxel RC or the tumor vessel data, but $r$ was greater for isotropic voxels than for anisotropic voxels for the tumor data at all $\Delta \chi$. Vessel geometry, however, did have a significant impact on VS$\text{ISIM}$ accuracy. With a few exceptions, the median VS$\text{ISIM}$ errors were significantly greater for the tumor data than for multi-voxel RC (Table 6.1). Interestingly, the median errors for the single-voxel RC data were significantly greater than both the tumor and multi-voxel RC data. This may have been because there were many more voxels with large $\langle R \rangle_{\text{TRUTH}}$ for single-
voxel RC compared to the other two cases, and VSI_{SIM} error increased with increasing \( \langle R \rangle_{\text{TRUTH}} \) due to a proportional bias as mentioned above.

**Figure 6.8b** shows scatter plots corresponding to those in **Figure 6.8a** but with \( R_{\text{SIM}} \) instead of VSI_{SIM} plotted on the vertical axis. Although there was still a systematic overestimation of the true average radius, one can appreciate that the \( R_{\text{SIM}} \) data lie closer to the dashed lines than the VSI_{SIM}, indicating that \( R_{\text{SIM}} \) was in better agreement with \( \langle R \rangle_{\text{TRUTH}} \). The median errors in \( R_{\text{SIM}} \) for all cases were significantly less than the corresponding errors in VSI_{SIM} (**Table 6.1**).

### 6.3.4 The Ugly: Vessel density

**Figure 6.11a** shows voxel-wise scatter plots of \( N_{\text{SIM}} \) vs. \( N_{\text{TRUTH}} \). The single-voxel RC plots only include datasets for which 3\% < FBV_{\text{TRUTH}} < 5\% and 6 \( \mu \)m < \( \langle R \rangle_{\text{TRUTH}} \) < 18 \( \mu \)m. The multi-voxel RC and \( \mu \)CT tumor plots are for images with isotropic 250 \( \mu \)m voxel dimensions. Corresponding scatter plots of \( Q_{\text{SIM}} \) vs. \( N_{\text{TRUTH}} \) are shown in **Figure 6.11b**.

For the single-voxel RC data, both \( N_{\text{SIM}} \) and \( Q_{\text{SIM}} \) correlated well with \( N_{\text{TRUTH}} \), with \( r \) ranging from 0.95 at 0.02 ppm to 0.63 at 0.2 ppm for \( N_{\text{SIM}} \) and from 0.93 to 0.64 for \( Q_{\text{SIM}} \) (**Figure 6.12**). The correlations were less strong for the multi-voxel RC data, with \( r \) ranging from 0.70 to 0.22 for \( N_{\text{SIM}} \) and from 0.77 to 0.37 for \( Q_{\text{SIM}} \). While the vessel density correlations for the RC data generally decreased with increasing \( \Delta \chi \), the correlations increased asymptotically for the tumor data from 0.06 to 0.6 for \( N_{\text{SIM}} \) and from 0.58 to 0.8 for \( Q_{\text{SIM}} \) (isotropic voxels).

Voxel size had little impact on \( r \) for the RC data, but isotropic voxels resulted in significantly stronger correlations than anisotropic voxels for the \( \mu \)CT tumor data. There was no significant differences in \( N_{\text{SIM}} \) errors between isotropic and anisotropic voxels for either the multi-voxel RC or tumor data.
Figure 6.11 Voxel-wise scatter plots of (a) $N_{SIM}$ vs. $N_{TRUTH}$ and (b) $Q_{SIM}$ vs. $N_{TRUTH}$ for the single-voxel random cylinders (RC) (top row), multi-voxel RC (middle row), and μCT tumor vessels (bottom row) at various values of $\Delta \chi$, corresponding to different contrast agent doses. For multi-voxel RC and tumor vessels, the data from isotropic voxel (250 μm) images are shown. The dashed lines represent the line of equality ($y = x$).
Figure 6.12 Plots of voxel-wise Pearson correlation coefficients between (a) $Q_{SIM}$ and $N_{TRUTH}$ and (b) $N_{SIM}$ and $N_{TRUTH}$ as functions of $\Delta \chi$.

For the single-voxel RC data, the correlations between $N_{SIM}$ and $N_{TRUTH}$ were slightly stronger than the correlations between $Q_{SIM}$ and $N_{TRUTH}$. The opposite was true for multi-voxel RC; and for the tumor vessels, $Q_{SIM}$ correlated with $N_{TRUTH}$ substantially better than did $N_{SIM}$.

