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THE EFFECTS OF A HIGH-FAT DIET ON MAMMARY GLAND BIOLOGY IN A MODEL OF TRIPLE-NEGATIVE BREAST CANCER

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List of Abbreviations

AT	adipose tissue
BC	Breast cancer
CM	conditioned media
DMBA	7,12-dimethylbenz[a]anthracene
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix proteins
FOV	field of view
H&E	hematoxylin and eosin
HAFD	High animal fat diet
HF	High fat
HFrD	High fructose diet
L	length
LFD	low fat diet
LPA	lysophosphatidic acid
m	moderately differentiated
MG	Mammary gland
MAT	mammary adipose tissue
MMPs	matrix metalloproteinase
MRI	Magnetic resonance imaging
NEX	number of excitation
n_t	number of tumors
p	poorly differentiated
p/s	penicillin/streptomycin
RARE	rapid acquisition with relaxation enhancement
ROI	region-of-interest
SV40 TAg mouse	C3(1)SV40 the simian virus 40 large T antigen (Tag) transgenic female mouse
TAM	Tumor-associated macrophage
TNBC	Triple-negative breast cancer
µl	microliter
W	width
w	well differentiated

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Abstract

Breast cancer is still the leading cause of death for women in the US. Triple-negative breast cancer (TNBC), which has been correlated with obesity in both pre and post-menopausal women, makes up only 10-20% of breast cancer cases but offers a poorer prognosis and lack of response to treatment. Diets high in fat plays a large role in the increase in obesity we therefore hypothesize that diets high in fat play a role in tumor development and progression due to changes in mammary gland microenvironment. More specifically, we hypothesize that changes in mammary adipose tissue (MAT) result in different more pro-tumorigenic secreted factors being released from the MAT and negatively affect the adjacent epithelial cells. HF feeding resulted in minimal changes in body weight but did increase fasting blood glucose as expected, pointing to the mice having become insulin resistant. MRI images of the inguinal mammary glands of mice fed HFD, additionally showed denser parenchyma compared to control-fed counterparts. H&E staining of the inguinal mammary glands demonstrated much higher adipocyte content and more active glands with HFD. Increases in secreted factors due to HFD, such as pentraxin 3, IL-6, and LPA, which have all been associated with poorer prognosis in TNBC, make the mammary microenvironment more invasive as which saw with the increase in poorly differentiated tumors with HF feeding at 20-21 weeks. These data suggest that the changes to the mammary adipose tissue due to high animal fat consumption not only leads to a more carcinogenic environment but also results in poorly-differentiated and higher grade tumors. This suggests that diets low in animal fat may be an effective treatment for TNBC but that inhibiting proteins pentraxin 3, like may also be an effective treatment for TNBC.

Chapter 1

Diet, Breast cancer, adipose tissue and how they are intertwined

1. A. Starving to supersized: the changes to the human diet over the last century

In the last 20 years we have seen a worldwide move toward diets containing an increase in calorie intake high in fat and 'empty' carbohydrates (Popkin and Gordon-Larsen 2004). Humans have evolutionarily been programmed to be hunter gathers but now have unlimited access to whatever food and food portions they desire. This has occurred over a shorter period of time then evolution could keep up with, so the human body is still programmed to store excess calories for times of low nutritional availability. The fruition of agriculture, animal husbandry as well as the industrial revolution occurred too quickly on the evolutionary scale so biology has not had enough time to adjust (Cordain et al. 2005). Our bodies are genetically not programed to consume our current diet. Diet was changed from the constant consumption of wild plants and some animal meat when it was available, to having an array of food that was previously unknown to pre- agricultural man.

Today in the US, 72.1% of the total calories consumed come from food that our body is not programmed to handle. Foods as normal to us as bagels are processed foods that evolutionarily our bodies do not understand. Americans consume about sixty percent more added sugars, which includes refined cane, beet sugar, corn sweeteners, edible syrups, honey and high fructose corn syrup and 40% more fat than recommended in their daily diets (Putnam, Allshouse, and Kantor 2002). Before the introduction of the domestication of animals, our bodies consumed lean meat unlike what is consumed today, which is rich in saturated fat. With the ability to "fatten up" animals, we are no longer eating mostly polyunsaturated fatty acids and

monounsaturated fatty acids like our ancestors had (Cordain et al. 2005; Simopoulos 2001). We are now consuming more grains and sugars than our bodies are genetically programmed to handle, as well as consuming fat in amounts that were not even possible before the 19th century.

The increase in grain consumption and therefore carbohydrates, combined with more saturated fat in our diets is just part of the problem. Humans now consume portions of food rich in sugars and fat that would have been unheard of and just unavailable before modern times. Studies investigating the calorie consumption of Americans show that about 25% more calories than are necessary for normal homeostasis are consumed. This is not only a problem in the US, it has become a global problem with obesity rates rising all over the world, even amongst lower income families (Popkin and Gordon-Larsen 2004). The worldwide increase in calorie consumption combined with the decrease in exercise are just two of the biggest factors that have lead to what is now known to be obesity epidemic (Simopoulos 2001; Cafaro, Primack, and Zimdahl 2006).

1. B. Obesity epidemic, adipose tissue and all its complexities

The obesity epidemic has become a huge concern all over the world. A person is classified as obese whose body mass index (BMI) is above 30. Overweight is classified as having a BMI between 25 and 29.9. Obesity is a complex disease that impairs peoples health and is not just a cosmetic problem as it was has previously been regarded (Kopelman 2000). Not only does obesity affect a person's health but it is also a major concern because of the increased mortality that is associated with it. Obesity was shown to decrease life expectancy by 7 years by the age of 40. More than 1.1 billon adults are overweight and 312 million of them are obese, with an estimated 155 million children being classified as either overweight or obese (Pataky, Bobbioni-

Harsch, and Golay 2010; Haslam and James 2005).

Obesity, where there is an excess of adipose tissue accumulation, is accompanied with many adverse health effects and overall profound change to the body's physiological state. In general, obesity causes alterations to total blood volume, cardiac function and even respiration depending on where the adipose tissue accumulates, but these are just some of the changes caused as a result of obesity (Kopelman 2000). The biggest changes that occur happen within adipose tissue, which then affect other peripheral tissues starting a cascade of problems.

Adipose tissue evolved to allow the body to store of energy as lipids for maintenance of whole body homeostasis when energy is needed. In times of starvation or low overall glucose levels, the stored triglycerides in adipose tissue undergo lipolysis and fatty acids are secreted to return the body to its homeostatic state. Though this was thought to be the only function of adipose tissue for a very long time, the discovery of leptin changed that thinking forever. The role of adipose tissue as a vital endocrine organ whose function is not only to regulate of energy balance but to also regulate other physiological processes like food intake, were brought to light (Trayhurn and Beattie 2001; Sethi and Vidal-Puig 2007).

Under normal healthy conditions adipose tissue functions to store excess energy and help keep energy homeostasis through its endocrine signaling and excess calorie storage. The continuous overloading of calories to a system that did not evolve to function in a state of excess nutrition causes the normally placid adipose tissue to become dysfunctional causing an array of problems. Over nutrition and therefore excess adipose tissue alone can lead to peripheral insulin resistance and metabolic disruption. Larger adipocytes, which have undergone hypertrophy, are actually less insulin sensitive than smaller adipocytes (Estrany et al. 2013; Lundgren et al. 2007).

The expansion of adipose tissue is a complex process which with an overload of caloric intake is forced to try and adapt to what they are not genetically programed to cope with.

The expansion of adipose tissue, due to excess caloric storage involves the development of not only new adipocytes but also the expansion of mature adipocytes already present (Sethi and Vidal-Puig 2007). All of the hyperplasia and hypertrophy that occur during AT remodeling effect adipose tissue ability to properly sustain its normal endocrine function. Nutritional overload triggers changes to adipokine secretion and signaling, leading to its eventual dysfunction (Ahima 2011; Sethi and Vidal-Puig 2007; Nieman et al. 2013). This nutritional overload which eventually leads to obesity, not only causes insulin resistance and changes to adipokine secretion as mentioned above but also results in chronic inflammation (van Kruijsdijk, van der Wall, and Visseren 2009). This chronic inflammation is thought to be important for the development of diseases associated with obesity, one of which is breast cancer (Pataky, Bobbioni-Harsch, and Golay 2010; Carmichael 2006).

1. C. Breast cancer & triple negative breast cancer, not just one type

Breast cancer (BC) is still one of the most common cancers diagnosed and remains the second leading cause of death amongst women (Siegel, Naishadham, and Jemal 2013; Lacey, Devesa, and Brinton 2002; Boyle 2012; Koboldt et al. 2012). Breast cancer is a heterogeneous disease that is clinically divided into different subtypes based on its expression profile. The four subtypes, luminal A, luminal B, basal-like (triple-negative) and HER-2 enriched, are classified by their expression levels of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (*HER2)* (Haque et al. 2012). Women with a body mass index (BMI) above 25 are at higher risk of developing all types of breast cancer including the triple

negative sub-type (TNBC). Basal-like/triple-negative, group is of most interest to us because of the demographic of women it affects and the lack of treatment options available (Koboldt et al. 2012).

Triple negative breast cancer (TNBC) is a subtype of BC that accounts for only 10-20% of all breast cancer cases, but accounts for a greater proportion of breast cancer because of its deaths has a high mortality rate. TNBC is molecularly, genetically and phenotypically different from other types of breast cancer (e.g. ER+). Patients diagnosed with TNBC are at a higher risk of early relapse compared to other subtypes (Criscitiello et al. 2012). It is a much more aggressive and difficult to treat subtype of cancer due to the lack of molecular drug targets (Rakha and Ellis 2009; Podo et al. 2010). TNBC has become a health disparity because of its association with women who are premenopausal, obese, of African descent and it is usually diagnosed at a more advanced stage (Criscitiello et al. 2012; Boyle 2012; Hall et al. 2000; Lacey, Devesa, and Brinton 2002).

With the lack of available pharmacological treatment to treat TNBC, any incite into what is increasing its risk and progression or what interventions could help patient outcome is extremely important. Over the last few years, there has been some evidence that diet may be playing a role in increasing TNBC risk. Long-term studies in women have shown that diets high in fat may play a role in TNBC. The WINS and the WHELS trials showed that woman who had ER-negative and ER/PR negative tumors were more responsive to dietary restrictions of fat, as well as exercise (Chlebowski and Blackburn 2015; Pierce 2009; Swisher et al. 2015). Additionally, it was shown that there was a significant decrease in association of ER-negative, PR-negative, and ER2/PR2 negative tumors with a prudent dietary pattern (whole grains,

vegetables, fruit, and fish) (Chlebowski et al. 2006). The association between TNBC incidence, diet, and obesity has been largely understudied and why we became so interested in further understanding the interplay between adipose tissue, diet and TNBC.

1. D. Mammary gland development, fat and breast cancer

The normal endocrine function of adipose tissue is not only essential for maintenance of whole body homeostasis but it is also very important for processes like breast and mammary gland development (Celis et al. 2005). Mammary adipose tissue is an active endocrine tissue whose direct crosstalk with mammary epithelial cells is central to the growth and morphogenesis of the mammary gland (Hovey, McFadden, and Akers 1999). Without white adipose tissue, mammary gland development is impaired. Studies with transgenic mice lacking white adipose tissue showed that inhibition of the normal interaction between epithelial cells and adipose tissue prevented normal mammary gland development (Couldrey et al. 2002). Additionally, studies showing improper signaling from the adipose tissue surrounding the mammary ducts also disrupts ductal development, once again bringing to the forefront the importance of the crosstalk between breast epithelial cells and adipose tissue (Celis et al. 2005).

The proximity of mammary epithelial cells to adipose tissue in the mammary gland has lead us and others to consider the role mammary adipose tissue plays in the development, incidence and progression of TNBC. Mammary epithelial cells are the cells invade proliferate and forms tumors but it's instructions and permissive signals to do so come from the tissue surrounding it. These facts once again point to the importance of mammary adipose tissue in the both the development of the mammary gland and of breast cancer (Wiseman and Werb 2002).

Normal mammary adipose tissue secrete adipokines and cytokines that promote

angiogenesis and invasion of the mammary epithelial cells into the mammary fat pad for the expansion of the mammary ductal tree during development. Obesity, which changes the normal physiology and function of adipose tissue, makes adipose tissue dysfunctional with improper signaling and feedback occurring. Dysfunctional adipose tissue results in chronic inflammation, insulin resistance, and abnormal release of adipokines, many of which have been linked to cancer. Interestingly, mammary gland development and breast cancer actually share many similarities. Both require the invasion, reinitiation of cell proliferation, angiogenesis and inhibition of apoptosis in order to occur (Wiseman and Werb 2002).

In normal mammary gland development, for branching to occur the distal epithelial cells of the ductal tree must break through the basement membrane in order to extend. Extracellular matrix (ECM) proteins like laminin-1 and other matrix metalloproteinase (MMPs), which cleave the ECM in the mammary gland, must function properly in order for extension of the ductal tree to occur. Some of these mechanisms have also been shown to be important for BC migration again showing the parallel between the two systems. MMP mediate cleavage of laminin-5 causing the release of bioactive laminin fragments shown to induce BC migration (Wiseman and Werb 2002). Obesity causes dysfunctional secretion of pro-inflammatory markers such as IL-6 and TNFα, which cause the induction of proteins like MMPs, allowing for the growth and survival of tumor cells. In breast cancer, all these mechanisms are turned back on through improper signaling and without proper regulation or feedback causing tumorigenesis to occur and progress.

The importance of the mammary gland microenvironment was further proven when metastatic breast cancer cells was mixed with adult mammary epithelial cells and transplanted

into epithelium- free fat pads of nude mice. All of the cells, including the human metastatic cells, proliferated and contributed to normal mammary gland development, again signifying the importance of the microenvironment and its role in cancerogenesis (Bussard and Smith 2012). In addition to once again showing the importance of the signaling between the mammary gland epithetical cells and adipose tissue, this also shows that correcting of improper signaling in the MG microenvironment could help with treatment and maybe even prevention of BC.

The reverse has also been shown, studies using media conditioned with adipocyte-secreted factors show the promotion of proliferation, tumorigenesis and induction of anti-apoptosis signaling in breast cancer cell lines (Iyengar et al. 2003; D'Esposito et al. 2012). Co-culture experiments with MCF-7 (ER+) or MDA-MB-231 (TNBC) breast cancer cell lines and either the murine 3T3-L1 adipocyte cell line or primary mammary human adipose tissue, showed that adipocytes cause more proliferation and anti-apoptotic signaling in breast epithelial cells. Additionally, adipocyte conditioned media from obese patients showed an even greater change in proliferation than conditioned media from lean patients (D'Esposito et al. 2012). All of the studies mentioned once again point to the importance of the mammary gland microenvironment and how changes to the mammary adipose tissue, more specifically its secretome, may determine the fate of epithelial cells within the MG. The precise alterations to the mammary adipose tissue secreted proteins and other molecules (e.g. specific lipid species) resulting from increased adiposity remain poorly understood and need further investigation.

1. E. Summary

The already established significance of the crosstalk in normal mammary gland development between the mammary fat and epithelial cells is what led others and us to further

interrogate the relationship between these two tissues. Additionally, the increase in BC risk associated with obesity, which disrupts normal adipose tissue function, is another indicator of importance of the role adipose tissue in plays disease progression when it is dysfunctional. The studies in the chapters that follow allowed us to further investigate the mechanisms underlying the increased risk of developing TNBC. We hypothesize that the changes to the mammary adipose tissue caused by the increased consumption of foods high in fat may cause and worsen TNBC prognosis. Understanding how a diet high in fat changes the mammary gland microenvironment may lead to the development of better therapies and preventions for triple negative breast cancer.

Chapter II

Dietary Fat Results in Increased Tumor Burden in a Mouse Model of Human Triple-Negative Breast Cancer Based on Magnetic Resonance Imaging and Histology

2. A. INTRODUCTION

Breast cancer is the most commonly diagnosed malignancy among women in the United States and other Western countries; it remains the second leading cause of cancer mortality worldwide (DeSantis et al. 2011; Hortobagyi et al. 2005). Epidemiological studies suggest an increase in the risk of triple-negative breast cancer (TNBC) (Yang et al. 2011; Agurs-Collins et al. 2010) in association with a high animal fat diet (Yang et al. 2011; Hauner et al. 2011; Kavanaugh and Green 2003; Sundaram et al. 2013; Sundaram et al. 2014). Although there is a statistically significant association between obesity and cancer risk, there is little clinical information about how diet composition influences female breast development, breast fat physiology, and corresponding cancer subtype risk independent of weight gain. Long term dietary intervention studies with recently diagnosed breast cancer survivors, show better prognosis and survival with reductions in fat intake in women with hormone receptor negative breast cancer (Chlebowski et al. 2006; Pierce 2009). But there is also little information about how the high animal fat diet influences the progression of pre-neoplastic and *in situ* cancers in the breast. Progression of pre-invasive neoplasms to invasive cancers is difficult to study in patients as these early lesions are typically resected after diagnosis. As a result, use of a high animal fat diet in animal models can provide important early insights into diet-induced changes in mammary gland biology and allowing for better insight of TNBC progression and a better analysis of possible preventative treatment where few are available.

The simian virus 40 large T antigen (SV40 Tag) transgenic FVB/N mouse line is a well-established model of human TNBC due to its similarity in cancer progression and tumor gene expression changes (Green et al. 2000; Maroulakou et al. 1994; Herschkowitz et al. 2007). Previous studies have shown that the consumption of a high fat diet does not significantly increase body weight in this model, but does shorten mammary tumor latency (Cleary, Grande, and Maihle 2004). High fat feeding also increased mammary cancer growth rate in these mice, as qualitatively estimated by palpation (Sundaram et al. 2013; Sundaram et al. 2014), but these measurements were made on larger, more mature tumors. In this study, the development of alterations to mammary ductal structures as well as formation of pre-palpable TNBC tumors were therefore studied by magnetic resonance imaging (MRI) in the SV40 Tag mouse model.

Due to their inability to provide precise soft tissue imaging, computed tomography and ultrasound have not been adequate to monitor neoplastic changes during early stage mammary cancer progression in mice (Cowen et al. 2015; Wirtzfeld et al. 2015). On the other hand, magnetic resonance imaging (MRI) provides excellent soft tissue contrast, can detect early cancer *in vivo* much more reliably than other imaging modalities, allows for the evaluation of the surrounding parenchyma, and allows accurate tumor volume measurements over time. Previous work from our laboratory demonstrated that *in vivo* T_2-weighted MRI reliably detects very early mammary tumors in SV40-TAg mice with high sensitivity and specificity, differentiating *in situ* from invasive cancer (Hipp et al. 2012; Jansen et al. 2009; Jansen et al. 2008; Mustafi et al. 2015). Precise correlations between *in vivo* MR with histology images have also been demonstrated (Mustafi et al. 2015). Furthermore, serial MR imaging directly demonstrated great heterogeneity in initiation time, growth rate, and time at which *in situ* cancers became invasive

(Fan et al. 2014).

The goal of the present study was to determine whether dietary fat altered the incidence of mammary cancer from SV40 Tag mice fed a HAFD from weaning. Using *in vivo* MRI and *ex vivo* MRI with excised glands along with histology, we found both an increase in early tumor incidence and tumor burden in SV40 TAg mice fed a HAFD compared to those fed a LFD.

