Published in final edited form as:

NMR Biomed. 2020 October ; 33(10): e4363. doi:10.1002/nbm.4363.

Magnetic Resonance Angiography Reveals Increased Arterial Blood Supply and Tumorigenesis Following High Fat Feeding in a Mouse Model of Triple-negative Breast Cancer

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Abstract

Breast cancer is the second most commonly diagnosed malignancy among women globally. Past magnetic resonance imaging (MRI) studies have linked a high animal fat diet (HAFD) to increased mammary cancer risk in the SV40Tag mouse model of triple-negative breast cancer. Here, serial MRI examines tumor progression and measures the arterial blood volume feeding mammary glands in low fat diet (LFD) or HAFD fed mice.

Virgin female C3(1)SV40Tag mice (n=8), weaned at 3-weeks old, were assigned to a LFD (n=4, 3.7-kcal/g; 17.2% kcal from vegetable oil) or a HAFD (n=4, 5.3-kcal/g; 60% kcal from lard) group. From ages 8–12 weeks, weekly fast spin echo MR images and time-of-flight (TOF) MR angiography of inguinal mammary glands were acquired at 9.4T. Following *in vivo* MRI, mice were sacrificed. Inguinal mammary glands were excised and fixed for *ex vivo* MRI and histology. Tumor, blood and mammary gland volumes for each time point were measured from manually traced regions-of-interest; tumors were classified as invasive by histopathology-blinded observers.

Our analysis confirmed a strong correlation between total tumor volume and blood volume in the mammary gland. Tumor growth rates from week 8–12 were 2-fold higher in HAFD-fed mice (0.42 ± 0.14 week⁻¹) than in LFD-fed mice (0.21 ± 0.03 week⁻¹), $p < 0.004$. Mammary gland blood volume growth rate was 2.2-fold higher in HAFD mice (0.29 ± 0.11 week⁻¹) compared to LFD mice (0.13 ± 0.06 week⁻¹), $p < 0.02$. The mammary gland growth rate of HAFD-fed mice (0.071 ± 0.011 week⁻¹) was 2.7-fold larger than LFD-fed mice (0.026 ± 0.009 week⁻¹), $p < 0.01$.

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This is the first non-invasive, *in vivo* MRI study to demonstrate a strong correlation between a HAFD and increased cancer burden and blood volume in mammary cancer without using contrast agents, strengthening the evidence supporting the adverse effects of a HAFD on mammary cancer. These results support the potential future use of TOF angiography to evaluate vasculature of suspicious lesions.

Graphical abstract

This manuscript reports that high animal fat diets (HAFD) produce higher tumor incidence, tumor burden and tumor aggressiveness in a mouse model of human triple-negative breast cancer. Mammary gland blood volume and vascular densities increased, showing the first direct correlation between tumor burden and arterial blood volume. This study provides insight into the HAFD effects on breast cancer incidence and aggressiveness and to understand the changes in the blood volume/vessels associated with *in situ* cancers and progression to invasive stages.

Keywords

High animal fat diet (HAFD); Magnet resonance imaging (MRI); Mouse mammary cancers; MR Angiography; Triple-negative breast cancer (TNBC)

1 Introduction

Breast cancer is the leading cause of cancer-related morbidity among women globally (1). One woman is diagnosed with breast cancer every two minutes on average in the US, making it the second most commonly diagnosed malignancy in women in the US, following non-melanoma skin cancers (2). Breast cancer is a heterogeneous disease with a variety of subtypes. Triple-negative breast cancer (TNBC) is an aggressive subtype that does not respond to many common treatments, giving women with the disease a poor prognosis (3). In addition to surgery and radiation, current standard-of-care treatments for TNBC are mostly limited to nonspecific chemotherapy (3). One potential new treatment is the use of vascular endothelial growth factor (VEGF) inhibitors (4). VEGF is responsible for stimulating angiogenesis, the growth of new blood vessels, and may protect tumor cells from apoptosis, thus preventing the efficacy of chemotherapy and radiotherapy (4). VEGF-positive lesions are associated with significantly higher vascular density measured by CD31 levels (5). Increased vascular density is in turn associated with a lower relapse-free survival rate, so inhibiting VEGF could potentially reduce angiogenesis and make current treatments more effective (4).