In general, $N_{SIM}$ underestimated $N_{TRUTH}$, especially at the lowest $\Delta \chi$ of 0.02 ppm. The underestimation was greatest for the tumor vessels and least for the single-voxel RC. Although the median $N_{SIM}$ error for single-voxel RC was positive at 0.15 ppm and 0.2 ppm (Table 6.1), $N_{SIM}$ underestimated $N_{TRUTH}$ when vessel density was low, even at higher values of $\Delta \chi$ (Figure 6.11a). This was likely caused by taking the cube of small values of $Q_{SIM}$, which initially increases linearly with $N_{TRUTH}$ (Figure 6.11b). The relationship became sublinear and $Q_{SIM}$ plateaued at higher values of $N_{TRUTH}$ when $\Delta \chi$ was large, similar to $FBV_{SIM}$. But, also like $FBV_{SIM}$, most of the tumor data lay in the linear portion of the $Q_{SIM}$ vs. $N_{TRUTH}$ curve (Figure 6.7c).
Table 6.1 Voxel-wise errors between simulated MRI parameters and their corresponding ground truth values.

<table>
<thead>
<tr>
<th>Δχ (ppm)</th>
<th><strong>Voxel-wise error (median±MAD)</strong></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single-voxel RC</td>
<td>Multi-voxel RC</td>
<td>μCT tumor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p√geometry</td>
<td>pvoxel</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Multi RC vs. tumor</td>
<td>Multi-voxel RC</td>
<td>μCT tumor</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>0.76±0.71</td>
<td>1.11±0.80 b</td>
<td>0.75±0.63</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.05</td>
<td>0.21±0.34</td>
<td>0.51±0.26</td>
<td>0.46±0.35</td>
<td>0.09</td>
</tr>
<tr>
<td>0.1</td>
<td>-0.05±0.39</td>
<td>0.41±0.21</td>
<td>0.39±0.28</td>
<td>0.52</td>
</tr>
<tr>
<td>0.15</td>
<td>-0.10±0.39</td>
<td>0.36±0.20</td>
<td>0.34±0.24</td>
<td>0.94</td>
</tr>
<tr>
<td>0.2</td>
<td>-0.22±0.43</td>
<td>0.31±0.19</td>
<td>0.29±0.22</td>
<td>0.90</td>
</tr>
<tr>
<td>0.02</td>
<td>216.62±79.00</td>
<td>42.99±25.69</td>
<td>40.68±16.62</td>
<td>0.51</td>
</tr>
<tr>
<td>0.05</td>
<td>114.54±45.66</td>
<td>11.46±5.80</td>
<td>15.87±5.89</td>
<td>0.17</td>
</tr>
<tr>
<td>0.1</td>
<td>87.27±33.09</td>
<td>11.15±4.92</td>
<td>18.28±6.72</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.15</td>
<td>73.92±29.47</td>
<td>12.33±5.02</td>
<td>18.02±6.49</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.2</td>
<td>54.20±24.17</td>
<td>13.08±5.63</td>
<td>18.11±6.97</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.02</td>
<td>10.48±4.21</td>
<td>3.00±1.38</td>
<td>1.01±1.82</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.05</td>
<td>8.63±3.67</td>
<td>0.06±1.04</td>
<td>0.24±1.61</td>
<td>0.18</td>
</tr>
<tr>
<td>0.1</td>
<td>10.30±3.70</td>
<td>1.54±1.30</td>
<td>2.62±1.79</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.15</td>
<td>10.73±4.11</td>
<td>3.15±1.65</td>
<td>4.41±1.98</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.2</td>
<td>9.93±4.06</td>
<td>4.40±2.10</td>
<td>5.64±2.33</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.02</td>
<td>-7.63±2.80</td>
<td>-30.61±12.11</td>
<td>-24.28±22.64</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.05</td>
<td>-3.07±1.60</td>
<td>-2.79±13.48</td>
<td>-19.73±19.73</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.1</td>
<td>-0.74±3.45</td>
<td>-3.44±15.49</td>
<td>-19.27±18.45</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.15</td>
<td>0.94±4.72</td>
<td>-8.43±14.77</td>
<td>-18.37±17.65</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0.2</td>
<td>1.10±4.59</td>
<td>-11.34±16.41</td>
<td>-17.13±17.13</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

a p√geometry and pvoxel are the p-values of two-tailed Mann-Whitney U tests of voxel-wise errors for different vessel geometries and for different voxel dimensions, respectively.