2. B. MATERIALS AND METHODS

Animal care: This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal work was approved by the University of Chicago Institutional Animal Care and Use Committee (IACUC). *In vivo* imaging studies of mice were performed under isoflurane anesthesia. All efforts were made to minimize any suffering and mice were humanely euthanized following the experiments.

Table 2-1: Selected nutrient information.

Diet	Diet name	Components	% by weight	% kcal
Low Fat	AIN-93G Purified Diet	Protein	17.7	18.8
		Carbohydrates	60.1	63.9
		Fat	7.2	17.2
High Animal Fat	High Fat (60% kcals) Diet	Protein	17.7	13.4
		Carbohydrates	34.9	26.5
		Fat	35.2	60.1

Animal and Diet: Sixteen 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 as previously described (Volden et al. 2013). At 4 weeks of age, mice were separated and randomly assigned to either a control low fat diet group (n=8, 3.7 kcal/g; 17.2% kcal from vegetable oil) or a high animal fat diet group (n=8, 5.3 kcal/g; 60% kcal from lard). These diets were purchased from Harlan Lab (Madison, WI). Four female mice were housed together per diet group; cage food intake and individual body weight were monitored weekly. Table 2-1 and Table 2-2 provide a list of nutrients and ingredients, respectively, for both control and high animal fat diets. After allowing these diets for 8 weeks, MR images were acquired on all 16 mice at 12 weeks of age. 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. Mice were anesthetized prior to *in vivo* MR imaging, and anesthesia was maintained during imaging with 1-2% isoflurane. Temperature, heart rate, and respiration rate were monitored by SA instruments (Stony Brook, NY), and the respiration was used to gate imaging.

Table 2-2: 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
Blue Food Coloring	--	0.1

Preparation of mammary glands for *ex vivo* MRI and histology: After *in vivo* MRI studies, the mammary glands, while still connected to the skin, were excised from the mouse body and placed in 10% formalin for tissue fixation for two weeks. Prior to *ex vivo* imaging the skin was 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. The gland was washed twice with fomblin (Solvay Solexis, West Deptford, NJ) and then saturated with fresh fomblin just prior to imaging. Plastic wrap was folded around the sample, and the sample was placed in the MRI coil for imaging. Fomblin is an oily, fluorinated polymer with no MRI signal, used to keep the tissue moist while imaging, and minimize susceptibility gradients. After *ex vivo* MRI, the sample was removed from the wrap, washed thoroughly in PBS, and put in a histology cassette in ethanol without any distortion of the tissue for immediate histological processing and H&E staining of slices. Since the *ex vivo* gland was scanned by MRI in the same orientation as the hematoxylin and eosin-stained tissue, the *ex vivo* MR images served as a 'bridge' between *in vivo* MRI and histology to facilitate accurate correlation.

In vivo MRI imaging: MR images were acquired on a 9.4 Tesla small animal scanner (Bruker, Billerica, MA) with 11.6 cm inner diameter, actively shielded gradient coils (maximum constant gradient strength for all axes: 230 mT/m). The mouse was placed supine on an animal holder and inserted into a 30 mm diameter quadrature mouse volume coil (Rapid MR International, Columbus, OH). Multi-slice RARE (Rapid Acquisition with Relaxation Enhancement) T_2-weighted images with fat suppression were acquired with the following parameters: $TR/TE_{effective}$=4000/20 ms, field-of-view (FOV) = 25.6 mm x 25.6 mm, matrix size=256^2, slice thickness=0.5 mm, RARE factor=4, number of averages (NEX) = 2. In-plane resolution was 100 microns. For the inguinal glands (left and right) two interleaved sets of images were acquired to cover the slice gaps of 1 mm and then combined together for a total of 62 slices.

Ex vivo MRI imaging: *Ex vivo* MR images were acquired on the same 9.4T scanner. 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). *Ex vivo* 3D images

were acquired with the RARE T_2-weighted sequence with fat suppression and with $TR/TE_{effective}$=4000/25 ms, FOV=30 mm x 25 mm x 5 mm, matrix size=384 x 320 x 64, isotropic resolution=78 microns, and NEX=4.

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 were 15 mm in length, 10 mm in width, and about 1.5 mm in thickness. During pathological sectioning, tissue was removed until a full face of tissue was found. Then one 5 microns thick slice was sectioned for H&E, the next 70 microns were discarded, the next 5 microns thick slice was sectioned for histology, and the next 70 microns were discarded, and so on until the tissue was exhausted. This meant that about 20 H&E slices were needed to cover a 1.5 mm thick slice of tissue. Histological slides were then evaluated by an experienced breast pathologist (J.M.) and tissue was classified as normal gland, *in situ*, or invasive cancer. H&E slides were then scanned using a fully automated Leica microscope (DM-6000B, Leica Microsystems, Weltzar, Germany) for visualization and stored as images in TIFF format.

Data analysis: MRI data were processed and analyzed quantitatively using software written in IDL (Exelis VIS, Inc., Boulder, CO). High resolution *ex vivo* MR images were used to facilitate correlation of *in vivo* images with histology as demonstrated previously (Mustafi et al. 2015). Two observers confirmed identification, Devkumar Mustafi with 10 years of experience in MRI and Erica Markiewicz with 10 years of experience in histology. The person who classified the cancers as *in situ* or invasive cancer based on MRI was blinded to histopathology assessments. An integrated software program, Amira (FEI Visualization Sciences Group, Burlington, MA), was used for volume rendering and 3D visualization of *in vivo* images. This program rotated the

in vivo MR images into the same orientation as *ex vivo* MR and histology images for precise MRI-histopathology correlations. This allowed correlation of all lesions identified by MRI in the entire gland with histology. Based on the sizes of *in situ* (150 to 400 microns in largest diameter) and invasive cancers (>400 microns in largest diameter) and their signal intensities on T_2-weighted MR images of 1.4 times that of muscle and of 2.3 times that of muscle, *in situ* cancers and invasive cancers, respectively, were accurately identified on *in vivo* MRI (Mustafi et al. 2015). In the work presented here, *all* invasive cancers identified on *in vivo* MRI in the entire inguinal gland were correlated with the corresponding cancers identified on histology to allow unbiased estimates of sensitivity and specificity. Using IDL, regions-of-interest (ROI's) of individual invasive tumors were drawn on every MR slice the tumor existed in. Each tumor was given a numerical label and the volume from each tumor's ROI's combined was calculated as mm^3. *In situ* cancers were also counted using the IDL software. Because *in situ* cancer is difficult to count throughout the entire mouse gland, 4 slices were chosen per gland; 2 on the right side of the mouse and 2 on the left side. The first and second slices chosen were located 3 slices prior to the last slice of the left and right lymph node, respectively. The third and fourth slices that were chosen were located 3 slices after the last slice of the left and right lymph node were seen, respectively. Individual ductal carcinoma *in situ* (DCIS) per chosen slice were individually counted and then combined as a total for that mouse. Student's t-Tests were performed for statistical analysis. A p-value <0.05 was considered significant.

2. C. RESULTS

Mammary ductal structures are altered following high fat feeding during puberty: Following weaning, SV40 TAg female mice were fed either a LFD or a HAFD for 8 weeks.

Mice given a HAFD, did not gain significantly more weight than the LFD fed mice (average body weight of 18.20±1.04 g on LFD, compared to an average body weight of 19.75±2.02 g on HAFD, $p<0.075$) (Figure 2-1). The FVB/N mouse strain was chosen for these studies since the females are resistant to high fat diet-induced obesity (Cleary, Grande, and Maihle 2004). This allows for the direct assessment of the high animal fat diet on the mammary gland, while avoiding the confounding factors of systemic hyperinsulinemia/hyperleptinemia arising from whole body weight gain and metabolic dysfunction. Analysis of axial T_2-weighted central MR images of mammary glands of mice fed LFD (Fig. 2-2a) versus HAFD (Fig. 2-2b) showed few areas of DCIS and relatively darker mammary glands in the LFD mice, indicative of denser parenchyma and shorter cancer latency in HAFD fed mice. HAFD mice show thicker and irregular ductal structure (Fig. 2-2b) suggestive of more active mammary ducts as well as of abnormal ductal development.

Figure 2-1:

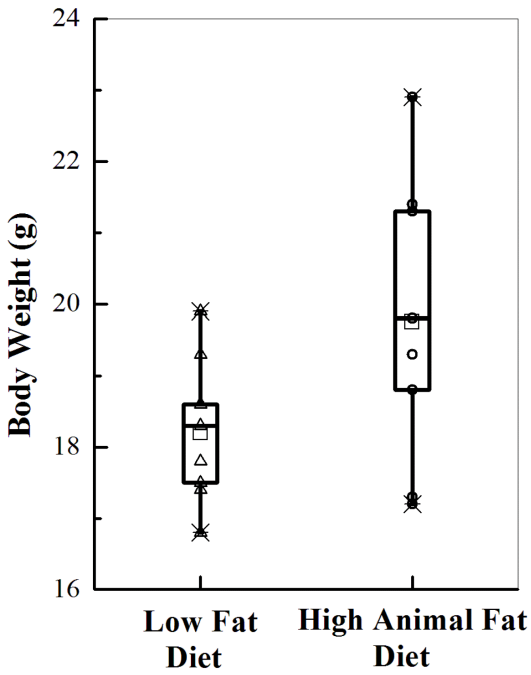

Figure 2-1. **Box plot of body weights for low fat-fed and high animal fat-fed SV40 TAg mice at 12 weeks of age.** An average body weight of 18.20±1.04 g per mouse (n=8) was found in the low fat diet group, compared to an average body weight of 19.75±2.02 g per mouse (n=8) in the high animal fat diet group; this difference was not statistically significant ($p<0.075$).

Figure 2-2:

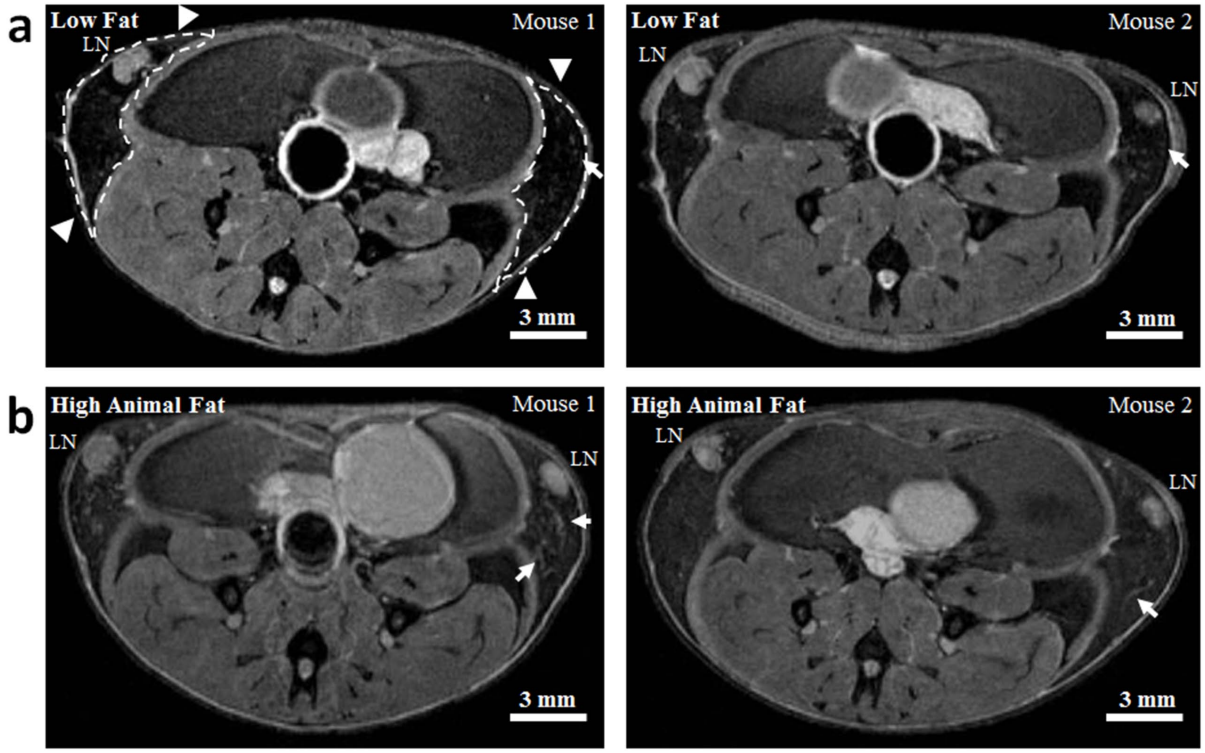

Figure 2-2. ***In vivo*** **MR images of low fat and high animal fat-fed SV40 Tag mice**. The top **(1a)** panel shows two central slices of mouse inguinal glands from two separate LFD mice at 12 weeks of age. Inguinal glands are indicated by white dashed lines with arrowheads in the left image; LN – lymph node. The bottom panel **(1b)** shows the corresponding slices from two separate HAFD mice at 12 weeks of age. Images are shown to illustrate that high animal fat-fed mice have denser parenchyma, and thicker and irregular ducts as indicated by white arrows, compared to LFD mice. Scale bars in all 2D T_2-weighted images are shown.

HAFD mammary fat MRI images cannot be fat-suppressed: Higher spatial resolution (isotropic resolution of 78 microns), 3D volume rendered, *ex vivo* MR images of LFD and HAFD fed SV40-TAg mouse mammary glands at 12 weeks of age (Fig. 2-3), further illustrate the dynamic diet-induced changes to the mammary glands of these mice. Figures 2a and 2b show *ex vivo* MR images with lymph nodes and invasive cancers indicated and labeled. Although the fat-suppressed fast spin echo sequence was used in each case (fig. 2-3), residual fat is still visible in HAFD fed mouse glands (fig 2-3b) due to the incomplete suppression of mammary fat, indicating multiple fat chemical shifts. Figures 2-3c and 2-3d show colored MR images of the mammary gland, comparing small areas as indicated by boxes in Figures 2-3a and 2-3b. A central slice containing the lymph node is seen in each image. The larger blue area in Figure 2-3d is due to denser parenchyma of the mice fed HAFD and was produced with the same intensity criterion (thresholding) as seen by the identically intense lymph nodes in 2-3c and 2-3d. The colored images in Figures 2-3c and 2-3d show much thicker and irregular ductal architecture in HAFD mouse mammary gland compared to LFD mouse mammary gland. Additionally, more branching points of mammary ducts are seen in the HAFD mice suggesting altered ductal tree development as indicated in Figure 2-3d.

Figure 2-3:

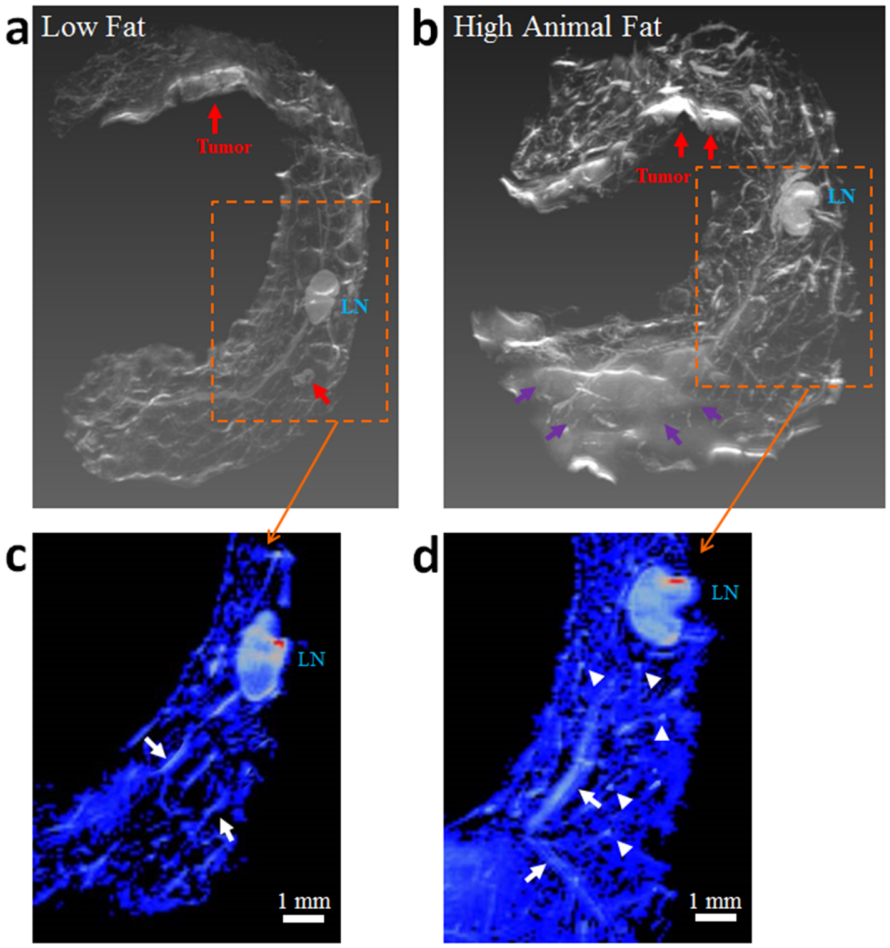

Figure 2-3. ***Ex vivo*** **MR images of mouse mammary glands of low fat and high animal fat-fed SV40 TAg mice**. 3D volume rendered *ex vivo* MR images of the excised right inguinal glands of LFD and HAFD SV40 TAg mice at 12 weeks of age are compared. In both images (**2a** and **2b**) invasive cancers are labeled and indicated by red arrows in both sets of images. LN-lymph node. In **2b**, purple arrows indicate the area that is only seen in HAFD mouse gland. This area was due to residual fat that failed to suppress fat completely although the fat-suppressed T_2-weighted protocol was used. *Ex vivo* MR images of LFD and HAFD mice are compared in **2c** and **2d** in the respective areas, indicated by boxes with dashed lines in **2a** and **2b** – in each image a central slice containing LN is shown here. Color images in **2c** and **2d** were produced with the same signal intensity of lymph nodes – images are showing to illustrate that HAFD mouse in **2d** has denser parenchyma as indicated by blue color throughout the mammary gland compared to that in **2c** for the LFD mouse. As indicated by white arrow in **2d**, thicker and irregular ducts are seen in a HAFD mouse mammary gland compared to those in a LFD mouse, also indicated by white arrows in **2c**. Also illustrated are more branching points as intense spots throughout the gland in the mammary gland of a HAFD mouse as indicated by white arrowheads in **2d**. A scale bar of 1 mm in both images of **2c** and **2d** is shown.