Epidemiological studies have determined a link between high animal fat diets (HAFD) and the increased incidence of TNBC (6–10). Laboratory studies done on mouse models report that increased consumption of high fat diets increase tumorigenesis and metastasis and lead to an increase in tumor size and malignancy (11). One potential explanation for this relationship is that consumption of a HAFD can result in inflammation that promotes the

growth of mammary cancers (12). Additionally, a study of mouse models with colon cancer determined a link between a HAFD and increased tumor angiogenesis due to an increase in angiogenesis-promoting growth factors like VEGF and CD31 (13), but this linkage has not been investigated in breast cancer and no studies have utilized non-invasive imaging to track the progression of cancer and associated vasculature. This study is the first to non-invasively and serially track changes in *in situ* and invasive cancers and angiogenesis in a study of dietary manipulations with a HAFD.

Studying the progression of the disease *in vivo* is difficult in humans as all cancers including pre-cancers are removed when discovered. However, the simian virus 40 large T antigen (SV40Tag) transgenic mouse model has been frequently used as an accurate representation phenotypically and genotypically of human TNBC (14–16). *In situ* cancers seen in these mice model human ductal carcinoma *in situ* (DCIS). In addition, this model is ideal for studying effects of a high fat diet because the overall body weight of these mice does not increase with a HAFD. Thus, the potential negative metabolic effects of obesity are eliminated and the effects of the HAFD on tumor growth can be studied without that confounding variable (17).

While past studies have demonstrated the impact of a HAFD on tumor burden (6–10, 12), results from these previous studies do not explain factors like the timing of the onset of *in situ* cancer or the progression from *in situ* to invasive cancers. Most previous work focuses on ultrasound as an imaging method or uses only immunohistochemical analysis or gene expression profiling to test for changes in *ex-vivo* (6–8). However, the current study utilizes serial MR imaging of mouse models to identify pre-invasive and *in situ* cancers and to track the growth and progression of both *in situ* and invasive tumors in the mammary glands.

MRI has been proven to sensitively and specifically detect and differentiate DCIS and invasive cancers *in vivo* (18–21). A past study from our laboratory has also shown the efficacy of noninvasive time-of-flight (TOF) MR angiography for detecting neoangiogenesis and demonstrated increased arterial recruitment to cancerous mammary glands in the same mouse model used here (22). However, in that study, mice were imaged at one time point and fed with regular chow (22). Here we take advantage of these characteristics of MRI to serially observe the effect of a HAFD on tumor growth and incidence in mice with TNBC, beginning the imaging at an earlier time point than the previous study (22) in order to better observe the earlier stages of cancer growth. Additionally, we investigate the effects of the HAFD on the blood volume within the mammary gland and the overall blood volume in order to determine the effects of the HAFD on angiogenesis as a potential cause of tumor growth.

2 Methods

2.1 Ethical Statement

All procedures involving mice were carried out by a veterinary technician in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The University of Chicago Institutional Animal Care and Use Committee (IACUC) approved all animal work and all efforts were made to

minimize any suffering of the mice. The mice were humanely euthanized following the experiments for purposes of histology.

2.2 Animal and Diet

Ten virgin female FVB/N mice homozygous for the SV40 TAg transgene (originally provided as hemizygous TAg mice by Dr. Jeffrey E. Green of the National Cancer Institute's Mouse Models of Cancer Consortium) were weaned at 3 weeks of age. At 4 weeks of age, mice were separated and randomly assigned to either a control LFD group (n=5, 3.7 kcal/g; 17.2% kcal from vegetable oil) or a HAFD group (n=5, 5.3 kcal/g; 60% kcal from lard). These diets contained the ingredients listed in Table 1 and were purchased from Harlan Lab (Madison, WI). Cage food intake and individual body weight were monitored weekly. Mice were caged with their cohorts to avoid any effects of isolation stress on the mice. After mice were on these diets for 4 weeks, MR images were acquired from all 10 mice at 8 weeks of age. One mouse from each cohort was sacrificed for histology using an overdose of isoflurane followed by cervical dislocation after this first scan at 8 weeks. The remaining 8 mice were imaged weekly until 12 weeks of age. Mice were anesthetized prior to and throughout *in vivo* MR imaging with 1–2% isoflurane. Temperature (36–38 °C), heart rate (375–400 beats/min), and respiration rate (50–60 breaths/min) were monitored by SA instruments (Stony Brook, NY), and the respiration was used to gate imaging. Following *in vivo* MRI studies at 12 weeks of age, mice were sacrificed by an overdose of isoflurane and cervical dislocation. Mammary tissues were then excised so that both *in situ* and invasive cancers detected by MRI could be correlated with histology.