b Data for isotropic voxels are in regular font, and data for anisotropic voxels in italics.
6.4 Discussion

The theory and simulations from which the SSC-MRI parameters were derived assume a vascular system modeled by randomly oriented and randomly distributed cylinders (Kiselev and Posse, 1999; Yablonskiy and Haacke, 1994). Real blood vessels, especially aberrant tumor vessels, can significantly deviate from this simplified geometry. Thus, we developed a computational platform capable of performing MRI simulations that incorporate arbitrary vascular networks, ranging from simple cylinders in a single imaging voxel to real blood vessels of whole tumors acquired by ex vivo μCT angiography. We found that correlations between simulated MRI parameters and their corresponding ground truth values were generally strongest for the single-voxel RC and weakest for the de facto tumor vessels. This confirms that vascular morphology has a considerable effect on the calculation of SSC-MRI parameters.

The dependence of SSC-MRI biomarker measurements within a voxel on the surrounding vasculature outside the voxel can have significant consequences on biomarker accuracy and, to the best of our knowledge, had not been investigated. Previously, it was assumed that the vasculature is contained in a single, isolated imaging voxel, but we performed large-scale simulations to generate multi-voxel images in which each voxel is affected by its neighbors. This can adversely affect the accuracy of SSC-MRI parameters, as demonstrated by the weaker correlation between simulated and ground truth values for multi-voxel RC compared to single-voxel RC data. This “neighboring voxel effect” may be especially large if there is high spatial heterogeneity in vascular morphology (as is typical in tumors) and neighboring voxels have very different vascular characteristics. For example, voxels that contain no or very few vessels can still contain nonzero or larger-than-predicted magnetic field perturbations due to fields generated by vessels in adjacent voxels. This is why the simulated MRI parameter values for
some voxels devoid of blood vessels were nonzero, even extremely large in the case of $VSI_{\text{SIM}}$ because of the $\Delta R_2$ in the denominator (Figure 6.4h).

The neighboring voxel effect may also be the reason that correlation and accuracy depended on the voxel size. The data from each simulation run were used to generate two images, the only difference being the voxel dimensions. The isotropic and anisotropic voxels were equal in volume but the anisotropic voxels had 42% more surface area. This means that protons were on average closer to voxel boundaries and more likely to move between voxels during the course of the simulations. Thus, the anisotropic voxel dimensions allowed for more “mixing” between neighboring voxels, which may have reduced the correlations between simulated and ground truth SSC-MRI biomarker values. This voxel size effect was greater for the tumor vessel dataset than for the multi-voxel RC dataset, likely because of the greater spatial heterogeneity of the former. This difference in heterogeneity is also reflected in the ranges of ground truth values for the multi-voxel RC and tumor data. Both had similar global $V_f$ and radius distributions (Figure 6.3b,d), but the range of values of voxel-wise $FBV_{\text{TRUTH}}$, $VSI_{\text{TRUTH}}$, and $N_{\text{TRUTH}}$ were much greater for the tumor data. (These large differences in the ranges of parameter values between the three vascular geometry cases were the reason for computing statistics using only the data that fell within the common range for all three cases.)

Our data suggest that the neighboring voxel effects may be mitigated by employing an isotropic or lower image resolution. In addition, the assumptions of the Yablonskiy and Haacke model that the vessels (cylinders) in a voxel are sufficiently numerous as well as uniformly distributed and oriented are more likely to be valid for large voxels (Yablonskiy and Haacke, 1994). Increasing the voxel size will also increase the signal to noise ratio for in vivo experiments. But of course, the trade off is a decrease in spatial information, which is important for investigating
highly heterogeneous systems such as tumors. The larger spatial scale of clinical imaging may actually be advantageous for translating SSC-MRI to humans. For example, a voxel size large enough to ensure the validity of model assumptions (e.g. ~1 mm isotropic) would be considered low resolution for small animal imaging but high resolution for clinical imaging.