Increased tumor size in mice fed a high fat diet: Because of the very large number of *in situ* cancers throughout the entire mouse gland, 4 slices were chosen per gland in each diet group as described in the 'Materials and Methods' section. 90 *in situ* cancers were found in 4 slices of both glands of the LFD group (n=8 mice) and 130 were found in the HAFD group (n=8 mice). Figure 2-4, a plot comparing the incidence of invasive cancers as a function of tumor volume in LFD and HAFD SV40 TAg mice show the distinct differences between the effects of both diets. Tumors found in the entire inguinal glands of both 12 week old LFD and HAFD groups (n=8 mice per group), were classified as either *in situ* or invasive based on the size and the relative signal intensity with respect to muscle and confirmed by histology, as previously described (Mustafi et al. 2015). The number of tumors with volumes larger than >0.16 mm^3 in LFD and HAFD was significantly different ($p<0.007$). Only one tumor was found in the LFD group with a larger tumor volume of 0.31-0.49 mm^3 compared to seven tumors in the HAFD group. No tumors were detected at the largest tumor volume of 0.50-2.59 mm^3 in the LFD fed group. Tumor incidences of larger tumor volumes were statistically significant as indicated by asterisks in Figure 2-4. The number of tumors in mice with LFD with a tumor volume of 0.08-0.15 mm^3 was not significantly different compared to the number of tumors in the same subgroup as the HAFD group. The volume of the largest tumor in the LFD group was 0.32 mm^3, while the volume of the largest tumor in the HAFD group was 2.59 mm^3.

Figure 2-4:

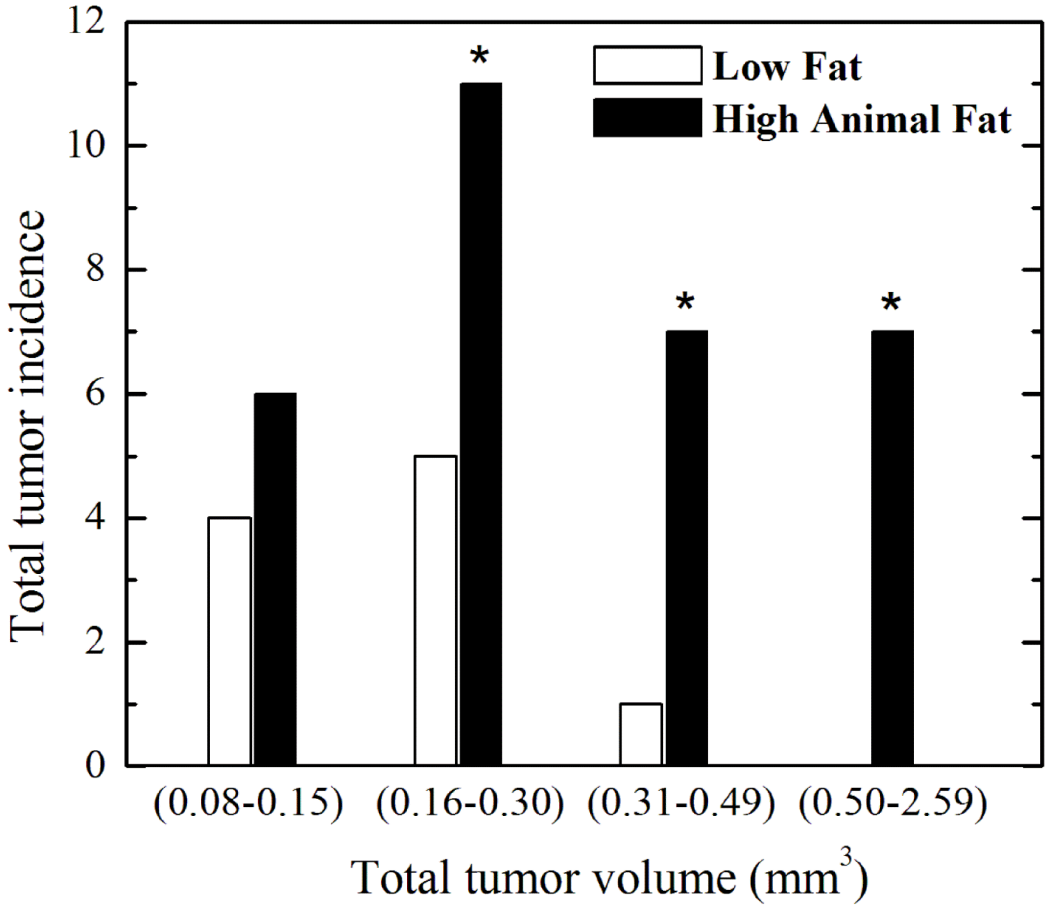

Figure 2-4. **Plot of tumor incidence as a function of tumor volume in low fat and high animal fat-fed SV40 Tag mice**. Tumor incidence and tumor volume in inguinal glands were measured from *in vivo* MRI in SV40 TAg mice fed LFD or HAFD for 8 weeks, n=8 12-week old mice in each group. The number of tumors for the smallest tumor volume subgroup of 0.08-0.15 mm^3 in the LFD group is not significantly different compared to the number of tumors in the HAFD group. However, the tumor incidences with larger tumor volumes of >0.16 mm^3 are statistically significant, as indicated by asterisks in the plot.

Table 2-3. **Tumor incidence and tumor volume in the inguinal gland of SV40 TAg mice at 12 weeks of age**.

	Low fat group	High animal fat group	p-value[1]
Total number of tumors	10	31	
Average ± Std Dev	1.25 ± 1.16	3.88 ± 2.03	0.0068
Total tumor volume	2.0	14.0	
Average ± Std Dev	0.20 ± 0.08	0.53 ± 0.45	0.0162

Eight mice were studied per diet group of low fat and high animal fat-fed mice.

[1]P-values were calculated from Student's t-Tests – at the level of 0.05, two means are significantly different, as listed.

In Table 2-3, the incidence and volume of all invasive cancers are listed for both LFD (n=8) and HAFD (n=8) groups at 12 weeks of age. The total number of invasive tumors was increased by a factor of 3 in the HAFD group compared to the LFD group, while the total tumor volume was increased by a factor of 7 in the HAFD group compared to the LFD group. The average number of tumors and the average tumor volume with standard deviations are also listed in Table 2-3. The mean values of the tumor incidence and tumor volume of invasive cancers, which was higher in the HAFD mice, were statistically significant between the two diet groups, as listed in Table 1 ($p < 0.0068$ for the tumor incidence; $p < 0.0162$ for the tumor volume).

Invasive tumors found in the *in vivo* MR images were then correlated to histology. Because *ex vivo* MR images could be acquired with very high spatial resolution, they served as a reliable bridge to better correlate each feature between *in vivo* MRI and histology. Figure 2-5 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. Major differences were noticed between the LFD and HAFD SV40 TAg mouse mammary glands when examined by a breast pathologist. Mammary glands from LFD mice show highly visible brown fat containing numerous mitochondria, while HAFD mice show primarily mature, white fat, distinguished by the single lipid droplet in the adipocyte, seen in the insets of Figures 2-5a and 2-5b and indicated by black arrows. Second, mammary glands from HAFD mice show more irregular and dilated ducts compared to the LFD group, as seen in MR images in Figure 2-3. Third, HAFD mice show increased invasion compared to LFD mice. Fourth, dilated blood vessels are seen in the gland of HAFD mice compared to LFD group. These HAFD-associated changes illustrate changes in normal development but in the progression

of cancer.

Figure 2-5:

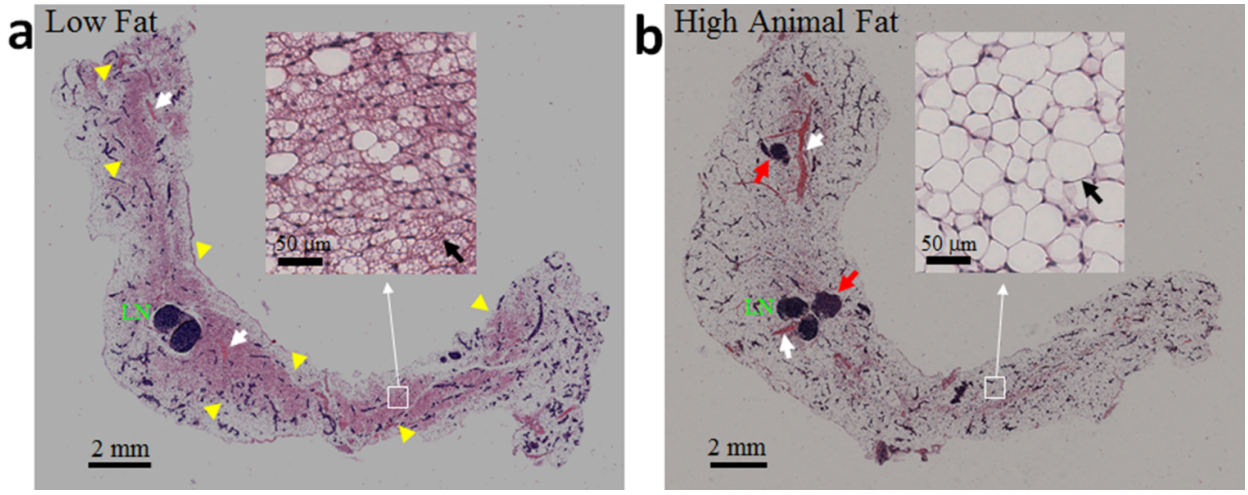

Figure 2-5. **Histological images of mouse mammary glands of low fat and high animal fat-fed SV40 TAg mice**. Hematoxylin and eosin (H&E)-stained images of the excised right inguinal glands of LFD and HAFD SV40 TAg mice at 12 weeks of age are compared. In each image a central slice of the mammary gland containing LN is shown here. In **4b**, two tumors are seen as indicated by red arrows. Blood vessels in both images are indicated by white arrows – dilated blood vessels are seen in HAFD mouse mammary gland. Highly visible brown fat throughout the gland in LFD mouse is indicated by yellow arrowheads in **4a**. Insets of panels **4a** and **4b** – areas corresponding to white boxes as indicated – show H&E-stained images with higher magnification. Highly visible brown fat, characterized by multiloculated adipocytes, predominates in the LFD mouse mammary gland. In contrast, the HAFD mouse mammary gland shows primarily mature fat, which is distinguished by the single lipid droplet in the adipocyte. These are indicated by black arrows in both images in insets. A scale bar in each image is shown.

2. D. DISCUSSION

The present MRI and histology study of a transgenic mouse model of human TNBC unambiguously demonstrates that high animal fat-fed mice develop significantly more invasive mammary cancers, with a much larger overall mammary cancer burden. At 12 weeks of age, the tumor incidence increased by a factor of 3 and the tumor burden increased by a factor of 7 in the HAFD group, compared to LFD group. The largest tumor volume increased by a factor of 8 in HAFD group compared to LFD group. Through the histology, we were able to show the increase in invasion in the HAFD fed mice, indicative of aggressive cancers.

SV40 TAg FVB/N mice on the HAFD did not significantly gain more weight compared to the mice on a regular diet (see, Figure 2-1), suggesting that critical changes affecting tumor growth occur in the mammary glands independently of systemic obesity (Cowen et al. 2015). It has been also suggested that an inflammatory state exists in animal models that is associated with excess systemic adiposity resulting from high fat diet consumption which then promotes mammary cancer progression (Duong et al. 2015; Iyengar et al. 2015; Stoll 2000). The connection between high fat diet/obesity and breast cancer has been attributed in part to adipose tissue dysfunction, which may occur locally within mammary fat pads (Morris et al. 2011) even in the absence of systemic increased adiposity. In our study, thicker dilated ducts as seen in both *in vivo* and *ex vivo* MR images (Figures 2-2 and 2-3) may be due to mammary adipose tissue inflammation induced by high fat diet. It has been shown that HAFD diet increases adiposity, as seen in Figure 2-5b, epithelial cell proliferation, and the number of terminal ducts per mouse mammary gland in female mice (Montales et al. 2014; Morris et al. 2011).

The MRI data reported here demonstrates that mammary glands can have localized increases in fat and overall parenchymal density although overall body weight is unchanged. Mammary gland histology shows that HAFD-fed mice have much less brown fat and increased white adipose tissue compared to LFD-fed mice. This is consistent with other work in this laboratory using chemical shift MRI, proton spectroscopy and mass spectrometry (data not shown) demonstrating that fat content increases consistently and fat composition changes significantly in the HAFD group. Overall, the HAFD diet significantly changes mammary fat content without causing significant obesity making it possible to study changes to mammary fat isolated from the systemic effects of obesity.

The precise correlation of *in vivo* and *ex vivo* MRI with histology in detecting early murine mammary cancers with high sensitivity, specificity, accuracy and the ability to distinguish between *in situ* and invasive cancer has previously been shown in this mouse model (Mustafi et al. 2015). Additionally, growth rates of *in situ* and invasive mammary cancers, as well as the transition from the *in situ* to invasive phenotype have been previously documented on the basis of longitudinal MRI data of SV40 TAg mice between 8-18 weeks of age (Fan et al. 2014). Around 12 weeks of age, *in situ* cancers progress to invasive cancers – the basis of which the present study was designed to evaluate the impact of HAFD on tumor incidence and tumor burden in this mouse model of human TNBC at this age.

High animal fat-fed mice display abnormal mammary ductal development, as seen in our mice, and an incomplete lining of the epithelium surrounding ductal lumen (Kamikawa et al. 2009). It has been previously shown that high fat-induced adipose tissue inflammation and leptin alter tight junction function and impair epithelial barrier (Khandekar, Cohen, and Spiegelman

2011; Kundumani-Sridharan et al. 2013; Le Drean et al. 2014). Multivariate analysis shows that negative claudin-1 expression, a tight junction protein, is an independent prognostic factor of both recurrence and death among TNBC patients (Ma et al. 2014). Therefore, we hypothesize that the increased cancer incidence observed in the HAFD fed mice in our study is due to the abnormal ductal structure and altered secreted factors from mammary adipose tissue. One intriguing possibility is the altered lipid composition of the mammary adipose tissue in the mice fed the HAFD could not be normally suppressed on MR images. Future studies are needed to confirm the mechanistic and metabolic links between HAFD, mammary gland biology and the cancer incidence seen in the mouse model of TNBC.

Due to high spatial resolution and excellent soft tissue contrast, MRI, compared to CT and Ultrasound, is an excellent tool for monitoring early-onset of *in situ* and invasive breast cancers and their progression in *in vivo*. MRI reliably detects smaller tumors and allows for more accurate volume measurements. In addition, MRI allows for the evaluation of the surrounding parenchyma, including fat composition, which we are very interested in investigating. Although the present study involved only one scan per mouse, MRI can be used to serially image and allow for the following of the effects of diet and changes in diet on cancer initiation and progression over time.

Although only one time point, 12 weeks of age, was selected, this was the best time to evaluate the effects of diet on tumor progression due to the advancement of *in situ* cancers to invasive cancer in this mouse model at 12 weeks of age. Future longitudinal MRI studies will provide improved understanding of growth rates of both *in situ* and invasive mammary cancers and the transition from the *in situ* to the invasive phenotype in LFD and HAFD groups. Other

dietary manipulations, e.g., high fructose diet would model effects of similar diets in children. Measurements of fat distribution and composition in the mammary gland and near the invasive tumor will also improve our understanding of mammary gland fat biology and its role in cancer biology.

In summary, the results presented here demonstrate that high animal fat diet fed mice significantly developed a higher number of aggressive cancers in a mouse model of human TNBC. This work is the first step towards using MRI to improve the understanding of the effects of diet on mammary/breast cancer risk and guide development of methods that reduce risk. We have also shown that diet-induced changes to the mammary adipose tissue microenvironment, which have been associated with cancer progression, can be monitored by MRI.

Chapter III

Diet high in fat alters mammary adipose tissue and the mammary gland microenvironment promoting ER-negative mammary cancer

3. A. Introduction:

The average diet in countries like the United States have become full of large portions of fat and sugar and are hugely lacking in healthier foods like fruits and vegetables, (Putnam, Allshouse, and Kantor 2002). Excess food consumption and lack of a corresponding increase in activity, are big proponents of what has led to the obesity epidemic (Cafaro, Primack, and Zimdahl 2006). Obesity is a complex disease but two known promoters driving it are lack of exercise and consumption of excess food, which includes an overall increase in fat intake (Cafaro, Primack, and Zimdahl 2006). These excess calories are for the most part it is stored in whites adipose tissue.

For many years adipose tissue was thought to be an inert, only storing excess calories as lipids. This all changed with more investigation into the physiology of adipose tissue. Adipose tissue is now known to by a dynamic endocrine tissue whose signaling impacts overall physiology (Scherer 2006). It is not only involved in maintaining overall body homeostasis but of particular interest to us it plays an integral role breast and mammary gland physiology. Without proper fat signaling between mammary epithelial cells and mammary adipose tissue (MAT) the ductal trees will not properly develop but with normal signaling, transplanted metastatic breast cancer (BC) cells take part in normal mammary gland development like normal

epithelial cells (Couldrey et al. 2002). Theses facts as well as the association of breast cancer risk with obesity, point to the importance of mammary adipose tissue and the role it may play in BC development.

Breast cancer is still one of the leading causes of death amongst women and subtypes of BC like triple negative breast cancer (TNBC) have a high mortality rate for women who are diagnosed. TNBC is an aggressive form of BC that is classified as molecularly, genetically and phenotypically different from other types of breast cancer (Criscitiello et al. 2012). It is associated with premenopausal woman, woman of African descent and is usually diagnosed at later stages (Criscitiello et al. 2012; Boyle 2012; Hall et al. 2000; Lacey, Devesa, and Brinton 2002). TNBC lacks the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (*HER2),* making it harder to treat than other subtypes due to the lack of targeted treatment availability (Haque et al. 2012).

Long-term diet intervention studies investigating how diet changes effect BC progression showed some promise with healthier diet choices having a positive impact and proving that diet change could be a possible treatment option. Women who ingested a healthier diet showed a decrease in association of ER-negative, PR-negative, and ER2/PR2 negative tumors (Chlebowski et al. 2006). Additionally, other long term trials showed that woman who had ER-negative and ER/PR negative tumors were more responsive to dietary restrictions of fat, as well as exercise (Chlebowski and Blackburn 2015; Pierce 2009; Swisher et al. 2015).

We hypothesize that high fat diet consumption changes the signaling crosstalk between mammary epithelial cells and mammary adipose tissue making the mammary gland microenvironment pro-tumorigenic and causing more malignant tumors. A decrease in tumor

latency with high fat feeding has been shown in adult SV40 TAg mice but to our knowledge this is the first study looking at the long term effects of pubertal exposure to high fat feeding in the SV40 TAg mouse model (Sundaram et al. 2013). In our studies we show that early exposure to HFD feeding not only results in more tumors but also more malignant tumors. Additionally, we hypothesize that these changes are occurring as a result of the high fat induced changes to the mammary adipose tissue (MAT), so we also interrogated the changes to the MAT secretome as well. Our studies show that diet does change the mammary gland microenvironment but that these HFD changes to MAT lead to a pro-tumorigenic microenvironment promoting a worse prognosis. Triple negative breast cancer is extremely hard to treat and has a high mortality rate so any incite such as this will facilitate finding other methods of not only prevention but possibly treatment.