2.3 In Vivo MRI Experiments

A 9.4 Tesla small animal scanner (Bruker, Billerica, MA) with 11.6 cm inner diameter and actively shielded gradient coils (maximum constant gradient strength for all axes: 230 mT/m) was used to acquire MR images. The mouse was placed supine on an animal holder and inserted into a 30-mm-diameter quadrature volume coil (Rapid MR International, Columbus, OH). Multi-slice RARE (Rapid Acquisition with Relaxed Enhancement) T₂-weighted images with fat suppression were acquired with the following parameters: TR/TE_{effective}=4000/20 ms, field-of-view (FOV)=25.6 mm × 25.6 mm, matrix size=256², slice thickness=0.5 mm, RARE factor=4, number of averages (NEX)=2, in-plane resolution=100 microns. Sixty-two slices were acquired by interleaving two sets of images of the inguinal glands (left and right) to cover the slice gaps of 1 mm. For time-of-flight (TOF) angiography, a flow compensated, T1-weighted sequence with a short TR and thin slices was used to maximize inflow effects and depict flowing blood as a bright signal. Parameters for TOF were: TR/TE_{effective}=10/3 ms, flip angle = 60°, FOV = 25.6 mm × 25.6 mm, matrix size = 256 × 256, slice thickness = 0.5 mm, 47 slices, with average of 4. In-plane resolution for TOF was 100 microns as in T2-weighted images. The acquisition time for each slice was 10.2 s and the total acquisition time for this TOF sequence was 8 minutes.

2.4 Preparation of Mammary Glands for Ex Vivo MRI and Histology

Following *in vivo* MRI studies at 12 weeks of age, the inguinal mammary glands were excised from the body while still connected to the skin. The mammary glands were placed in 10% formalin for tissue fixation for two weeks and then removed from formalin and rinsed

daily with phosphate buffered saline (PBS, Fisher Scientific, Waltham, MA) for 3 days. The right inguinal gland from each mouse was chosen for *ex vivo* MRI and histology. The selected gland was then excised from the skin and placed between two layers of pathology foam, mimicking the exact position of the *ex vivo* mammary gland on the skin, and the exact position of the mammary gland in hematoxylin and eosin (H&E) stained slices, as previously described (21). Since the *ex vivo* gland was scanned by MRI in the same orientation as the H&E-stained tissue, the *ex vivo* MR images served as a 'bridge' between *in vivo* MRI and histology to facilitate accurate correlation.

2.5 Ex Vivo MRI Experiments

The *ex vivo* 3D images were obtained on the 9.4 T scanner with the RARE T₂-weighted sequence with fat suppression and with TR/TE_{effective}=4000/25 ms, FOV=30 mm × 25 mm × 5 mm, matrix size=384 × 320 × 64, isotropic resolution=78 microns, and NEX=4. The wrapped, single mammary gland prepared as described above was placed in a homebuilt 8-leg, low-pass, half-open birdcage coil (length=3 cm, width=3 cm, height=2 cm).

2.6 Histology

Following *ex vivo* MRI studies, an intact right inguinal gland from each mouse was placed in a histology cassette and paraffin embedded. Typical tissue dimensions from which about 20 H&E slices were sectioned were 15 mm in length, 10 mm in width, and about 1.5 mm in thickness. These histological slices were then examined by a breast pathologist (J.M.) and classified as normal gland, *in situ*, or invasive cancer; the glands were further examined for any abnormalities in the ductal structure or blood vessels and for the presence of apoptotic figures.

2.7 Data Analysis

MRI data were then processed and analyzed quantitatively using software written in IDL (Exelis VIS, Inc., Boulder, CO). Three observers who were blinded to histopathology assessments identified mammary cancers as *in situ* or invasive based on their sizes and signal intensities on T₂-weighted MR images. A cancer was identified as *in situ* if it was 150 to 400 microns in diameter and had a signal intensity of 1.4 times that of the muscle, or invasive if it was more than 400 microns in diameter and had a signal intensity of 2.3 times that of muscle (21). High resolution *ex vivo* MR images were used to aid the correlation of *in vivo* images with histology to confirm the accuracy of the identification. Using IDL, regions-of-interest (ROI's) for individual invasive cancers were drawn on every MR slice in which the invasive cancer was detected. Each invasive cancer was given a numerical label and the volumes from each invasive cancer's ROI's in all slices were combined and reported in mm³. *In situ* cancers were also individually counted and then combined as a total number of *in situ* cancers for that mouse for each of the five time points.