The importance of the criterion that a voxel must contain a large number of vessels is further illustrated by the single-voxel RC VSI\textsubscript{SIM} measurements (Figure 6.8a). For a given $V_f$, voxels with larger $\langle R \rangle_{\text{TRUTH}}$ had fewer cylinders and thus had greater variance in cylinder orientation and location. This may have led to the increase in the variance in VSI\textsubscript{SIM} with increasing $\langle R \rangle_{\text{TRUTH}}$. In previous simulation studies, e.g. by Boxerman et al., the voxel size was allowed to vary depending on $\langle R \rangle_{\text{TRUTH}}$ to accommodate a large number of large cylinders (Boxerman et al., 1995). Here, the voxel size was kept constant to more closely emulate realistic imaging situations. Thus, the condition that the number of cylinders in the voxel is large could not be met when $\langle R \rangle_{\text{TRUTH}}$ was large. Even though a set of voxels had the same $V_f$ and $\langle R \rangle_{\text{TRUTH}}$, the number and arrangement of cylinders varied greatly from voxel to voxel, resulting in high variance in the MR signal.

Another assumption of the underlying SSC-MRI model is that the $V_f$ is small and thus the intravascular signal can be neglected. Theory predicts a linear dependence of $\Delta R_2^*$ on $V_f$ when $V_f$ is low enough such that cylinders do not overlap. As $V_f$ increases, so too does the degree of overlap, which introduces nonlinear behavior (Yablonskiy and Haacke, 1994). Consistent with theory, we found that the linear relationship between FBV\textsubscript{SIM} and FBV\textsubscript{TRUTH} diminishes for large blood volumes for both the single-voxel RC and tumor vessel data (the maximum FBV\textsubscript{TRUTH} for the multi-voxel RC was only 5%) (Figure 6.5a). The impact of this nonlinear behavior was mitigated by the fact that most voxels in the tumor data were in the linear regime of FBV\textsubscript{TRUTH}. 

116
But even in the linear regime where $FBV_{\text{TRUTH}}$ was low, $FBV_{\text{SIM}}$ generally overestimated $FBV_{\text{TRUTH}}$. This bias in $FBV_{\text{MRI}}$ has been observed in previous simulations (Christen et al., 2012b) as well as studies comparing in vivo SSC-MRI to histology (Lemasson et al., 2013; Persigehl et al., 2013) and to high resolution ex vivo $\mu$CT angiography (Kim et al., 2013; Ungersma et al., 2010).

Similarly, $VSI_{\text{SIM}}$ systematically overestimated both $\langle R \rangle_{\text{TRUTH}}$ and $VSI_{\text{TRUTH}}$ in all cases. This bias was noted by Tropres et al. in their original paper on vessel size imaging and corroborated by several subsequent studies (Kim et al., 2013; Kiselev et al., 2005; Lemasson et al., 2013; Persigehl et al., 2013; Ungersma et al., 2010; Valable et al., 2008). Interestingly, the error in $VSI_{\text{SIM}}$ for the $\mu$CT tumor vessel data was very large in voxels with small $FBV_{\text{TRUTH}}$ (Figure 6.10c). This may be partially caused by the neighboring voxel effect as mentioned earlier and also by the fact that voxels with low blood volume tend to have a small number of vessels. Thus, there can be a tradeoff between the two model assumptions that the number of vessels is large and that fractional blood volume is small. And while the fractional blood volume is assumed to be small, there is also a lower bound defined in the criterion for the static dephasing regime (Eq. 23). Violation of this criterion may have contributed to the large $VSI_{\text{MRI}}$ error in voxels with small $FBV_{\text{TRUTH}}$. The extreme overestimation of VSI was only observed in the tumor vessel case where there were regions of sparse vascularization.

Tropres et al. also demonstrated using Monte Carlo simulations that the overestimation of $VSI_{\text{MRI}}$ decreases with increasing $\Delta\chi$ (Tropres et al., 2001). This is consistent with the results presented here – with the exception of $R_{\text{SIM}}$, the error in all simulated SSC-MRI parameters decreased as $\Delta\chi$ increased. This may be related to the static dephasing and slow diffusion approximations made in modeling the SSC-MRI gradient and spin echo signals, respectively, and in deriving the SSC-MRI vascular parameters (Kiselev and Posse, 1999; Yablonskiy and Haacke, 2000).
These approximations, however, are not inherent to the simulations. The static dephashing regime holds for large $\Delta \chi$ and large vessel radii, and thus the model is more accurate when these conditions are met. Although the errors of the simulated parameters decreased with increasing $\Delta \chi$, their correlations with the true values also tended to decrease. The reason for this trend is not clear but may be attributed to nonlinear effects created by increasingly overlapping magnetic fields as well as greater neighboring voxel effects at higher $\Delta \chi$.