3. B. Materials and Methods:

Animal model and diets: All animal protocols conformed to NIH and the University of Chicago Animal Care guidelines. Female FVB/N mice homozygous for the SV40 TAg transgene, which is driven by a fragment of the promoter of the rat prostatic steroid binding protein gene, were originally provided by Dr. Jeffrey E Green of the National Cancer Institute's mouse model of cancer consortium. All mice in our study were weaned at 3 weeks of age and at 4-5 weeks of age, virgin female mice were randomized and assigned to either a control (low fat) diet (3.7 kcal/g; 17.2% kcal from fat, TD 94045, Harlan Laboratories, Madison, WI) or a high (animal) fat diet group (5.3 kcal/g; 60% kcal from lard, TD 140023, Harlan Laboratories, Madison, WI). Diet details are provided in Tables 2-1 and 2-2. Mice were housed in groups of 4 and on a 12-hour light/dark cycle. Food and water were consumed *ad libitum*. Food consumption for each diet

group, as well as individual body weight, was monitored weekly. To minimize hormonal effects due to estrous cycle, the estrous cycle was monitored via vaginal cytology at least a week prior to scheduled sacrifice date. All mice were sacrificed in the estrus phase of the estrous cycle. (Caligioni 2009)

Tumor Measurements: *In vivo* tumor growth was monitored and measured upon detection of tumors, with digital calipers. All 10 mammary glands were palpated for tumors beginning at 10 weeks of age. Length and width of tumors were measured at least once a week and tumor volume was approximated using the formula $(L^2)(W)(0.52)$ as previously described (Skor et al. 2013). Tumor weight was measured immediately following dissection and separation of surrounding tissue mammary gland tissue and recorded for further evaluation.

Tumor Histology: Tumor tissue was fixed at room temperature in formalin solution (10% neutral buffer; sigma Aldrich ref# HT501260ml) for one to two days, depending on the size of the tissue, with larger amounts of tissue being left to fix for longer periods of time. Tissue was then placed in 70% ethanol at room temperature for 1-2 days and stored in at 4° C until it was processed by the human tissue resource center at University of Chicago, where it was embedded in paraffin and slides were prepared. Tissue was cut, placed on slides and stained with Haematoxylin and Eosin for further analysis of tumor differentiation.

Tumor scoring: Tumor samples were evaluated and scored as previously described, as well as by the Annapolis mouse models pathology panel by a trained pathologist Daniel Johnson (Cardiff et al. 2000; Williams et al. 2009). A total of tumors (n_t) = 51; from 13 control diet fed mice and a total number of tumors (n_t) = 75 tumor from 15 high fat diet fed mice were evaluated for degree of glandular differentiation. Well-differentiated (w) tumors were defined as having more than

80% normal glandular and papillary structure. Moderately differentiated (m) tumors were defined as having 20-80% normal glandular and papillary structure. Poorly differentiated (p) tumors were defined as having less than 20% normal glandular papillary structure remaining in the tissue examined.

Conditioned media: All mammary gland tissue was removed in sterile conditions and minced for crude mammary adipose tissue (MAT) extraction, in 500 µl of phenol red free, serum free (HyClone; cat no: SH30284.01) Dulbecco's Modified Eagle Medium (DMEM) high glucose media with 0.1% BSA and 1% penicillin/streptomycin (p/s). Mammary gland (MG) tissue was finely minced and spun for separation of tissue at 100 × *g* for one minute to separate stromal tissue from adipose tissue in the MG. Floating mammary adipose tissue was collected and re-spun to remove all remaining media. Amount of MAT was weighed in a sterile pre-weighed tube and was placed in 10 ml of serum free DMEM media with 0.1% BSA and 1% penicillin/streptomycin per 1 gram of tissue for an 8-hour incubation and for collecting of adipose secreted factors and making of conditioned media (CM). Following an 8-hour incubation, media is filtered using PVDF membrane 0.22-µm syringe filters, aliquoted in sterile tubes and stored at -80° C. Media was thawed at 4° C and re-filtered before experimental use.

LPC/ LPA analysis: CM was prepared as described above an sent on dry ice to Wayne State Lipidomics Core Facility for directed analysis of LPA and LPC species via mass spectrometry as previously described (Volden et al. 2016; Volden 2014). Extraction of lipid species was done at Wayne state. Four samples per diet group from 12 weeks old mice that were analyzed.

Adipokine array: Conditioned media (CM) made from mammary fat (MF) separated from all the mammary glands of each mouse as described above, 3 mouse samples per diet group, were

thawed at 4° C and analyzed as described in instructions for the adipokine antibody array kit (ARY013; R&D Systems). Each membrane was incubated on a rocker with 250 µl of MF CM, 125 µl of array buffer 4, and 1.125 ml of array 6 overnight at 4° C. Membranes were then imaged after incubation with the corresponding secondary antibody, as well as with streptavidin-horseradish peroxidase and chemiluminescent detection reagents provided. Adipokine levels in the MF-CM were determined with BioRad imager using Quantity One software to image blots and determine densitometry of each antibody spot. Adipokine antibodies were captured in duplicate and the average for each duplicate pair minus the densitometry value of the surrounding background was used to determine the peak average signal density of each antibody.

Proliferation and cell death: Cell lines derived from SV40 TAG mice were used to evaluate the effects of CM derived from MF (Holzer et al. 2003). M6 (mammary carcinoma epithelial cells), M27 (weakly tumorigenic, ductal carcinoma in situ cells), as well as morphologically normal cells M28 (normal mammary epithelial cells from 2 month old SV40 TAG FVBN mouse) were seeded at 2.5 x 10^3 cells per well in 96 well plates (Sigma-Aldrich Z707902-96EA) (Holzer et al. 2003; Volden et al. 2016). Cells were left to adhere overnight in phenol red free 10% FBS 1% P/S DMEM media (HyClone; cat no: SH30284.01) and were treated the following day. Each well was washed twice with 1x PBS prior to addition of treatment groups. 100 µl of CM per condition was added in triplicate for each media group tested. 100 µl Yo-Yo dye, which only stains cells with a compromised nuclear and plasma membrane, was additionally added to determine cell death over time per condition after being diluted 1:1000. Two Images (1.90 x 1.52 mm) per well were taken every 4 hours over a 60-hour period in which the plates were in the Incucyte or Incucyte zoom imaging system. Images were analyzed for effects on proliferation and apoptosis (cell death) of mammary epithelial cells using Image J as previously described using cell specific coded analysis (see below) (Skor et al. 2013).

Cell count and cell death: Total cell count and cell death (green fluorescent-labeled) were

quantified via Image J macros using digitally captured IncuCyte images, as previously described with modifications for each cell line described below (Skor et al. 2013). Images were taken every 4 hours with a 10x objective using the IncuCyte FLR HD system at two different locations in each experimental well. Cells (objects) in each image were corrected for background and contrast intensity. Phase-contrast images measured cell size to determine object number per image and were normalized to initial time point images.

M6 Image J Macros:

Cell (Object) count:

```
//Image processing
//Example M6
//Phase contrast
run("Despeckle");
run("Despeckle");
run("Smooth");
run("8-bit");
//run("Threshold...");
setThreshold(0, 67);
run("Convert to Mask");
run("Outline");
run("Fill Holes");
run("Analyze Particles...", "size=60-Infinity show=Outlines display summarize");
```

Dead cell (fluorescent) count:

```
//Image processing
//Example M6 cells
//Fluorescence
run("Despeckle");
run("Despeckle");
run("Smooth");
run("8-bit");
//run("Threshold...");
setThreshold(33, 255);
run("Convert to Mask");
run("Outline");
run("Fill Holes");
run("Analyze Particles...", "size=45-Infinity circularity=0.00-1.00 show=Outlines display
```

summarize");

In vitro adipokine signaling: 2500 M6 cells, mammary carcinoma epithelial cells derived from SV40 mice, were seeded as described above. Prior to treatment, wells were washed twice with 1x PBS and treated with recombinant MCP1, Pentraxin 1 (Cat # 479-JE-010/CF, 2166-TS-025/CF; R&D systems) and LPA 18:1 (CAS# 65528-98-5, Cayman chemical) the following day in serum free (HyClone; cat no: SH30284.01) DMEM high glucose media with 0.1% BSA and 1% penicillin/streptomycin. Both MCP1 and Pentraxin 1 were reconstituted in PBS before addition to SF media. Cells were followed over time for 60 hours using IncuCyte or IncuCyte zoom live cell imaging system and analyzed as described above for cell proliferation and cell death with macro for Image J below.

In vitro inhibition of LPAR 1/3 signaling: 2500 cells were seeded onto 96 well plates as described above and allowed to adhere overnight. Cells were then washed with 1x PBS for removal of any remaining serum and 100 µl MAT-CM from control and HFD fed mice were added to each well. LPAR inhibitor (Ki16425, Cayman chemical, item #10012659) was dissolved in DMSO and was added at concentrations of 0 nmol/L, 10 nmol/L, 100 nmol/L, and 1000 nmol/L with the concentration of DMSO diluted down to below 1% per well. Effect of inhibitor on cell proliferation and cell death was monitored using the IncuCyte or IncuCyte zoom over 40 hours with images taken every 4 hours and later evaluated using the software provided and Image J M6 macro (see below).

M6 In vitro Image J Macros:

Cell (Object) count:

```
//Image processing
//Example M6
//Phase contrast
run("Smooth");
run("Smooth");
run("Smooth")
run("Despeckle");
run("Despeckle");
run("Despeckle")
run("8-bit");
setThreshold(0, 98);
run("Convert to Mask");
run("Outline");
run("Fill Holes");
```

run("Analyze Particles...", "size=55-Infinity circularity=0.00-1.00 show=Outlines display summarize");

Dead cell (fluorescent) count:

```
//Image processing
//Example M6
//Fluorescence
run("Smooth");
run("Smooth");
run("Smooth")
run("Despeckle");
run("Despeckle");
run("Despeckle")
run("8-bit");
setThreshold(4, 255);
run("Convert to Mask");
run("Outline");
run("Fill Holes");
run("Analyze Particles...", "size=45-Infinity circularity=0.00-1.00 show=Outlines display summarize");
```

M6 in vitro LPA inhibitor Image J Macros:

Cell (Object) count:

```
//Image processing
//Example M6
//Phase contrast
run("Despeckle");
run("Despeckle");
run("Smooth");
run("8-bit");
//run("Threshold...");
setThreshold(0, 67);
run("Convert to Mask");
run("Outline");
run("Fill Holes");
run("Analyze Particles...", "size=60-Infinity show=Outlines display summarize");
```

Dead cell (fluorescent) count:

```
//Image processing
//Example M6
//Fluorescence
```

```
run("Smooth");
run("Smooth");
run("Smooth")
run("Despeckle");
run("Despeckle");
run("Despeckle")
run("8-bit");
setThreshold(4, 255);
run("Convert to Mask");
run("Outline");
run("Fill Holes");
run("Analyze Particles...", "size=45-Infinity circularity=0.00-1.00 show=Outlines display summarize");
```

MR *in vivo* and *in vitro* imaging and whole gland histology: As described in chapter 2, following 13-14 weeks on control (low fat) or HF diets, *in vivo* fast spin echo MR images of inguinal mammary glands were acquired at 9.4T from 4 mice per diet group at 17-18 weeks of age (Mustafi et al. 2015). Following *in vivo* MRI, inguinal mammary glands were excised and fixed in formalin for *ex vivo* MRI following an overdose of isoflurane and cervical dislocation. 3D volume-rendered MR images were then correlated with histology. Prior to *in vivo* imaging, mice were anesthetized. Anesthesia was maintained during imagining with 1-2% isoflurane, with temperature, respiration and heart rate monitored through out. Mammary glands for *ex vivo* imaging were removed with the skin and fixed in 10% formalin for 2 weeks. Before imaging of the right mammary gland, it was washed with phosphate buffered saline (PBS, Fisher Scientific, Waltham, MA) for three days and the skin was removed. Glands were then placed between two layers of pathology foam (Fisher Cat. # 22038221) in the same alignment as the position of the gland in the mouse. The mammary glands were washed twice with fomblin (Solvay Solexis, West Deptford, NJ) and saturated with fresh fomblin prior to imaging. Following imaging, glands were thoroughly washed with PBS and placed in 70% ethanol for histology processing

and H&E staining.

3. C. RESULTS

SV40 Tag mice on a high fat diet consume more kcals, become hyperglycemic and show signs of metabolic change but do not become obese

Female mice are overall more resistant to weight gain and diet-induced obesity than their male counterparts but the FVBN strain of mice in particular has been shown known to remain lean and to be more resistant to weight gain than other strains (Boudina et al. 2012; Haluzik et al. 2004; Berglund et al. 2008; Jo et al. 2009). Given our interest in studying the effects of high fat diet on TNBC, the SV40 TAg transgenic strain on the FVBN background was best model available to study the effects that HFD, causes without having to also worry about the effects of obesity. SV40 TAg transgene causes female mice to develop tumors in their mammary glands that follow a similar molecular and pathological progression to human TNBC. Invasive tumors from Tag mice have been shown to be genetically similar to human TNBC making this an ideal model for our studies of TNBC (Maroulakou et al. 1994; Green et al. 2000; Herschkowitz et al. 2007). Both control and high fat fed groups consumed very similar amounts of food by weight (grams), with the control mice actually consuming a non-significant bit more, fig 3-1A. Even with the slight increased food consumption by the control mice, the HFD fed mice consumed much more calories overall due to the higher caloric density per gram of the HFD, 3.7 kcal/gram vs. 5.3 kcal/gram respectively, fig 3-1B. The HFD group did gain a little more overall weight than the control group, average a total of 4.6 grams ($\pm$ 0.375 standard error) more than the control fed mice, but as can be seen in figure 3-2, that weight difference can be accounted for by the increase in adiposity of the all adipose depots of each mouse. Particularly of interest to us,

there was a visible increase in white adipose tissue in the mammary glands of the HFD group, as well as an apparent increase in adipocyte size compared to the control glands, as can be seen in the first panel in fig 3-4a. Larger adipocytes, like those seen with HFD feeding, have previously been associated with insulin resistance (McLaugblin et al. 2007; Weyer et al. 2000) as well increased lipid efflux from the adipocytes into the general circulation. These changes can further worsen systemic insulin sensitivity and in agreement with previous studies, we saw increased fasting blood glucose levels with HF feeding, fig 3-1D. The results suggest these mice have become hyperglycemic, glucose intolerant and possibly insulin resistant due to their elevated glucose levels (fig 3-1D).

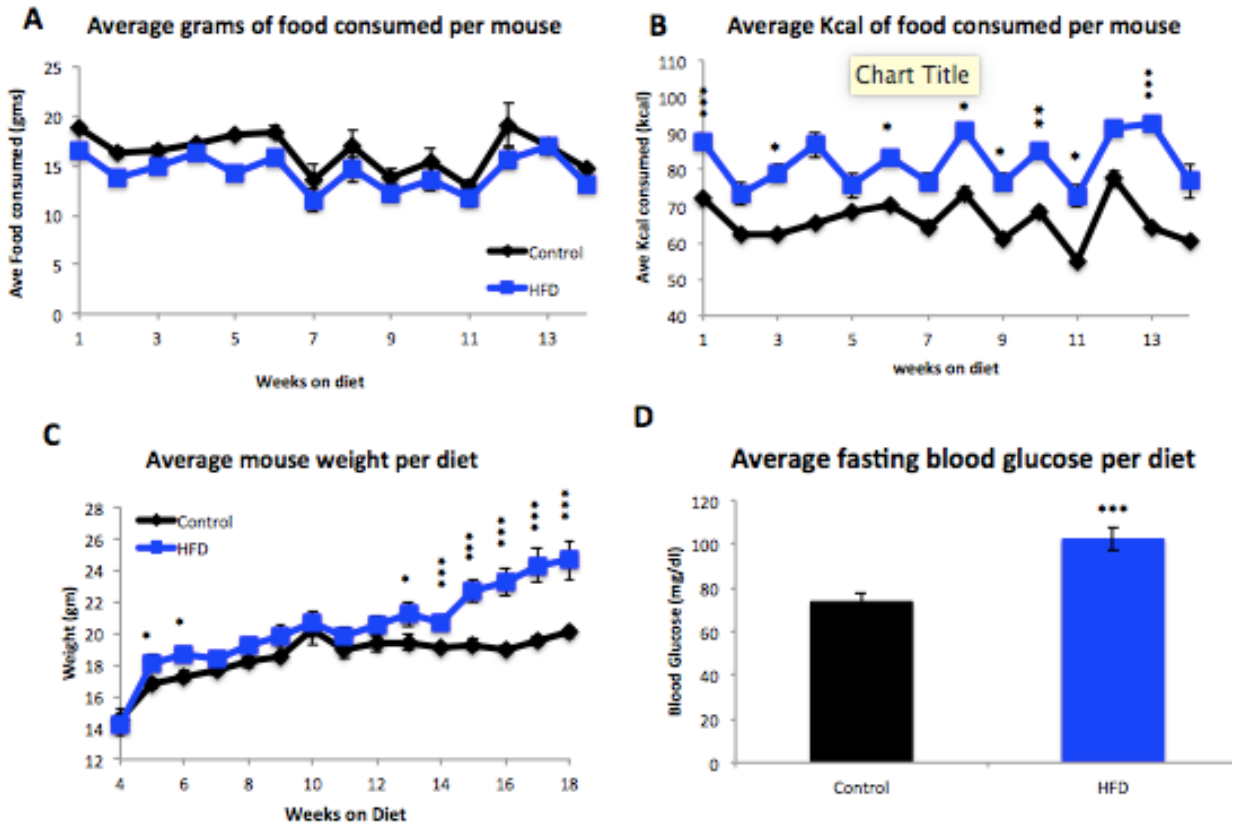

Figure 3-1: HF feeding minimally increases overall body weight but increases fat depot sizes and causes systemic metabolic changes to glucose metabolism. Body weight and food consumption (A-C) were monitored weekly throughout our studies. Fasting blood glucose was evaluated at 17-18 weeks of age following a 6 hour fast and measured using a glucometer (D).

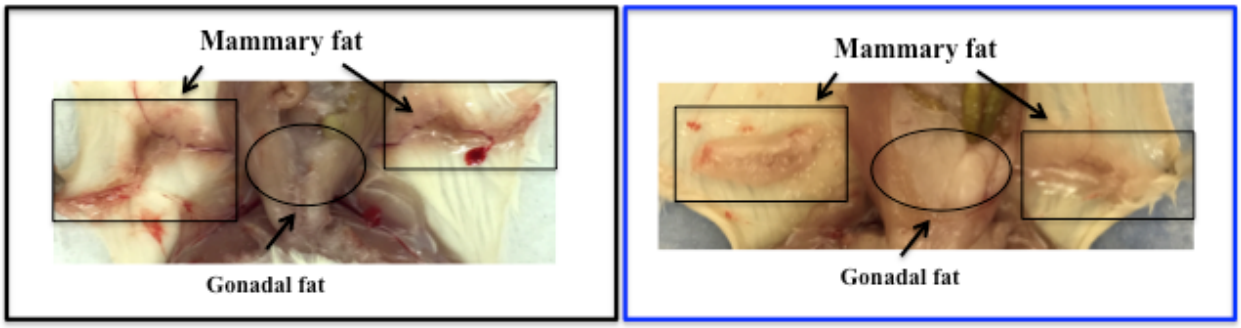

Figure 3-2: Greater adiposity gained in all fat depots of mice fed HFD

Images taken during dissection show differences the size of fat pads in control (black outer box) and HFD (blue outer box) fed mice. HFD fed showing a much larger amount of WAT present at 20-21 weeks of age.