An integrated software program, Amira (FEI Visualization Sciences Group, Burlington, MA), was used for volume rendering and 3D visualization of *in vivo* images, including TOF images demonstrating the blood volume. Amira was used to create labels of ROIs of the mammary gland and the whole abdomen in 32 slices of each set of TOF images. The lymph nodes viewed on the T2-weighted images were used as markers to consistently identify the

center slices and the ROI's were drawn on the corresponding TOF images. ROI's were also drawn on 2 slices of the muscle to be used to normalize the signal intensity and reduce extraneous signal when calculating the blood volume within each ROI. The muscle was chosen for this normalization process because the muscle does not exhibit change due to the change in diet. The labels of the ROI's were then entered into a software program, Matlab (Natick, MA), in order to calculate the blood volume in each region of interest. Using the ROI's from the muscle, a constant threshold for the signal intensity was set at 2.3 times that of the muscle. The blood volumes in the mammary glands and the total blood volumes were calculated for each mouse for each week from 8 to 12 weeks. The same was done to calculate the overall mammary gland volume. ROIs were drawn around the mammary glands using AMIRA and the volumes were calculated through Matlab.

When conducting statistical analysis of individual tumor growth, individual (tumor) sample sizes of tumors of $n=6$ for LFD-fed mice and $n=16$ for HAFD-fed mice were treated as independent samples, since they were distinct, clearly separated cancers. For the mammary gland volume and the mammary gland blood volume, the volumes in the right and left mammary glands were calculated individually and used as independent samples, making the sample size $n=8$ for both cohorts. The specific growth rates (ρ) were calculated by fitting the mammary gland volume, mammary gland blood volumes, and tumor volumes as a function of time ($V(t)$), according to equation 1.

$$V_{fit}(t) = V_0 \exp(\rho(t - t_0)) \quad (1)$$

In equation 1, t (in weeks) is the age of the mouse and V_0 is the respective volume detected at initial time t_0 (in weeks). Mann Whitney U-tests were performed for statistical analysis and a p -value <0.05 was considered significant.

3 Results

3.1 High animal fat diet alters mammary ductal structures

In vivo axial T2-weighted MR images allow analysis of the ductal structure in the mammary glands. Changes in this structure can be observed with increasing distinction from 8 to 12 weeks of age, 4–8 weeks on dietary intervention. The ductal structures of the HAFD mice became increasingly thick and abnormal compared to the clear outline of the ducts in the LFD mice. Figure 1 demonstrates the changes of the ductal structures from week 8 to week 12 *in vivo* as well as at week 12 *ex vivo*. In Figure 1, for *in vivo* images of inguinal glands all mice fed with LFD ($n=4$) and HAFD ($n=4$) are shown, while for *ex vivo* images of inguinal glands two representative images from a LFD-fed mouse and from a HAFD-fed mouse are shown. Higher spatial resolution 3D volume-rendered, *ex vivo* MR images further confirm these changes to the mammary ductal structure. Additionally, these images show an increase in branching points of mammary ducts in HAFD mice compared to LFD mice. Figure 2 identifies another example of dilated ducts in a HAFD-fed mouse, indicated using yellow arrows, as well as showing the increase in arterial blood volume in the mammary gland of HAFD-fed mice (Figure 2c). Based on these changes in the mammary ductal

structures, it can be inferred that the HAFD is associated with more abnormal and more active ductal development.

3.2 Increase in mammary gland volume is observed in mice on a high animal fat diet

Prior to imaging at each week, the body weight of each mouse was recorded. At 12 weeks of age, mice fed a HAFD for 8 weeks (average body weight= 19.75 ± 2.02 g) did not gain significantly more weight than LFD-fed mice (average body weight= 18.20 ± 1.04 g), $p=0.075$. More significant than changes in body weight, though, was the increase in mammary gland volumes from week 8 to week 12. In addition to showing the increased blood volumes in the mammary glands, the 3D volume rendering models of one HAFD-fed mouse and one LFD-fed mouse at 8 and 12 weeks in Figure 3 demonstrate the increase in mammary gland volume. The average initial volume of the mammary glands at 8 weeks was larger in the HAFD than the LFD mice, with the average of the HAFD-fed mice being 1.4-fold larger than that of the LFD-fed mice at 8 weeks.