In general, there appeared to be a tradeoff between greater linearity but less absolute accuracy at low contrast agent doses and less linearity but greater absolute accuracy at high doses. Thus, the utility of SSC-MRI may be maximized by using some optimal intermediate contrast agent dose, which can depend on factors such as contrast agent type, field strength, and the characteristics of the vascular system being investigated. It may be advantageous to use a lower contrast agent dose, which produces greater linearity and is more clinically applicable, and introduce empirical scaling factors or modifications to the established SSC-MRI vascular biomarkers to improve their quantitative accuracy. The computational platform developed here would be a powerful new tool for optimizing these vascular biomarkers.

As previously mentioned, $R_{\text{SIM}}$ was the one parameter for which the error did not decrease with increasing $\Delta \chi$. For the single-voxel RC data, $R_{\text{SIM}}$ was relatively independent of $\Delta \chi$. For the multi-voxel RC and tumor data, the error actually increased with $\Delta \chi$. Interestingly, $R_{\text{SIM}}$ was a significantly more accurate measure of the true average vessel radius in microns compared to $\text{VS}_{\text{SIM}}$. $R_{\text{SIM}}$ also correlated better with $\langle R \rangle_{\text{TRUE}}$. Similarly, Farrar et al. previously determined that $R_{\text{MRI}}$ was in better agreement with vessel radius measurements made in U87 brain tumors with intravital microscopy compared to $R_{\text{MRI}}^{3/2}$ (Farrar et al., 2010). We have also found using cross validation analysis that there was no significant difference between $R_{\text{MRI}}$ and and $\text{VS}_{\text{MRI}}$ in
their abilities to predict μCT-measured vessel radius in MDA-MB-231 breast tumor xenografts (Kim et al., 2013) (Chapter 4.3.4).

\( N_{\text{MRI}} \) is not an independent measure of vessel density, but an estimate of histologic vessel density that is a function of blood volume fraction and average vessel cross sectional area (Jensen and Chandra, 2000). The equation for \( N_{\text{MRI}} \) (Eq. 10) is derived from Eq. 9 by substituting FBV\(_{\text{MRI}}\) for \( f \) and VSI\(_{\text{MRI}}^2\) for \( \langle R_v^2 \rangle \). Thus, errors in FBV\(_{\text{MRI}}\) and VSI\(_{\text{MRI}}\) will propagate to \( N_{\text{MRI}} \). Because VSI\(_{\text{MRI}}\) significantly overestimates the true average vessel radius, it is expected that \( N_{\text{MRI}} \) will significantly underestimate the true vessel density. Indeed, previous studies have shown that \( N_{\text{MRI}} \) correlated well with but underestimated histologic vessel density (Lemasson et al., 2013; Ungerma et al., 2010; Wu et al., 2004), consistent with our finding that \( N_{\text{SIM}} \) systematically underestimated \( N_{\text{TRUTH}} \).

The results presented here suggest that many of the assumptions made by the underlying model of the SSC-MRI vascular biomarkers may not be valid for in vivo tumor studies. The tumor VSI\(_{\text{SIM}}\) measurements illustrate the adverse effects when these assumptions are violated. Interestingly, FBV\(_{\text{SIM}}\) showed to be fairly robust and much less sensitive to model assumptions. Some assumptions are also made by the FPM and Monte Carlo simulations. The first is that the vasculature is homogeneously magnetized due to a constant concentration of a strictly intravascular contrast agent (i.e., \( \Delta \chi \) is constant in time throughout the intravascular space). This assumption is likely valid for USPIOs within the timeframe of an SSC-MRI protocol (~10-20 min). For a dose of 1 mg Fe/kg and given a blood volume of 70 mL/kg, 1 mL of blood would contain \( 1.5 \times 10^{17} \) iron oxide nanoparticles. While they have been shown to extravasate from leaky tumor vessels, this process is slow, and the plasma half-life of ferumoxytol is 14.5 hours for a dose of 4 mg/kg in humans (Landry et al., 2005). Other assumptions of our method include no blood flow;
impermeable vessel walls; and uniform, isotropic water diffusion. Boxerman et al. showed that blood flow and vascular permeability had little effect on transverse relaxation rates (Boxerman et al., 1995). For simplicity, only uniform and isotropic diffusion was considered here; but any arbitrary anisotropic diffusion map, including in vivo ADC maps, can be used. The effect of nonuniform diffusion on SSC-MRI measurements may be significant, especially in tumors that may have both densely cellular and necrotic regions and in brains where white matter tracts create highly anisotropic diffusion patterns. The computational tools presented here are ideally suited for addressing these and other problems in the effort to develop clinically translatable MRI biomarkers of angiogenesis.