High fat feeding causes changes to mammary gland architecture

The effects diets high in fat have on mammary biology or more specifically mammary gland architecture have not been very well studied. Studies with pubertal exposure to a diet in high fat in a carcinogene 7,12-dimethylbenz[a]anthracene (DMBA)-induced tumorigenesis model but no studies to our knowledge have been done using the SV40 Tag model of TNBC examining the tumorigenic effect of pubertal exposure to a HFD (Zhao et al. 2013). Studies using this model were done with adult female mice and HFD exposure in adulthood, which did show changes to tumor latency (Sundaram et al. 2013). Our mice began HFD at 4-5 weeks of age and characterized at different of ages of adulthood, show drastic differences in their mammary gland histology and biology overall. Mice fed a control diet (fig 3-3A) show much less white adipose tissue than their HFD fed counterparts, where barely any stromal tissue is observed (fig 3-3a). As mentioned earlier, in figure 3-5A, left control and HF panels, the remarkable difference in adipocyte size can be seen when HFD histology samples are compared to control samples, with HFD fed mice displaying much larger adipocytes.

Exposure to either carcinogens or dietary factors during the in utero or during pubescent years largely alters how the mammary ductal tree continues to differentiate and form. The ductal tree, but more specifically the terminal end buds, are still differentiating and highly proliferated during puberty making the ductal tree most susceptible while at this stage of differentiation (Hilakivi-Clarke 2007). The diet-induced changes to the normally structured and organized mammary ductal tree can be seen in the ex vivo MR images of 17-18 weeks of age as in fig 3-3B, but can also be seen as early on as 12 weeks of age in Fig 2-3. The ductal tree of mice fed a high fat diet were irregular in size and in branching, leading us to conclude that normal

mammary gland development was perturbed with the HFD.

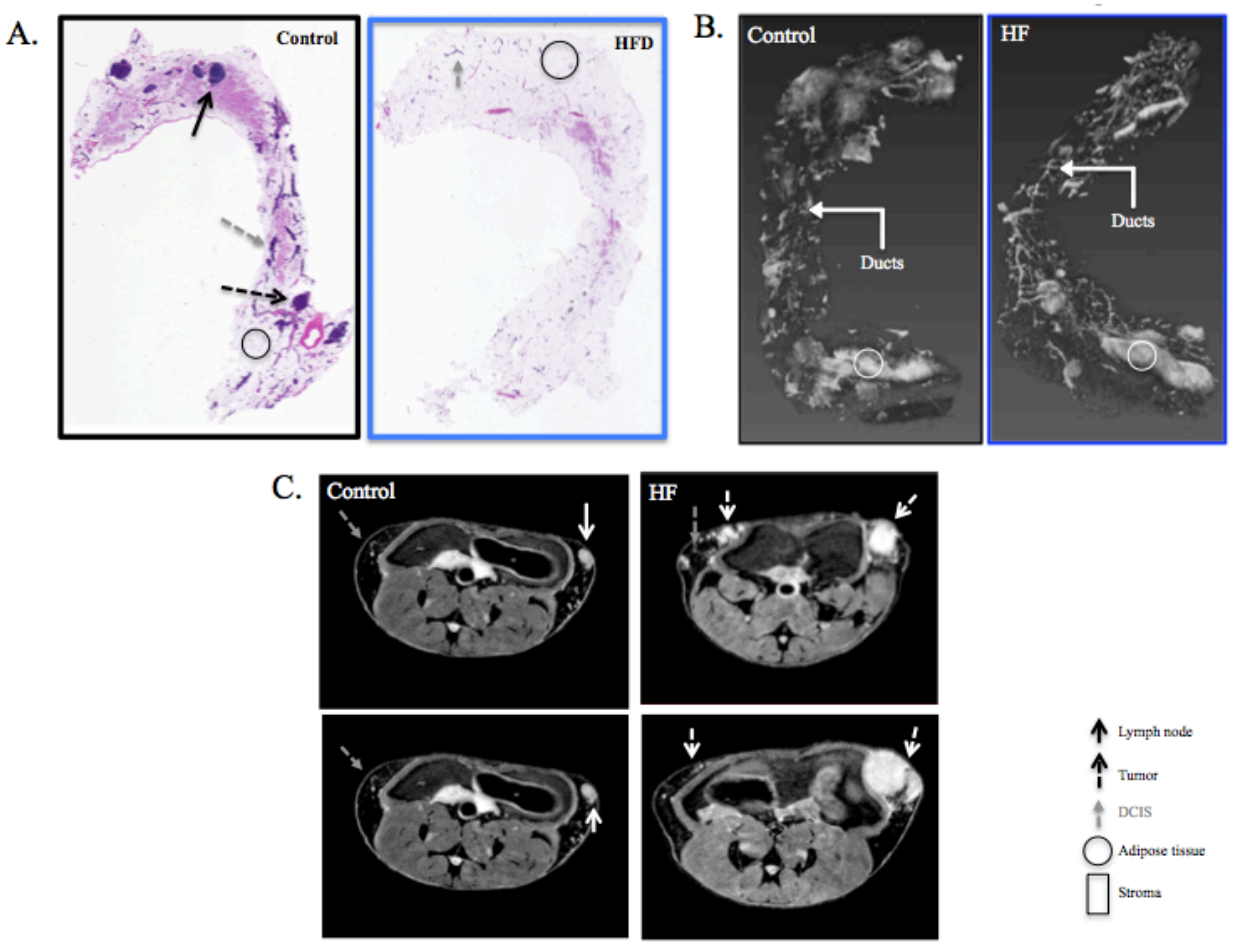

Figure 3-3: HFD causes morphological changes to the SV40 Tag mammary gland. Whole mammary glands from 17-18 week old mice were collected for H&E staining (A) and ex vivo (B) imaging following in vivo imaging of lower mammary glands (C). Following dissection, whole mammary glands were fixed and imaged via high resolution MRI imaging (B). Following imaging, glands were stained with H&E for comparison.

Tumor grade, incidence and burden were increased and worsened with high fat diet consumption

The importance of pubertal exposure to stressors and their effect on breast cancer risk later on in life has long been debated. To date, studies looking at pubertal stressors have only been done in DMBA carcinogenic models and to our knowledge studies looking at the effect of non-carcinogenic of stressors like social isolation are still ongoing. In our model, where tumors are caused through genetic inhibition of p53 and RB, tumors have been characterized as being close in progression and genetic similarity to human TNBC, we have not only seen changes to the ductal tree but we hypothesize that these diet induced changes to the mammary gland are resulting in more tumor incidence and higher grade tumors. The drastic difference in appearance of the mammary glands of mice fed the two different diets can seen in fig 3-3C. One very apparent difference is the size and the number of the tumors that can be seen in vivo MR images (fig 3-3c) from mice fed both diets. There are plenty more bright white areas in the HFD MR images mammary glands, both in vivo and ex vivo (fig 3-3b,c), indicating more tumors and DCIS.

Quantitative analysis of the tumors (fig 3-4) collected in both groups of mice, show not only an increase in tumor number (fig 3-4A) with HFD feeding but also an increase in tumor burden (Fig 3-4B) and size (weight; fig 3-4C). Additionally, grading the differentiation states of the H&E stained tumors from both diet groups showed a drastic shift towards more high-grade, poorly differentiated tumors with HFD feeding (Fig 3-5). Therefore showing that HFD feeding not only causes a greater number of tumors with a high fat diet but also results in worse tumors.

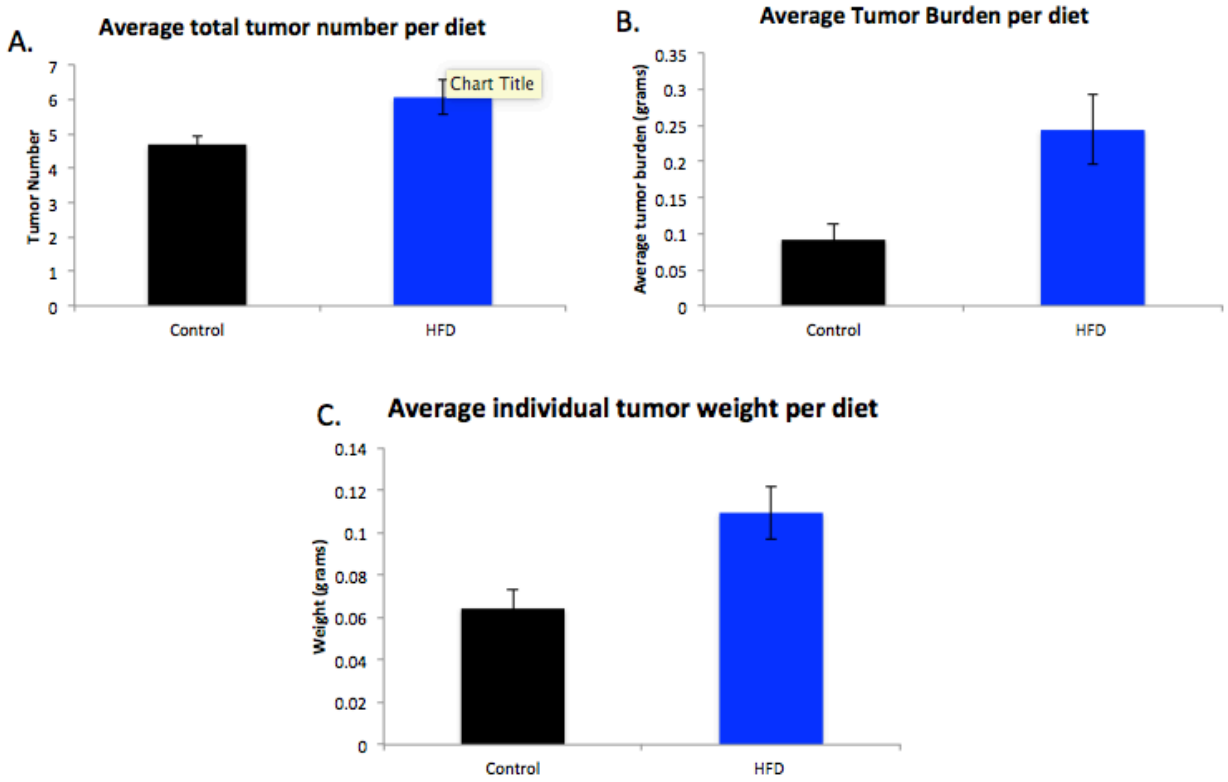

Figure 3-4: Diets high in fat cause increases tumor number, burden and weight.
At 20-21 weeks of age, mice were sacrificed and tumors individual tumors were collected and evaluated upon dissection. HFD (blue) showed a higher amount of tumor number, burden and weight compared to the control group (black).

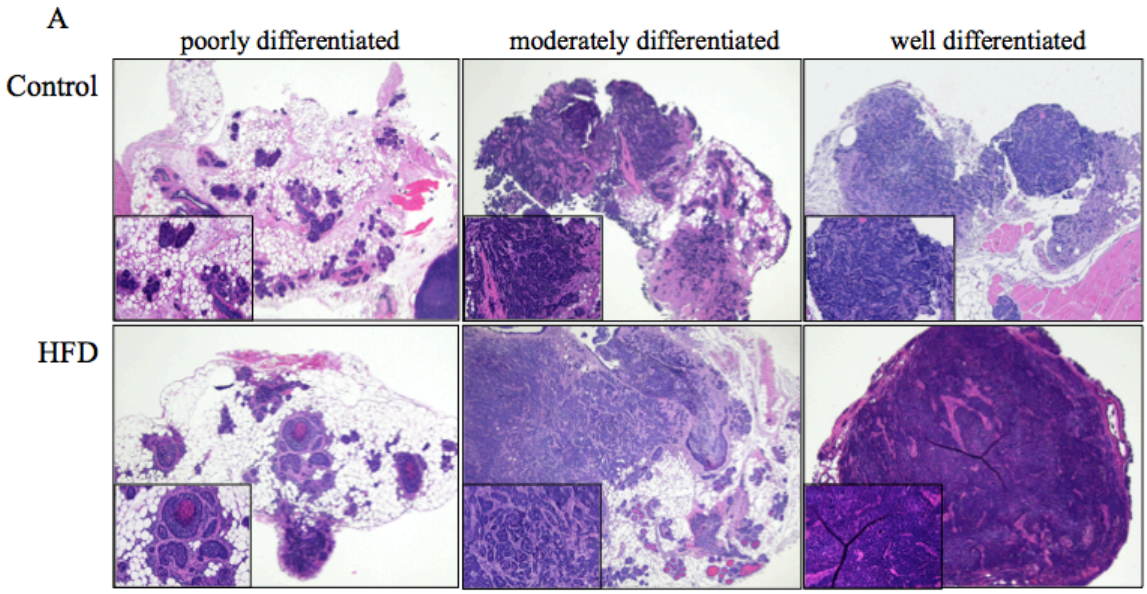

Figure 3-5: High fat diet results in higher-grade tumors than control diets. Examples of fixed and H&E stained tumors for each differentiation state and diet groups are shown in (A), as well as the results of the grading of tumor slides by Dr. Dan Johnson are shown in (B). Black representing control tumors and blue representing HFD tumors. N=51 control tumors and N=75 for HFD tumors.

HFD fed mice secrete more tumorigenic adipokines and lipids than their control counterparts

Our overarching hypothesis starting these studies was that the changes occurring due with HFD were occurring mainly if not completely in the mammary adipose tissue and were increasing BC risk. To investigate this, we collected the MAT secretome by making conditioned media (CM) to determine the changes occurring to the MG secretome. The secreted factors collected in the CM are those that would normally be secreted into the MG microenvironment by the MAT and therefore interacting directly with the mammary epithelial cells, which would later

go on to form DCIS and tumors.

Previous studies have shown that a family of lipid species, lysophosphatidic acid (LPA), have been found at higher levels in higher grade and higher stage of ovarian cancers (Shen et al. 2001). Additionally, previous work from our lab using the same mouse model has shown that LPC, the precursor of LPA, is increased with chronic social isolation (Volden et al. 2016). Social isolation has not only been associated with increased tumor burden and invasiveness in the SV40 TAg mouse model, but social isolation in humans has also been linked to worse prognosis. Given the association of LPA and LPAR signaling with several types of cancer (ovarian, cervival, breast, bladder, and head to name a few) and the ability to detect changes to LPA & LPC in this mouse model, we integrated if there were any diet induced changes to the LPC and LPA levels in the MG microenvironment (Mills and Moolenaar 2003; Houben and Moolenaar 2011). As we had hypothesized, HF feeding caused an overall increase in LPA levels compared to their control counterparts. We also saw a statistically significant increase in LPA, the active form of lysophosphatidic acid, levels with HFD feeding and a decrease in LPC with HF feeding when looking at both LPA and LPC species (fig 3-6 A, B).

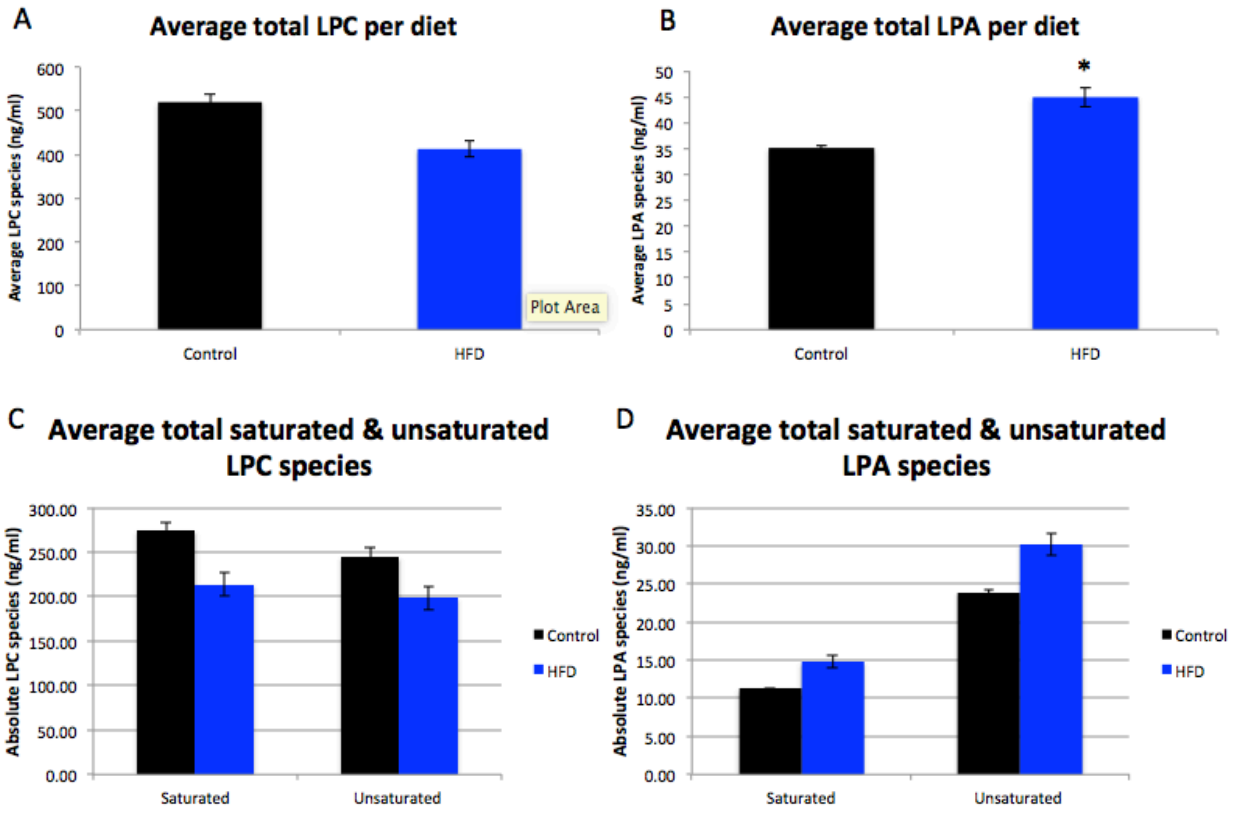

Figure 3-6: HFD causes increase in LPA species and decrease in LPC species in MAT secretome. Frozen CM media samples from 12-week-old mice, 4 per group, were sent to Wayne State for mass spectrometry analysis of individual LPA and LPC species. Total average LPC (A) and LPA species (B) were examined per control (black) and HF (blue) diet. Average total saturated and unsaturated LPC (C) and LPA (D) species were also evaluated.

In figure 3-7 where all the LPA and LPC measured a re shown, it is easier to see that indeed all the LPA species are up and all the LPC species have decreased with HFD. Additionally, when evaluating the types of LPA and LPC that were changed, saturated vs. unsaturated, we noticed an increase in not only the unsaturated but also of particular to us, the saturated forms of LPA (fig 3-6 C, D). A study investigating if individual species of LPA could be used as markers for ovarian cancer showed that there was not one particular LPA species that was increased in correlation with ovarian cancer stage but an increase in the overall levels of saturated LPA, which is what we also saw increased MAT from HFD mice (Shen et al. 2001). In control mice, overall levels (saturated and unsaturated included) of the inactive LPC were increased (fig 3-6). A heat map demonstrating individual mouse values for each diet for each species also demonstrate the overall changes mentioned above as well as individual mouse differences and where there could be some discrepancies between biological replicates (fig 3-8).