3.3 Mammary gland blood volume increases in mice fed a high animal fat diet

TOF images tracked the blood volume in each mouse from week 8 to 12. Analysis of the blood volume within the mammary glands shows an increased blood volume in mice fed the HAFD. Figure 2 depicts the TOF image superimposed on the T2-W image, qualitatively demonstrating the increased blood volume in the mammary glands of the mice fed the HAFD. The increase in blood volume in the mammary gland can also be seen through 3D volume rendering models of the TOF images, as depicted in Figure 3. These 3D models show a significant increase in blood volume and blood vessel branching points in the HAFD-fed mouse at week 12. The blood volumes for each mouse were also plotted as a function of age (Figure 4). At week 12 the average blood volume in mammary glands for HAFD-fed mice (2.56 ± 0.27 μ L) was 7-fold higher compared to LFD-fed mice (0.36 ± 0.10 μ L), $p<0.0001$. The total blood volume of the entire mouse increased more in the HAFD as well, but this increase was not statistically significant.

3.4 Tumor burden and incidence are greater in high animal fat diet-fed mice than in low fat diet-fed mice

The tumor volumes of invasive cancers were measured and recorded for each week as stated in the “Methods and Materials” section. At week 8, only 1 mouse on the LFD had an invasive cancer, whereas 3 mice in the HAFD group already had invasive cancers. As shown in Figure 5, all of the HAFD mice experienced invasive cancer by week 9, but still only 3 of the LFD mice developed invasive cancer by week 12. At week 12 the largest individual invasive cancers recorded were 0.6 mm³ in the HAFD group and only 0.2 mm³ in the LFD group and the largest total invasive cancer volumes – the sum of the individual invasive cancer volumes for both glands in each mouse – were 1.02 mm³ in the HAFD group and only 0.2 mm³ in the LFD group. The average total invasive cancer volumes were 0.55 mm³ in the HAFD group and only 0.09 mm³ in the LFD group. A total of $n=16$ invasive cancers were identified in the HAFD group and $n=6$ in the LFD group (Figure 5a). These results show that the HAFD produces not only more invasive cancers, but also larger invasive cancers than the LFD group.

The *in situ* cancers were also counted in each week. Analysis of these data shows that the growth rates of the number of *in situ* cancers in the LFD and the HAFD mice were similar from week 8 to week 12. Still, at week 8 the total *in situ* cancer count in the HAFD mice was 1.8 times greater than that in the LFD mice. While the growth of *in situ* cancers was similar from week 8 to week 12, the HAFD mice exhibited a much larger initial amount of *in situ* cancer at week 8.

3.5 Growth rates of mammary gland volume, mammary gland blood volume, and invasive cancers are greater in high animal fat diet-fed mice than low fat diet-fed mice

The growth rate of the mammary gland of the HAFD-fed mice ($0.071 \pm 0.011 \text{ week}^{-1}$) was 2.7-fold larger than that of the LFD-fed mice ($0.026 \pm 0.009 \text{ week}^{-1}$), $p < 0.01$. From week 8 to week 12 the growth rate of the blood volume within the mammary gland was 2.2-fold greater in the HAFD group ($0.29 \pm 0.11 \text{ week}^{-1}$) compared to the LFD group ($0.13 \pm 0.06 \text{ week}^{-1}$), $p < 0.02$ (Figure 4). The growth rate of the invasive cancer in the HAFD group ($0.42 \pm 0.14 \text{ week}^{-1}$) was 2-fold larger than that of the LFD group ($0.21 \pm 0.03 \text{ week}^{-1}$), $p < 0.004$, as shown in the graphs of the tumor volumes in Figure 5b.

3.6 Histological correlations with MR findings

Figure 6 compares histological H&E-stained images of the excised right inguinal mammary gland of LFD and HAFD fed mice at 12 weeks of age. Each image shown is a central slice containing the lymph node of the mammary gland. An experienced breast pathologist (J.M.) found several major differences between the LFD and HAFD SV40Tag mouse mammary glands. First, mammary glands from LFD mice showed highly visible brown fat containing numerous mitochondria, while HAFD mice showed primarily mature, white fat, distinguished by a single lipid droplet in the adipocyte, seen in the insets of Figures 6a and 6b. Second, mammary glands from HAFD mice showed more *in situ* and invasive cancers compared to the LFD group. Third, HAFD mice showed increased tumor invasiveness compared to LFD mice. Fourth, more dilated blood vessels were seen in the gland of HAFD mice compared to LFD group.