The salient findings of the numerical simulations are summarized below:

- Our data confirms that the performance of SSC-MRI vascular biomarkers is affected by vascular systems that deviate from model assumptions, as is the case for tumors.
- \( FBV_{\text{MRI}} \) is a more accurate measure of true blood volume than \( VSI_{\text{MRI}} \) is of average vessel radius; \( FBV_{\text{MRI}} \) is also more robust to deviations from model assumptions.
- There is evidence of a "neighboring voxel effect", whereby the performance of SSC-MRI vascular biomarkers depends not only on the morphology of vessels in the voxel of interest but in adjacent voxels as well.
- There is a tradeoff between the linearity and quantitative accuracy of SSC-MRI biomarkers, as a function of contrast agent concentration (i.e., \( \Delta \chi \)).
7 Conclusion

SSC-MRI is an in vivo imaging technique with the potential to provide clinical biomarkers of tumor angiogenesis. Our goal was to systematically assess the accuracy of these SSC-MRI vascular biomarkers, as well as their utility for monitoring early responses to anti-angiogenic therapy. We developed an ex vivo multimodal vascular imaging platform that integrated the unique advantages of μCT and μMRI to obtain co-registered, high-resolution vascular morphometric data, which was used to validate in vivo SSC-MRI data from an orthotopic MDA-MB-231 human breast cancer model. The μCT vascular data was also incorporated into a large-scale computational model of susceptibility contrast that we developed and used for in silico validation of SSC-MRI biomarkers.

The experimental and simulated data produced consistent results. Both indicated that FBV_{MRI} and VSIm_{MRI} systematically overestimate the true blood volume and vessel size; but FBV_{MRI} had a much smaller bias, which is in agreement with previous studies. We also found that FBV_{MRI} correlated more strongly with the ground truth fractional blood volume and exhibited greater robustness to various biophysical factors compared to VSIm_{MRI}. This suggests that FBV_{MRI} may be the more reliable biomarker of tumor angiogenesis.

Both experimental and computational results also showed that the relative biomarker R_{MRI} was actually a more accurate measure of average vessel radius than VSIm_{MRI} was. While the cross-validation analysis of the experimental data indicated that the absolute biomarker N_{MRI} was a better predictor of MVD_{μCT} compared to the relative biomarker Q, the Q-Q plot of Q vs. MVD_{μCT} exhibited a stronger linear correlation than that of N_{MRI} vs. MVD_{μCT}. Similarly, the voxel-wise correlation between Q_{SIM} and MVD_{TRUTH} was stronger than that between N_{SIM} and MVD_{TRUTH}. Given that the relative biomarkers are easier to compute, these results suggest that the relative
biomarkers \( R_{\text{MRI}} \) and \( Q \) may be more suitable angiogenic biomarkers than their absolute analogs, \( VSI_{\text{MRI}} \) and \( N_{\text{MRI}} \). Alternatively, the computational method developed here could be employed to help identify correction factors to improve the accuracy of the absolute biomarkers.

Numerical simulations indicated that the biases in the SSC-MRI biomarkers result from the deviation of \textit{in vivo} conditions from the theoretical assumptions made in deriving the biomarkers. Tumors, with their aberrant and highly heterogeneous vasculature, are especially liable to violate these assumptions. Due to the demonstrated biases in the SSC-MRI biomarkers, it is necessary to be mindful of pertinent biophysical factors at the time of their acquisition. Given the clinical limitations on field strength and contrast agent dose, these factors will be particularly important in the effort to translate SSC-MRI to the clinic. And given the high inter-tumor variability in vascular phenotypes, the robustness and generalizability of these biomarkers must be assessed with care.