Our interest in understanding the overall changes occurring with HF feeding led us to evaluate the changes to the adipokine species secreted from MAT in CM (fig 3-9). Using adipokine arrays with a span of antibodies already bound to the membrane, we investigated the change to the overall secretome of MAT with a HFD. We saw an overall increase in growth factors like IGFB 2, which at elevated levels has been associated with increased risk of developing colorectal cancer, prostate, lung and breast cancer (Livingstone 2013). Increased signaling and expression through IGF2 not only promotes growth and is anti-apoptotic but also increases the risk of transformation. Additionally, transgenic mice over-expressing IGF2 showed increased risk of developing MG adenocarcinoma as well as lung cancer making IGF2 (Livingstone 2013; Bates et al. 1995; Moorehead et al. 2003).

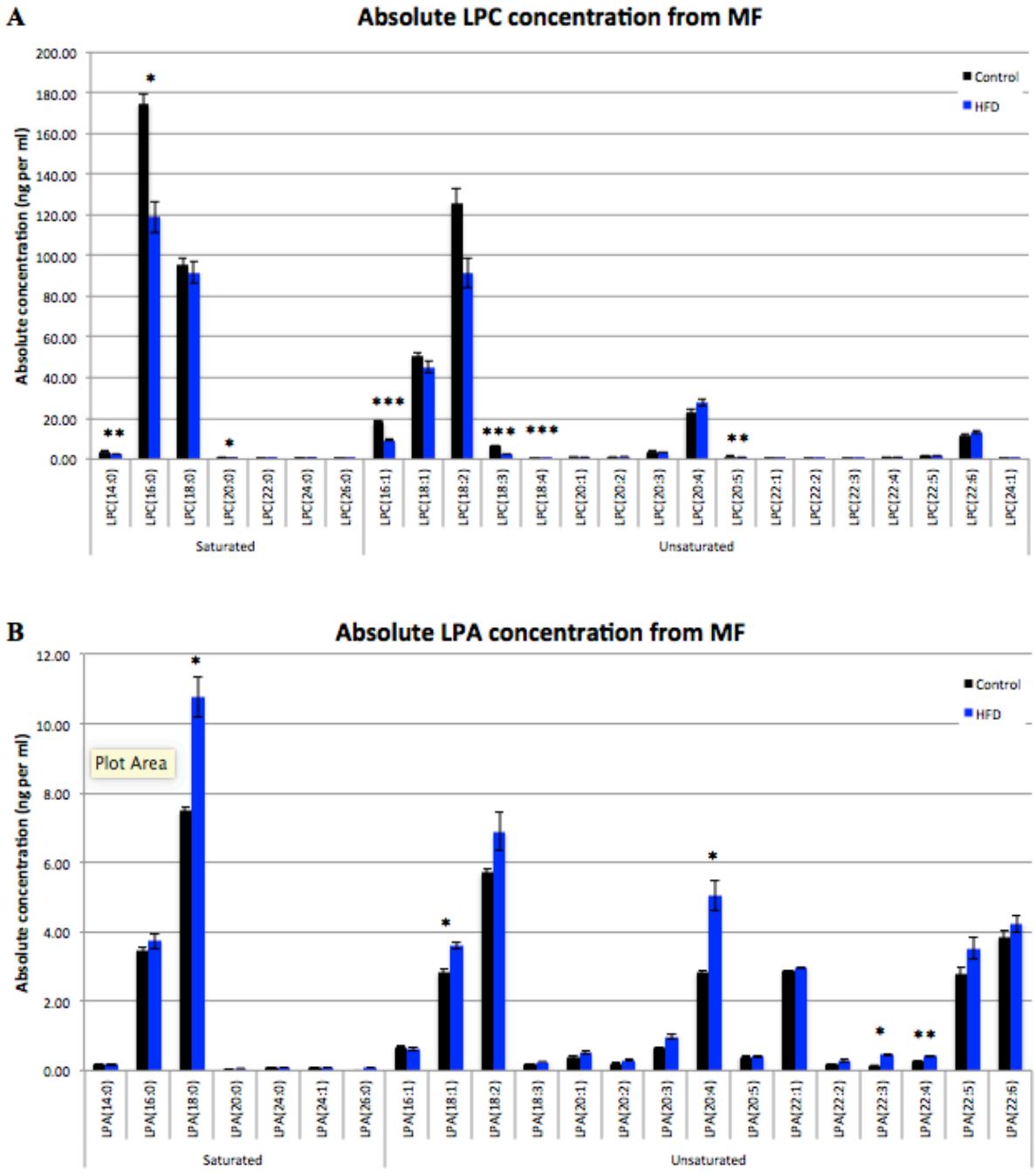

Figure 3-7: HFD increases active form of lysophosphatidic acid secreted from MAT. Absolute LPC (A) and LPA (B) concentrations from mammary fat conditioned media derived from 12-week-old mice, were evaluated via mass spectrum analysis. Significance was evaluated using a student t-test. Error bars represent standard deviation and in both panels * = p value <0.05, ** for p value <0.005 =, *** for p value <0.0005.

A

		Control				HFD			
***	LPC(14:0)	3.19	3.27	3.79	4.30	2.31	1.80	2.25	2.79
	LPC(16:0)	167.90	151.22	176.53	201.04	132.19	78.37	113.92	151.33
	LPC(16:1)	18.31	16.44	18.75	18.97	11.18	6.37	9.19	10.34
	LPC(18:0)	83.24	85.63	99.76	112.07	111.12	61.46	94.20	99.88
	LPC(18:1)	42.35	48.11	51.69	60.53	52.18	28.01	44.82	55.86
	LPC(18:2)	109.41	96.62	138.74	157.85	102.23	48.90	98.70	116.30
***	LPC(18:3)	6.61	5.11	5.86	7.49	2.70	1.51	2.66	3.24
***	LPC(18:4)	0.07	0.07	0.06	0.08	0.02	0.04	0.03	0.04
*	LPC(20:0)	0.56	0.65	0.73	1.06	0.42	0.25	0.36	0.31
	LPC(20:1)	0.87	0.89	1.31	1.44	0.98	0.69	0.81	0.91
	LPC(20:2)	0.87	0.95	1.08	1.29	1.48	0.91	1.12	1.37
	LPC(20:3)	3.32	3.15	3.29	4.63	3.78	2.46	3.22	4.44
	LPC(20:4)	21.54	21.85	19.54	29.81	29.64	20.50	23.61	36.48
**	LPC(20:5)	1.27	1.14	1.13	1.64	0.90	0.72	0.63	0.85
	LPC(22:0)	0.12	0.21	0.21	0.25	0.22	0.15	0.17	0.16
	LPC(22:1)	0.13	0.14	0.15	0.16	0.15	0.15	0.14	0.19
	LPC(22:2)	0.03	0.06	0.05	0.08	0.07	0.05	0.05	0.06
	LPC(22:3)	0.02	0.02	0.02	0.02		0.03	0.04	0.07
	LPC(22:4)	0.53	0.58	0.44	0.83	0.89	0.70	0.63	1.18
	LPC(22:5)	1.56	1.84	1.24	2.03	1.98	1.66	1.28	2.39
	LPC(22:6)	11.03	12.49	8.24	13.55	14.02	12.68	10.17	15.77
	LPC(24:0)	0.23	0.32	0.38	0.53	0.41	0.36	0.30	0.36
	LPC(24:1)	0.07	0.08	0.14	0.15	0.11	0.12	0.07	0.08
	LPC(26:0)	0.05	0.08	0.09	0.07				0.04

B

		Control				HFD			
	LPA(14:0)	0.15	0.11	0.19	0.24	0.13	0.12	0.23	0.18
	LPA(16:0)	3.43	3.88	3.31	3.29	3.52	4.96	2.98	3.45
	LPA(16:1)	0.69	0.79	0.56	0.64	0.57	0.97	0.47	0.48
*	LPA(18:0)	7.95	7.91	6.99	7.06	10.32	14.26	8.99	9.52
*	LPA(18:1)	2.59	3.27	2.96	2.48	3.35	4.08	3.15	3.85
	LPA(18:2)	5.44	5.23	5.85	6.30	6.11	5.54	5.81	10.13
	LPA(18:3)	0.18	0.15	0.17	0.19				0.26
	LPA(20:1)	0.41	0.44	0.20	0.47	0.41	0.66	0.28	0.67
	LPA(20:2)	0.31	0.10	0.24	0.12	0.18	0.47	0.35	0.20
	LPA(20:3)	0.80	0.65	0.46	0.71	1.01	1.19	0.62	1.08
	LPA(20:4)	2.73	2.95	2.63	3.01	3.96	4.68	3.89	7.66
	LPA(20:5)	0.34	0.58	0.33	0.33	0.28	0.50	0.27	0.58
	LPA(22:1)	2.78	2.94	2.86	2.87	3.00	3.05	2.86	2.96
	LPA(22:2)	0.18	0.15	0.30	0.08	0.11	0.54	0.34	0.18
*	LPA(22:3)	0.13		0.17	0.16	0.31	0.47	0.60	
*	LPA(22:4)	0.26	0.36	0.29	0.16	0.38	0.44	0.37	0.44
	LPA(22:5)	2.74	3.83	2.12	2.49	2.39	4.88	2.58	4.24
	LPA(22:6)	3.66	5.12	3.22	3.34	3.31	5.48	3.56	4.50
	LPA(24:0)	0.10		0.06	0.05	0.10		0.08	
	LPA(24:1)	0.06	0.04	0.04	0.13	0.07		0.12	

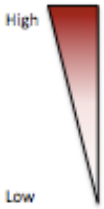

Figure 3-8: Individual mouse LPA and LPC species changes per diet. Absolute LPC (A) and LPA (B) concentrations from mammary fat conditioned media derived from 12-week-old mice, were evaluated via mass spectrum analysis. Significance was evaluated using a student t-test. Error bars represent standard deviation and in both panels * = p value <0.05, ** for p value <0.005 =, *** for p value <0.0005.

In addition to growth factors and their binding partners being increased, many immune signaling adipokines like IL6, MCP1 and Pentraxin 3 were also elevated all over which have been positively correlated to worse prognosis, metastasis and poor tumor grade not only in BC, but specifically in TNBC (Choi et al. 2014; Celis et al. 2005; Dirat et al. 2011; Knupfer and Preiss 2007; Salgado et al. 2003). Pentraxin-3, one of the adipokines we saw highly increased in the HFD CM, has been positively correlated with bone metastasis (Choi et al. 2014; Magrini, Mantovani, and Garlanda 2016). MCP-1, which was also highly increased with HFD, has been positively associated with increase tumor-associated macrophage (TAM) infiltration and increased lung metastasis (Yoshimura et al. 2013; Fujimoto et al. 2009). Increased TAM infiltration has been linked to worse prognosis, increased angiogenesis and metastasis, where inhibition of TAM infiltration decreased tumor progression and metastasis (Fujimoto et al. 2009; Leek et al. 1996; Leek et al. 2000; Condeelis and Pollard 2006). All of the changes that occurred with HF feeding in the MAT secretome, point to the mammary gland microenvironment becoming very pro-tumorigenic.

In vitro analysis of adipokines secreted in CM with HFD and its effect on proliferation and cell death

In trying to understand the dynamic and complicated changes occurring in the MG microenvironment and how the HFD induced changed to the MAT secretome affected mammary epithelial cells, we attempted to reconstruct the MG secretome in vitro with cell lines that were previously derived from the SV40 Tag mouse model (Holzer et al. 2003). We first attempted to examine if the CM from HFD caused any changes to proliferation or cell death compared to the control CM. In preliminary experiments investigating both proliferation and cell death showed

similarly results with both control and HFD CM. HFD CM looks like it may be slightly inhibiting cell death more than the control CM, but this needs to be further investigated (Fig 3-10). Using recombinant proteins for MCP-1 and Pentraxin-3, adipokines that were highly elevated in our adipokine array, and the IncuCyte imaging system to monitor cell proliferation and cell death, we attempted to investigate whether signaling from these individual proteins would cause changes to proliferation or inhibited cell death (fig 3-11). Additionally, we used LPA 18:1, which was previously shown to effectively increase cell proliferation to try and recreate the MG microenvironment in vitro. Analysis of cell death and proliferation over time, showed no conclusive changes in proliferation or in cell death with MCP-1 and Pentraxion-3 or with recombinant LPA 18:1, which was significantly elevated in CM from the HFD secretome. We also attempted to test if inhibiting LPA 18:1 signaling was important for HFD induced changes to the microenvironment using an inhibitor for the LPA receptor corresponding to LPA 18:1. Unfortunately, preliminary experiments investigating how changes to the mammary gland microenvironment with a HFD were inconclusive and therefore further analysis of what is occurring need to be done.

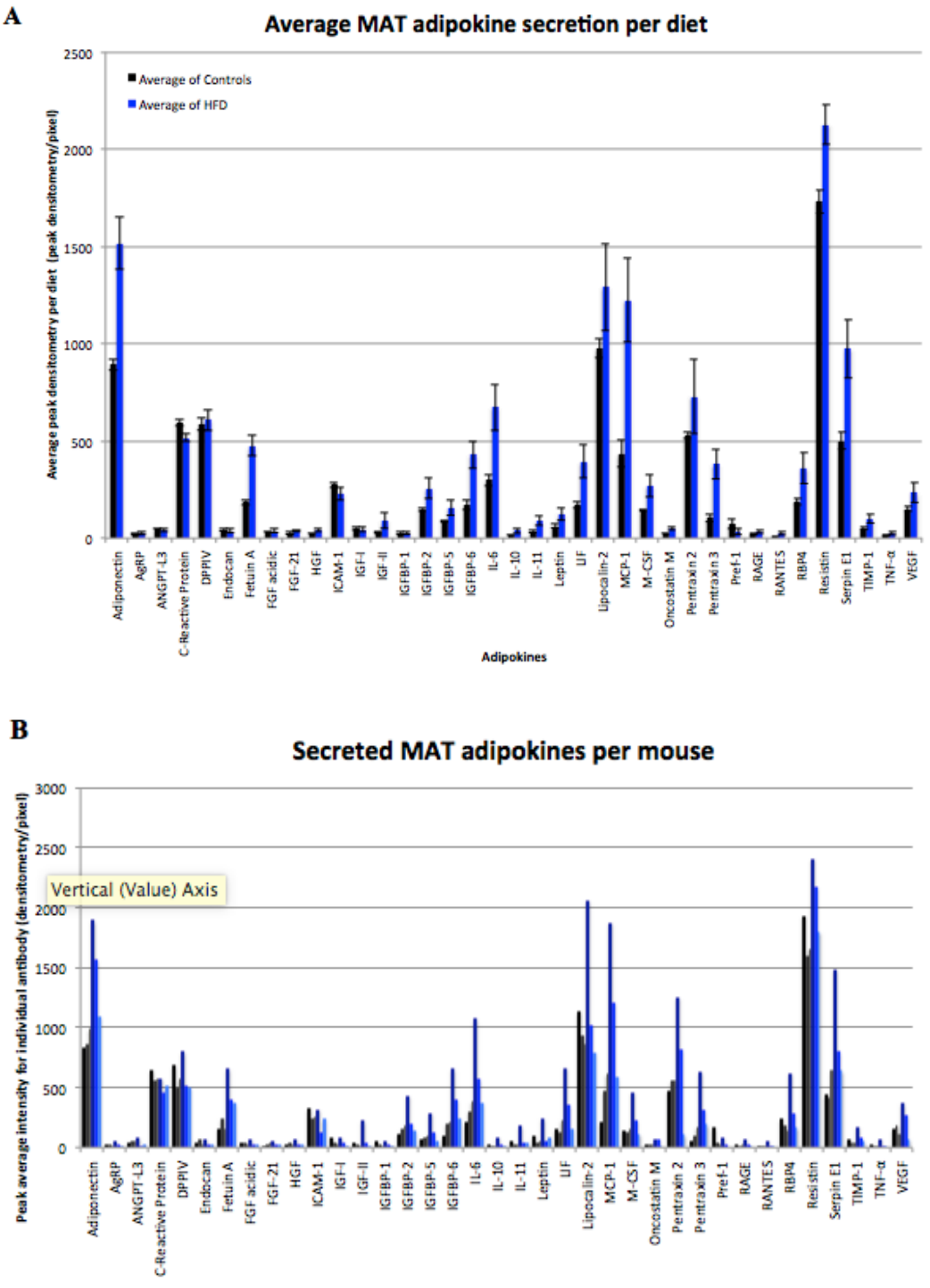

Figure 3-9: HFD feeding leads to increased secretion of growth factors and cytokines. CM from 15-16 week old mice was analyzed using an adipokine array (R&D) to evaluate changes to the MAT secretome. Average MAT adipokine levels can be seen in (A) and individual adipokine levels per mouse are shown in B. Shades of grey and black represent control mice and shades of blue represent HFD fed mice. N = 3 per diet group.

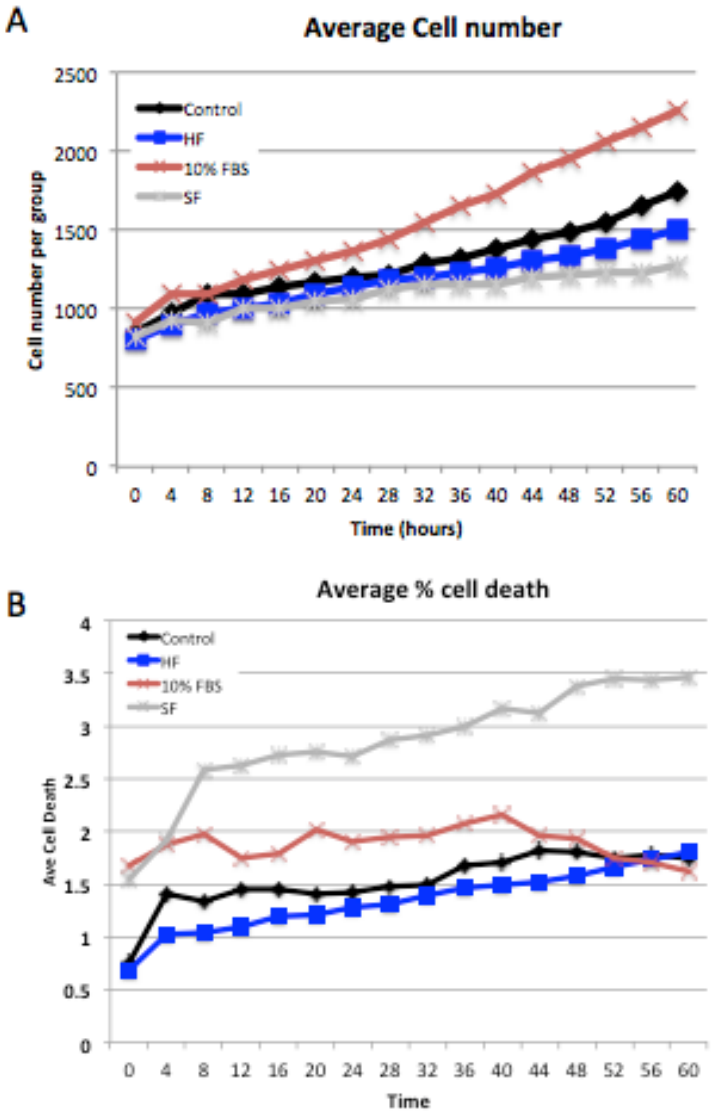

Figure 3-10: CM from HFD fed mice cause a small decrease in cell death but not on cell proliferation. Cell proliferation (A) as well as cell death (B) of M6, mouse mammary carcinoma cells, were evaluated using the InCucyte imaging system. Images were taken of the 96 well plates with M6 cells every 4 hours and then analysized with Image J and the macro codes provided. Each data point represents the average of 3 technical replicates.