In week 8, H&E images of LFD-fed mouse ($n=1$) gland show a higher brown fat and a lower white fat compared to HAFD-fed mouse mammary glands ($n=1$). However, the development of abnormal ducts or dilated blood vessels in mice fed with a LFD or a HAFD at week 8 is not statistically significant.

4 Discussion

Past MRI studies from this laboratory have demonstrated both an increase in the recruitment of arteries to cancerous mammary glands in SV40Tag transgenic mice with TNBC (22) and an increase in invasive mammary cancers due to a high fat diet in this mouse model (12). Using serial MRI, this study confirms that increase and observes the pattern of the increase over 5 weeks, beginning the scans at an earlier time point to observe any changes in the mammary glands throughout the development of the cancer. Additionally, this study shows the corresponding increase in mammary gland volume, as well as blood volume in the mammary glands, using TOF MRI. The tumor growth rate of HAFD-fed mice was found to

be 2-fold greater than that of LFD-fed mice and the mammary gland blood volume growth rate of HAFD-fed mice was 2.2-fold greater than that of LFD-fed mice. This similar magnitude of growth rates of HAFD- versus LFD-fed mice for tumor and blood volumes suggests a direct correlation between total invasive cancer volume and blood volume in the mammary gland.

While there has been some evidence in the past that a HAFD causes angiogenesis (23), this is the first study to quantitatively measure the increase in arterial blood volume and analyze the trend in the growth (13). Our data clearly demonstrate that the HAFD-fed mice exhibit a significantly greater increase in mammary gland arterial blood volume than the LFD-fed mice. The overall abdominal blood volume increased slightly in the HAFD as well, but this increase was not significant and was likely due to the large increase in blood volume concentrated in the mammary gland. Although the images depicted increased blood volume in fairly large arteries using angiography, it is very likely that increases in arterial blood flow supplying the areas where cancers formed are associated with an increase in the density of smaller blood vessels, especially capillaries. Future studies must be done to determine whether this increase in blood volume due to angiogenesis is caused directly by the HAFD or indirectly because the fats promote tumor growth and tumors promote angiogenesis (24). Previous work suggested a direct connection between angiogenesis and HAFD based on a study of colon cancer that found increased levels of growth factors like VEGF and CD31 in the tumor tissues of HAFD-fed mice; these growth factors activate transcription factors that promote angiogenesis (13). Still, our finding that the overall abdominal blood volume did not significantly change and that the HAFD only significantly increased blood volume in the mammary glands suggests that the increased angiogenesis may be the result of the tumor growth or the change in fat composition and not the other way around. Further exploring this connection between diet and breast cancer could aid in the improvement of current treatments aimed at blocking angiogenesis to inhibit cancer growth.

In addition to the increase in mammary gland blood volume, the average mammary gland volume increased in the HAFD-fed mice, likely due to expansion of mammary gland adipose tissue mass. The average mammary gland volume of the HAFD mice was 1.6-fold larger than that of the LFD mice at 12 weeks. The average mammary gland volume of the HAFD mice at 8 weeks was 1.4 times greater than that of the LFD, suggesting a change in the mammary gland volumes even prior to 8 weeks. These mice are resistant to obesity and did not gain significantly more weight overall, so the increase in volume was specific to the mammary glands rather than the abdominal volume of the mice. This gland-specific increase suggests that the HAFD directly affected the composition and volume of fat in the mammary gland. In addition to promoting growth factors that promote angiogenesis, a HAFD has been linked to increased expression of growth factors that cause inflammation and proliferation of cells and block apoptosis (13). These growth factors may have contributed to the overall growth in volume of the mammary glands, as well as any tumor growth.

Correlations of MR and histological (H&E-stained) images were based on *in situ* and invasive cancers, ductal morphology and blood vessels – identified from the red blood cells, endothelial cells and often thick wall. The mammary glands of HAFD-fed mice exhibited irregular and enlarged ducts, dilated blood vessels, and more mature white adipose tissue,

while the mammary glands of LFD-fed mice exhibited fewer/smaller blood vessels, and more abundant brown adipose tissue (12, 25). The connection between high fat diet/obesity and breast cancer has been attributed in part to adipose tissue dysfunction (26). The increase in blood volume and vascular density in the mammary glands of mice fed a HAFD as determined by MRI in this study has been corroborated with histological images using H&E staining. A detailed analysis of co-registrations of MR images with images from histology and immunohistochemistry will be published elsewhere.