Despite all this, anti-angiogenic treatment studies, including the one presented here, have demonstrated the ability of SSC-MRI to detect therapy-induced changes in tumor vascular morphology. We showed that SSC-MRI was sensitive to these early vascular changes before any changes in tumor volume or cellularity were detectable by more conventional methods. With the growing body of preclinical data, recent implementation of the USPIO ferumoxytol as an MR contrast agent in clinical feasibility trials, and ongoing development of other clinically relevant superparamagnetic blood pool contrast agents, SSC-MRI has the potential to fill the need for clinical biomarkers of angiogenesis. Ultimately, the goal would be to not only establish SSC-MRI biomarkers as indicators of anti-angiogenic treatment response but as \textit{predictors} of response as well. Such noninvasive and repeatable imaging biomarkers could increase the quality of patient care while helping to cut costs by identifying patients most likely to benefit from anti-angiogenic
treatment, aiding customization of treatment regimens, and enabling more responsive treatment evaluation.
8 References


Carmeliet P, 2005. VEGF as a key mediator of angiogenesis in cancer. *Oncology* 69 Suppl 3, 4-10.


MET and VEGFR2 inhibitor, simultaneously suppresses metastasis, angiogenesis, and tumor growth. Mol Cancer Ther 10, 2298-308.


## Curriculum Vita

### PERSONAL INFORMATION

<table>
<thead>
<tr>
<th>Name</th>
<th>Eugene Kim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date/location of birth</td>
<td>6 July 1986 / Montgomery County, PA</td>
</tr>
</tbody>
</table>

### EDUCATION

<table>
<thead>
<tr>
<th>Date/Time</th>
<th>Institution</th>
<th>Degree</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aug. 2008 – Feb. 2014</td>
<td>Johns Hopkins University School of Medicine</td>
<td>Ph.D.</td>
<td>Biomedical Engineering</td>
</tr>
<tr>
<td>Aug. 2004 – May 2008</td>
<td>Duke University, Pratt School of Engineering</td>
<td>B.S.E.</td>
<td>Biomedical Engineering Magna Cum Laude</td>
</tr>
<tr>
<td>Sep. 2006 – Dec. 2006</td>
<td>Queen Mary, University of London</td>
<td>Non-degree Associate</td>
<td></td>
</tr>
</tbody>
</table>

### RESEARCH EXPERIENCE

<table>
<thead>
<tr>
<th>Date/Time</th>
<th>Institution</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Conduct <em>in vivo</em> susceptibility contrast MRI experiments on mouse models of human breast cancer.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analyze the <em>in vivo</em> MRI data to generate vascular biomarker maps using AFNI.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Develop image processing strategies for segmentation and morphological quantification of 3D vasculature from micro-MRI and micro-CT images.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Use MATLAB to perform large-scale simulations of susceptibility contrast MRI that incorporate real vascular networks from micro-CT images.</td>
</tr>
</tbody>
</table>
| Feb. 2009 – June 2009 | Johns Hopkins University                          | Rotation student Advisor: Prof. Xingde Li, Biomedical Engineering
|                     |                                                   | Image tissue-mimicking, fluorescent, gelatin phantoms with a two-photon endoscope. |
|                     |                                                   | Analyze the depth dependence of fluorescence intensity in the phantoms using ImageJ. |
**Duke University**  
*Pratt Undergraduate Research Fellow*  
Advisor: Prof. Mark Oldham, Radiation Oncology  
- Use optical-CT to image vasculature and fluorescent reporter gene expression in unsectioned breast tumor xenografts.  
- Apply attenuation correction to improve quantitative accuracy of fluorescence optical-CT images.

May 2006 – Aug. 2006  
**Duke University**  
*3D Computer Graphics Modeler/Animator*  
Advisor: Rachael Brady, Computer Science  
- Model and animate virtual objects and environments in Maya for a psychology experiment.  
- Assist in the operation of the Duke Immersive Virtual Environment (DiVE), a six-sided virtual reality environment used for multidisciplinary research and education.

**TEACHING EXPERIENCE**

**Johns Hopkins University**  
*Teaching Assistant*  
EN.580.221: Molecules and Cells  
- Conduct weekly recitation sections and office hours.  
- Supervise grading of weekly homework assignments.  
- Hold review sessions for and grade exams.

**Johns Hopkins University**  
*Teaching Assistant*  
EN.580.111: BME Freshman Modeling and Design  
- Supervise lab sections.  
- Give lectures and prepare students for lab projects.  
- Grade and provide feedback on student lab reports.

**Duke University**  
*Teaching Assistant*  
BME 153L: Biomedical Electronic Measurements I  
- Supervise lab sections.  
- Support lab manager in lab preparation and upkeep.  
- Assist students with problems regarding lab experiments.
SELECT PUBLICATIONS