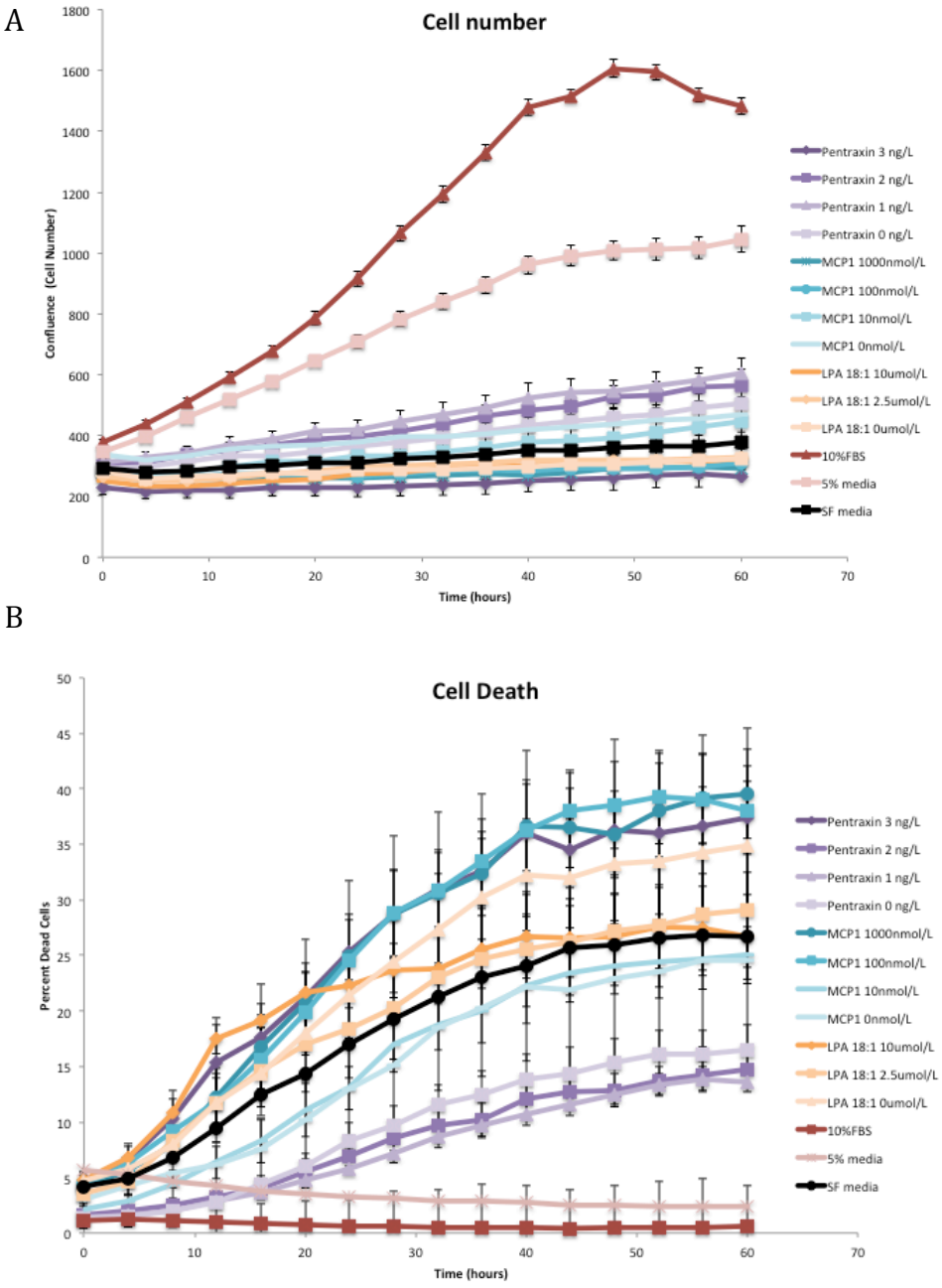

Figure 3-11: Pentraxin 3 may have an effects on proliferation and cell death but not LPA 18:1 MCP1. Cell proliferation (A) as well as cell death (B) of M6, mouse mammary carcinoma cells, were evaluated using the InCucyte imaging system. Images were taken of the 96 well plates with M6 cells every 4 hours and then analysized with Image J and the macro codes provided. Each data point represents the average of 3 technical replicates.

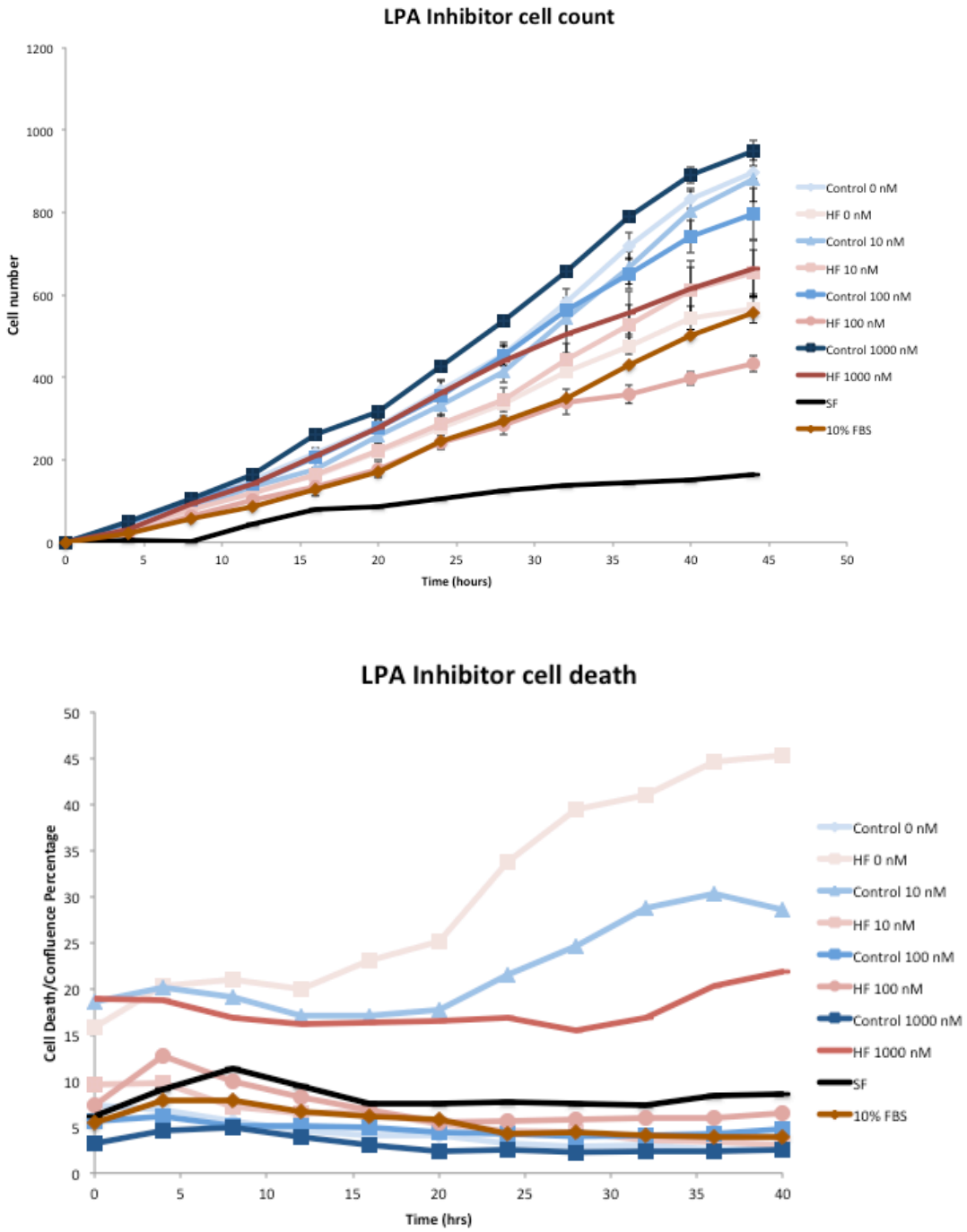

Figure 3-12: Inhibiting the LPA 18:1 receptor had inconclusive effects on proliferation and cell death of M6 cells. Cell proliferation (A) as well as cell death (B) of M6, mouse mammary carcinoma cells, were evaluated using the InCucyte imaging system. Images were taken of the 96 well plates with M6 cells every 4 hours and then analysized with Image J and the macro codes provided. Each data point represents the average of 3 technical replicates.

3. D: Discussion:

Cancer is still the second leading cause of death in the United States, with breast cancer accounting for one-third of cancers diagnosed and the most common form of cancer in women (Siegel, Naishadham, and Jemal 2013; Lacey, Devesa, and Brinton 2002; Boyle 2012). Triple negative breast cancer (TNBC) is a devastating disease that is not only is hard to treat but is also aggressive and an invasive subtype of breast (Maiti et al. 2010; Davis and Kaklamani 2012). TNBC is molecularly, genetically and phenotypically different from other types of breast cancer which is why it is so difficult to treat (Rakha and Ellis 2009). It only accounts for a small proportion of breast cancer cases, but it is responsible for a large proportion of breast cancer deaths making it extremely important to better understand (Podo et al. 2010). TNBC is even more devastating because it effects pre-menopausal, African–American and obese woman making this subtype a health disparity (Boyle 2012; Criscitiello et al. 2012; Hall et al. 2000; Lacey, Devesa, and Brinton 2002). Because there are such few options for women diagnosed with TNBC, understanding how to prevent TNBC and how factors such as obesity and diet, are causing an increased risk of developing TNBC and worse prognosis is crucial.

The increase in breast adipose tissue mass as well as alterations in function to the adipose tissue that occur with excess energy consumption its links to increased TNBC progression is potentially very important, yet is not well understood and why we were so interested in investigating it further. Understanding how a high fat diet affects TNBC progression could lead to not only possible drug targets that would target pathways activated causing the poorer differentiation we saw in our studies but will lead to simple preventive measures becoming available to those groups at risk.

In our study we saw that in a mouse model who's tumors are genetically and phenotypically similar to human malignancy, high fat diet not only made the microenvironment in the MG more tumorigenic but that the consumption of the HFD caused not only more tumors but tumors which were more poorly differentiated. This is the first study to our knowledge that has begun feeding the mice the HFD before adulthood and seen these changes in tumor number and burden, as well as grade. Given the rise in childhood obesity our study is an important finding that shows that children should be eating healthier diets because it will affect them later on in life. There is still so much that needs to be understood about the changes occurring the mammary adipose tissue but this study is just more proof of the importance of adipose tissue in disease.

Our results show that just diet without being accompanied with obesity, can cause changes to the mammary gland, more specifically the adipose tissue, making the mammary gland microenvironment more tumorigenic. We hypothesize these changes to the mammary gland secretome allow for easier transformation of the mammary ductal tree when the epithelial cells are most susceptible before adulthood. These studies point to simpler treatments and preventative measures that could be taken to decrease the risk of developing TNBC.

Chapter IV

Future Studies further investigating the connection between a HFD and triple-negative breast cancer

4. A. Introduction:

The exact impact of changes to our overall diet in the last century has been controversial. There are many conflicting studies on the effect diet has on breast cancer, particularly HFD, with some saying it has no effect. With the increased ingestion of fatter and high sugar containing foods has followed the huge increase of many diseases, including cancer. The link between food higher in fat and sugar and its effects on human physiology was not really considered until recent years. Through the ingestion of higher caloric intake, the body has had to adjust to find ways to deal with the excess nutrition that our bodies have not evolved to deal with (Simopoulos 2001). Some is stored in the liver but for the most part the excess calories, which are turned into lipid, are stored in the adipocyte.

For a long time adipocytes were thought as just storage sites for the body but over the years it was finally realized that adipose tissue is a dynamic endocrine organ. In breast tissue particularly, studies investigating the importance of the crosstalk between adipose tissue and its neighboring epithelial cells have shown the importance of this relationship not only in normal development but also in breast cancer (Wang et al. 2012; Couldrey et al. 2002). The importance of the relationship between both of these tissues was what lead us to investigate MAT as discussed in previous chapters. The studies discussed in previous chapter were only the beginning of a very complicated story that still needs a lot more investigation. These proposed future studies would allow us to continue enriching our knowledge of the effects of diet and

TNBC and help us better understand the interplay between diet and triple negative breast cancer.

4. B. Wide spread analysis of changes to secreted signaling lipids and proteins species derived from mammary adipose tissue after high fat diet feeding:

Understanding the wide spread importance of lipids in normal and disease physiology is extremely vital in being able to better understand all disease states. It is known that lipids are involved in normal physiological signaling but their role in disease states still needs to be widely understood. Most signaling lipids are not studied in the wider context of how they effect and interact with overall signaling pathways in disease states but studied in isolation (Wymann and Schneiter 2008). The intercellular signaling of many lipids like sphingosine, which when converted to sphingosine-1-phosphate (S1P) promotes cell growth and proliferation, are poorly understood (Wymann and Schneiter 2008).

By studying and identifying the lipid and proteins species being secreted from MAT and changed with diet, we will be able to better understand how secretome from these changes to the MG microenvironment are ultimately effecting the neighboring mammary epithelial cells possibly causing cancer later on. Preliminary data (not shown, done in collaboration with the Karczmar lab) showed differences in overall lipid species with HFD as well a MS peak that had not been seen before and was unable to be completely suppressed for clearer imaging of the ducts as had been done previously. We did see some changes to the LPA and LPC lipid species (data shown in chapter 3) but further analyze the all changes to the lipidome would give us a better understanding of the lipid changes from HFD since our LPAR inhibitor studies were inconclusive.

To investigate the lipidomic changes occurring due to HFD we would send CM samples from control and HFD fed mice to the Kansas State Lipidomics Research center as has previously been done (Volden et al. 2016). CM media would be prepared as described in chapter 3, with an 8-hour incubation of MAT with serum free media containing only 1% P/S and 0.1% BSA. CM will be flash frozen in liquid nitrogen after filtration and separation from any remaining tissue. Tissue will be stored in -80° C freezer until it is sent in dry ice to Kansas state. Because of our interest in changes to the MAT before tumors form, we would collect tissue from 12-week-old mice, controlling for hormonal changes by sacrificing all the mice when they are in estrus. Cycle stage will also be evaluated as described in chapter 3. Automated electrospray ionization/tandem mass spectrometry analysis will be done to evaluate the individual lipid changes to the MG secretome. Samples will also be prepared as describe in Volden et al. 2016.

Because of our interest in understanding the overall changes occurring in the MG secretome, we will also send CM media samples to the proteomics core at the University of Chicago to begin to put together the pieces of the big puzzle that is the MG environment. The proteomics data and lipidomics data will be a big data set to analyze but by using this data we may be able to take a more broad approach to really understanding what changes are occurring. We will have a better understanding of the MG milieu, in addition to being able to start mapping out the signaling occurring in the MG microenvironment with changes in diet. Mapping out the MG secretome we allow us to possibly identify some possible target pathways that may be used for treating TNBC, as well as possibly figuring out preventive measurements that can be taken to avoid activating pro-tumorigenic signaling in the mammary gland.

4. C. Analysis of HF diet-induced changes to mammary gland development

Proper development of the breasts, or of mammary glands, has been shown to be very important in inhibiting overall breast cancer risk. In humans, a study evaluating the susceptibility of transformation of tissue samples from the ductal tree at different developmental stages showed that the degree of differentiation of the ductal structure and its cells had a big impact on the cells susceptibility to transformation via carcinogens. The less differentiated the cells and the structure was the more susceptible it was to the carcinogen (Russo and Russo 1994). Previous studies, in carcinogen-based rodent models have shown the similar results. In rodents, mammary epithelial cells are most susceptible to transformation when they are pre-pubescent, before the ductal tree is differentiated opposed to when it has completed differentiation (de Assis et al. 2010).

Most interesting to our group are studies showing changes to the transformation susceptibility in the ductal tree with changes to lipid content of the diet. Rodents fed diets with large amounts of high fat n-3 polyunsaturated fatty acids (PUFA) during puberty and even prenatally were more susceptible to breast cancer and had a decrease in latency in the DMBA carcinogen based mouse model (HilakiviClarke et al. 1997; Hilakivi-Clarke 2007). In our studies and in images shown in chapter 2 & 3 we are able to see we deem are changes to the mammary gland ductal tree morphology. Most of the studies investigating ductal tree development and breast/mammary cancer risk have been done in carcinogen-based models. Using our transgenic mouse model of TNBC, we would be able to study how diets high in fat change not only development but also BC risk by serially evaluating ductal development and investigating if there are changes to BC risk or latency with a diet high in animal fat.

To do these studies, we would have to assess whole mounts of the mammary gland at different stages of puberty and ages to determine if changes are occurring to the mammary gland

ductal tree with a high fat diet. Quantitative analysis of the amount of terminal end buds, where the MG is most susceptible to transformation, would need to be accessed as well as evaluation of the number of mature ductal structures, lobules and alveolar buds, later on in life (de Assis et al. 2010). By determining the types of structures present in the mammary gland, we will be able to access if the increased risk we have seen, as well and the differences in tumor differentiation state were the consequence of improper development due to the high (animal) fat diet. These studies will also help to further understand the importance of a proper diet for children and how the lack of a proper diet may effect their health later on given the high levels of fat ingestion and obesity rates currently observed in children (Cunningham, Kramer, and Narayan 2014).

4. D. Evaluation of epigenetic changes to mammary epithelial cells resulting from changes to the MAT secretome as a result of high fat feeding

The importance of the mammary gland microenvironment and the crosstalk between mammary epithelial cells has already been discussed in chapter 1 but in short, without proper crosstalk between the mammary epithelial cells and the MAT proper development does not occur (Couldrey et al. 2002). This important crosstalk between the MAT and MEC and its role in the differentiation of epithelial cells in the mammary ducts, makes us hypothesize that one of the ways that MAT could be causing the increased risk we are seeing with HFD is through changes to the epigenome of the neighboring mammary epithelial cells. Studies have shown that high fat diet can change the methylation state, therefore down regulating expression, of particular genes but we are interested in what genes are acetylated, therefore up regulated and turned on as a result of high fat feeding (Choi and Friso 2010). Additionally, studies looking at other dietary components, like glucose, have shown epigenetic changes linked cancer, therefore understanding

the effects that high animal fat has on TNBC progression would be pivotal to better understanding just how detrimental not eating healthy can really be (Carrer and Wellen 2015; Hardy and Tollefsbol 2011).