The results of this study indicate that the HAFD promotes the growth of individual tumor volumes, which supports a recent study from our lab that showed that a HAFD promotes aggressive cancer growth and incidence (12), but that the increase in tumor incidence from week 8 to 12 is more pronounced for larger invasive cancers than *in situ* cancers. Still, while the growth of the number of *in situ* cancers is similar in LFD and HAFD mice, the initial amount of *in situ* cancers at week 8 was 1.8-fold higher in HAFD mice than LFD mice. This difference suggests quicker or earlier growth of *in situ* cancers in the first few weeks of the diets. We began scanning the mice at 8 weeks of age and concluded the scans at 12 weeks. Twelve weeks of age is the average age at which *in situ* cancers become invasive (27), but the mice on the HAFD in this study all developed invasive cancers by 9 weeks of age, suggesting earlier invasive cancer growth and possibly earlier *in situ* cancer development as well, resulting in the greater number of *in situ* cancers seen at week 8 for the HAFD group. A future study will include MRI scans of the mice at earlier time points to provide more insight into the effects of a HAFD on *in situ* cancer growth and on the transition from *in situ* to invasive cancers.

While the results of this study provide concrete evidence that supports the effects of a HAFD on angiogenesis and tumor growth, the number of mice in each cohort that were scanned for all weeks was small at only n=4 for each group. Future studies will include a larger sample size to increase the statistical power of the results. In addition, it will be important to begin serial scanning earlier and scan for a longer time period to improve sampling of the natural history of *in situ* and invasive cancer.

Future studies should evaluate effects of specific types of fats, such as effects of omega-3 versus omega-6, and other dietary choices such as high fructose to determine why the HAFD increases mammary gland and cancer growth and to determine the effects of other dietary manipulations to improve dietary recommendations. Additionally, they should further explore the correlation between the abnormality of the mammary ducts seen in our images of HAFD mice and the increased tumor growth and blood volumes. A better understanding of the changes that occur in the mammary gland as aggressive cancers form could open translational opportunities for the use of MRI in earlier detection of cancer risk and in dietary recommendations for patients.

5 Conclusion

The results demonstrate that a high animal fat diet produces higher tumor incidence, tumor burden and tumor aggressiveness in a mouse model of TNBC. The MR images also demonstrate a change in the mammary ductal structure and an increase in mammary gland

volume due to the HAFD. The blood volume and vascular densities within the mammary gland increase significantly in the HAFD mice. This is the first direct correlation between the tumor burden and arterial blood volume in the mammary gland in mice fed with a HAFD. This study provides insight into the effects of a HAFD on breast cancer incidence and aggressiveness and a foundation for further work using MRI to understand the changes in the blood volume and blood vessels associated with *in situ* cancers and cancer progression to the invasive stage.

Acknowledgments

This research is supported by grants from the National Institutes of Health (R01-CA167785, R01-CA218700, U01-CA142565, and P20-CA233307), Department of Radiology at the University of Chicago, Florsheim Foundation, Segal Foundation, and VPH prism grant from the European Union. The Lynn S. Florsheim Magnetic Resonance Laboratory subcore of the Integrated Small Animal Imaging Research Resource is partially supported by funds from the University of Chicago Comprehensive Cancer Center from the National Cancer Institute Cancer Center Support Grant P30CA014599.

Abbreviations

FOV	field of view
HAFD	high animal fat diet
H&E	hematoxylin and eosin
LFD	low fat diet
NEX	number of excitation
RARE	rapid acquisition with relaxation enhancement
ROI	region-of-interest
SV40Tag mouse	C3(1)SV40 the simian virus 40 large T antigen (Tag) transgenic female mouse
TOF	time-of-flight
TNBC	triple-negative breast cancer

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Figure 1. *In vivo* and *ex vivo* T2-W MR images of the right inguinal mammary gland of the SV40Tag mice at 8 and 12 weeks of age.

Panel **a** shows *in vivo* T2-W images of all 4 LFD-fed (top) and HAFD-fed (bottom) mice at 8 weeks (left) and 12 weeks (right). The *ex vivo* MR images of the excised mammary glands of one LFD-fed (left) and one HAFD-fed (right) mouse at week 12 in panel **b** show more active and abnormal ductal development in the HAFD-fed mouse. Scale bars of 1 mm are shown.

Figure 2. *In vivo* T2-W and TOF MR images of the inguinal mammary gland of SV40Tag mice at 12 weeks of age.

Images of LFD-fed (a) and HAFD-fed (b) mice are shown: left, T2-W images; middle, TOF images; right, TOF image overlaid on T2-W image – blood vessels in red. Dilated ducts in HAFD mouse are indicated by yellow arrows on T2-W image. The magnified images of the right gland in panel c highlight the dilated ducts, indicated by yellow arrows, and increased arterial blood supply, shown in red, in HAFD mouse compared to LFD mouse. Scale bars of 3 mm are shown.

Figure 3. Three dimensional volume-rendered images show mouse mammary glands and vessel densities of SV40TAg mice.

Images of a LFD-fed mouse at week 8 (top) and week 12 (bottom) are shown in panel **a**, while panel **b** shows the corresponding images for a HAFD-fed mouse, as labeled. Only the inguinal mammary glands from a T_2 -weighted RARE sequence are shown in blue-to-green color. Blood vessels constructed from TOF datasets are shown in red. By week 12, blood vessels in the inguinal glands of the HAFD-fed mouse grew significantly as invasive cancers developed. Mammary gland volumes are also shown to be larger at both time points for the HAFD-fed mouse compared to the LFD-fed mouse. Lymph nodes (LN) are labeled in all images.

Figure 4. Plots of blood volumes as a function of age in LFD- and HAFD-fed SV40Tag mice. (a) The blood volumes in inguinal mammary glands are shown for SV40Tag mice from 8 to 12 weeks of age fed with LFD (n=4) or HAFD (n=4). From week 8 to week 12 the growth rate of the blood volume within the mammary gland was 2.2-fold greater in the HAFD group ($0.29 \pm 0.11 \text{ week}^{-1}$) compared to the LFD group ($0.13 \pm 0.06 \text{ week}^{-1}$), $p < 0.02$. (b) The total blood volumes in the entire abdomen, including in the inguinal mammary glands, are shown for the same mice fed with LFD (n=4) or HAFD (n=4) as in a. Blood volumes were measured from *in vivo* time-of-flight MR angiography.

Figure 5. Plots of initial and total tumor volumes as a function of age in LFD- and HAFD-fed SV40Tag mice.

Only invasive cancers, not *in situ* cancers, were used to calculate tumor volumes. Initial tumor volume (a) and total tumor volume (b) in inguinal glands were measured from *in vivo* MRI in SV40Tag mice from 8 to 12 weeks of age fed with LFD (n=4) or HAFD (n=4).

Each dot in panel (a) represents a new initial invasive tumor volume. The growth rate of the invasive cancer in the HAFD group ($0.42 \pm 0.14 \text{ week}^{-1}$) was 2-fold larger than that of the LFD group ($0.21 \pm 0.03 \text{ week}^{-1}$), $p < 0.004$.

Figure 6. Histological images of mouse mammary glands of low fat and high animal fat-fed SV40TAg mice.

Hematoxylin and eosin (H&E)-stained images of the excised right inguinal glands of LFD (a) and HAFD (b) SV40TAg mice at 12 weeks of age are compared. In each image a central slice of the mammary gland containing the lymph node is shown here. Insets of panels **6a** (*in situ* cancer) and **6b** (invasive cancer) – areas near the lymph nodes corresponding to black boxes as indicated – show H&E-stained images with higher magnification. Blood vessels in both images are indicated by red arrows and a higher number of dilated blood vessels are

seen in HAFD mouse mammary gland. Highly visible brown fat predominates in the LFD mouse mammary gland. In contrast, the HAFD mouse mammary gland shows primarily mature fat. In the inset of **6b**, a number of blood vessels are seen surrounding an invasive cancer, also indicated by red arrows. Scale bars of 4 mm and 0.2 mm for normal and magnified images, respectively, are also shown.

Table 1:

Key ingredients in low fat and high animal fat diets.

	Low Fat	High Animal Fat
Formula	Diet Components (g/Kg)	
Casein	200	200
L-Cystine	3	3
Corn Starch	397.5	117.4
Maltodextrin	132	132
Sucrose	100	100
Soybean oil	70	70
Lard	--	280
Cellulose	50	50
Mineral Mix (AIN-93G-MX; 94046)	35	35
Vitamin Mix (AIN-93VX; 94047)	10	10
Choline Bitartrate	2.5	2.5
TBHQ (antioxidant)	0.014	0.014