To investigate our hypothesis, we would like to use the normal epithelial, M28, and the ductal carcinoma in situ (DCIS), M27, cell lines derived from the SV40 Tag model (Holzer et al. 2003). Use of these cell lines representing the stages before and the beginning of cancer progression, we will get a better idea of the changes occurring which push epithelial cells over the threshold to become carcinogenic. We would treat each cell line with CM containing MAT secretome from both diet groups for 24-hours, following 24-hour serum starvation, for later evaluation of the changes to the epigenome of the MEC resulting from changes by the HFD group mammary adipose tissue secretome. Before considering changes to the acetylation state of specific genes, we would analyze the overall acetylation changes with HFD compared to control. To evaluate overall acetylation changes we would look at at lysine 12 in H4 histone, lysine 18 in H3 histone and lysines 9 and 14 in H3 histone. These sites were selected because they have already been shown to be modified in cancer, as well as being shown to be associated with transcriptional activation (Puppin et al. 2011). If we do see overall acetylation changes, we would go ahead and do CHIP-seq to determine exactly what genes are being turned on with high fat feeding. Determining what genes are being turned on could lead to possible drug targets later on for the already hard to treat TNBC if after validation of the sequencing results is done.

4. E. Effects of a high fructose diet on a model of TNBC

The worldwide shift towards a "Western diet" higher in fat and fructose that has occurred over the last 20 years has had a major effect on global health (Popkin and Gordon-Larsen 2004).

A huge increase in caloric intake since the 1990s, with no corresponding increase in physical activity has resulted in a worldwide increase in rates of obesity. Epidemiological studies looking at patient outcomes have shown that not only is the risk for breast cancer increased with higher adiposity, but that the odds for successful treatment worsens and mortality increases with obesity especially in patients with TNBC (Sinicrope and Dannenberg 2011) (Carmichael 2006) (Yang et al. 2011) (Whiteman et al. 2005).

Studies analyzing the foods accounting for the increase in caloric intake have shown that Americans consume about 40 percent more fat and about 60 percent more added sugars than recommended. These added sugars include refined cane, beet sugar, corn sweeteners, edible syrups, honey and high fructose corn syrup are consumed leading to the current obesity epidemic (Putnam, Allshouse, and Kantor 2002). It is largely believed that the fats in diet are efficiently stored in adipose tissue, resulting in weight gain and secondary metabolic complications that have been associated with increased cancer risk (Sung et al. 2011; Prieto-Hontoria et al. 2011; Louie, Roberts, and Nomura 2013). In contrast, fructose, whose consumption has also been implicated in the obesity epidemic, has very different systemic effects on the body than other sugars like glucose, sucrose (Port, Ruth, and Istfan 2012) or dietary fats. Fructose results in the generation of differential overall metabolites compared to fat. Fructose is metabolized directly into triglycerides and precursors necessary for proliferation, particularly protein synthesis, which may underlie its contribution to TNBC progression (Frayn and Klingman 1995; Koo et al. 2008; Port, Ruth, and Istfan 2012). The unique alterations in gene expression and potential contributions to cell proliferation with fructose metabolism are why we are interested in understanding the role of fructose in the development of TNBC and its divergent effect from

high fat consumption. The role of diet in BC may be a key factor which we don't yet fully understand and grasping it's overall role could lead to better prognosis and treatment given that poorer BC prognosis is associated with a Western diet, high in fat and fructose (Hauner et al. 2011).

With so much unknown, we began to look at the effects of high fructose diet (HFD) in the SV40 TAg mice but have not been able to come to any particular conclusions as to how fructose may be causing more tumors and worse type of tumors than our control diet but have seen that it (Fig 4-3). We did not see any significant changes to weight, food consumption or even fasting blood glucose after weeks of being on both diets (Fig 4-1) but we did see more tumors and worse types (grade) of tumors with HFrD (Fig 4-3). Ex vivo images of the entire MG show an abnormal signal displayed (fig 4-2). Something is very different about the HFrD fed glands but exactly what it is needs to be further investigated. Initial impressions are that this abnormal signal we are seeing in the MRI is a different type of lipid species that has not been seen before because of our inability to remove its signal through the previously used fat signal removal protocol. These results left us puzzled to what was occurring, so we again turned to the MAT secretome to try and understand what could be causing these less differentiated tumors with HFr feeding (Fig 4-4). We did not see any apparent clues with evaluation of the LPA and LPC species because unlike HFD, the only statistically significant changes were to LPA species and they were all decreased with HFrD (Fig 4-4B) We did see some elevation of cytokines (IL-10 & IL 11), growth factors (FGF-21) and other proteins like Fetuin A, & Endocan increased compared to the control group. Some of the proteins we saw elevated in the HFrD MAT secretome have already been linked to breast cancer, so these could be possible paths to evaluate

in the future. Endocan has been associated with tumor prognosis, metastasis and angiogenesis and IL-11 has been linked to breast cancer metastasis to the bone. Adipokines like these two proteins could be good targets to begin trying to comprehend how HFrD is effecting signaling in the epithelial cells and help us begin to better understand what is occurring with diets high in fructose and TNBC (Yang et al. 2015; Johnstone et al. 2015).

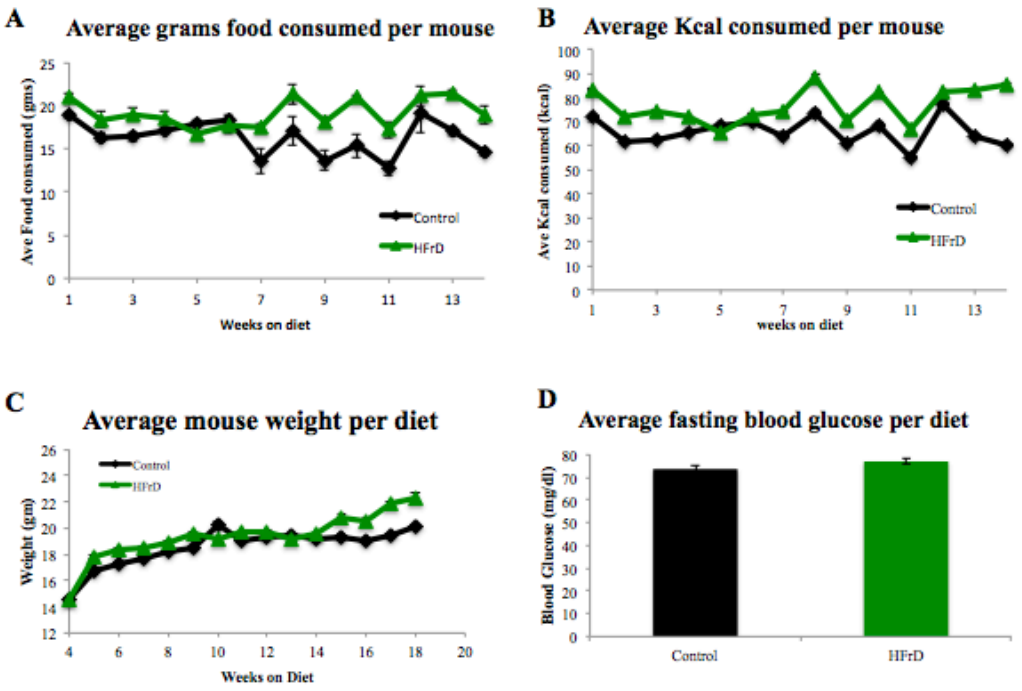

Figure 4-1: Physiological effects of a diet high in fructose. Body weight and food consumption (A-C) were monitored weekly throughout our studies. Fasting blood glucose was evaluated at 17-18 weeks of age following a 6 hour fast and measured using a glucometer (D).

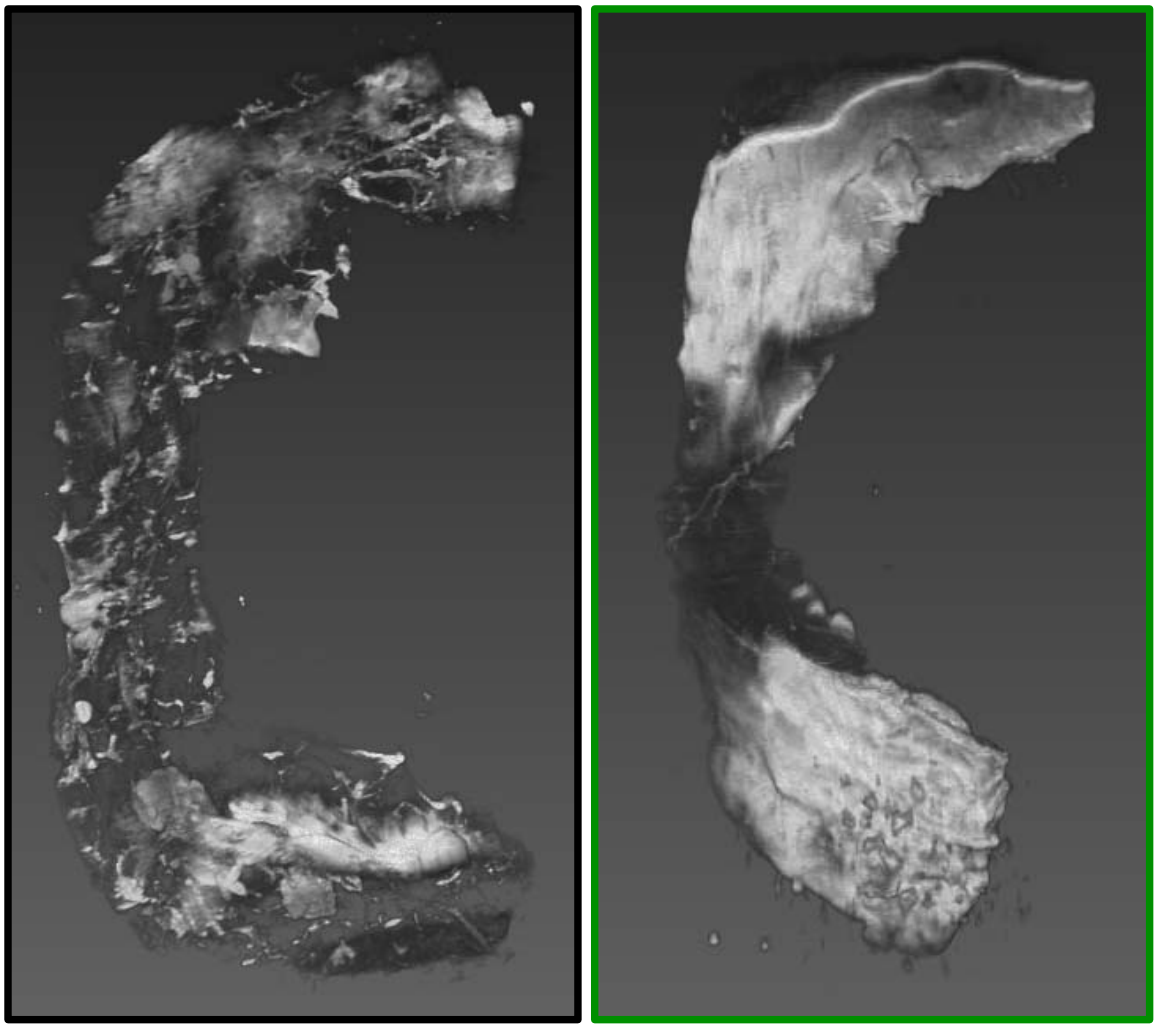

Figure 4-2: Changes to the mammary gland with high fructose diet. Whole mammary glands from 17-18 week old mice were collected ex vivo imaging following in vivo imaging of lower mammary glands. Following dissection, whole mammary glands were fixed and imaged via high resolution MRI imaging. Black boxed control whole gland and green boxed being HFrD.

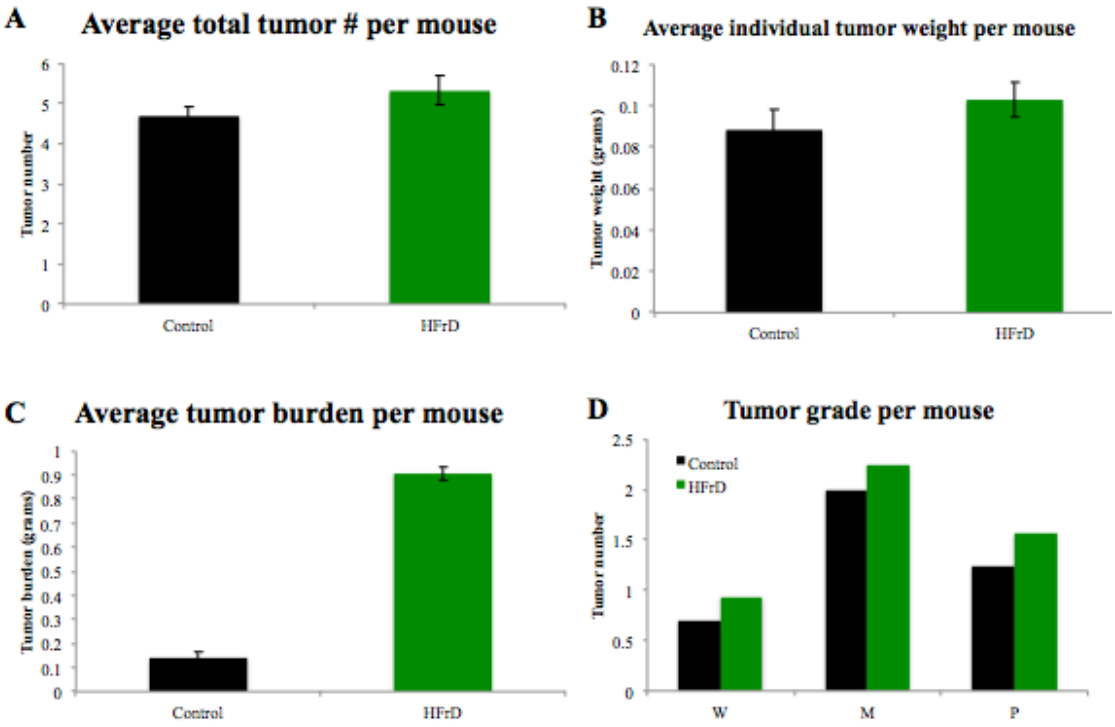

Figure 4-3: HFrd causes increases in tumor weight, burden and incidence and well as worse tumor grade. At 20-21 weeks of age, mice were sacrificed and tumors individual tumors were collected and evaluated upon dissection. HFrD (green) showed a higher amount of tumor number, burden and weight compared to the control group (black). Results of the grading of tumor slides by Dr. Dan Johnson are shown in (D). Black representing control tumors and blue representing HFD tumors.

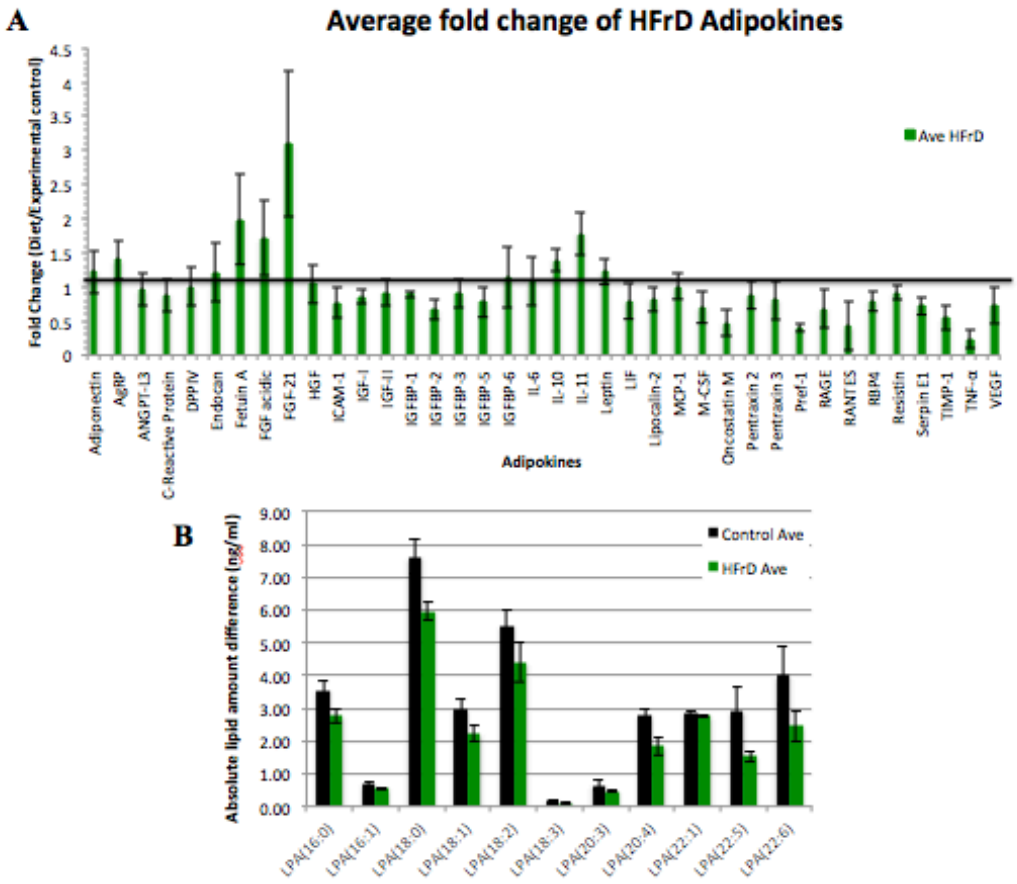

Figure 4-4: Secretome of HFr mammary adipose tissue. CM from 15-16 week old mice was analyzed using an adipokine array (R&D) to evaluate changes to the MAT secretome. Average MAT adipokine levels can be seen in (A). Absolute LPC (A) and LPA (B) concentrations from mammary fat conditioned media derived from 12-week-old mice, were evaluated via mass spectrum analysis. Significance was evaluated using a student t-test. Error bars represent standard deviation and in both panels. N = 3 per diet group.

4. F. Conclusions:

With the data shown we can conclude that HFD (and possibly HFrD) plays a role in causing the mammary gland microenvironment to become more tumorigenic, but there are many questions that still need to be answered. The role of HFD and how it changes the mammary epithelial cell epigenome needs to be better understood so we can understand what genes are being upregulated causing the poorly differentiated tumors we have seen. Additionally, really understanding the network of adipokines and lipids being secreted into the microenvironment of the MG could lead us to finding targets to treat TNBC.

Consumption of large amounts of fructose has become the new norm around the world, but how it is changing overall physiologically and its role in tumor progression is unknown. We saw some leads as to how the microenvironment is changed with HFr consumption but lots of work still has to be done to get a better understanding of what is occurring. We saw very different results with HFrD feeding compared to HFD feeding pointing to very different mechanisms of how they could that are affecting the mammary gland microenvironment. With HFrd we saw the up regulation of proteins adipokines like FGF21, which is up regulated with stressors like fasting to increase gluconeogenesis(Kim and Lee 2015). FGF21 is also interesting because it is up regulated in renal cancer and is associated with worse prognosis but to our knowledge has not been studied in the context to TNBC or BC (ME et al. 2016). Understanding the role of FGF21 in respect to cancer would be really important to understand because down regulating its signaling may be used to treat cancer by cutting off nutrient supply to tumors. Understanding the molecular basis for the differences we are seeing with HFD and HFrD will be key tying together how diet itself is effecting TNBC and through changes to mammary adipose

tissue.

The increase in childhood obesity has left us with many questions of how the horrible diet children are consuming is effecting there overall physiology and what permanent damage they can be causing that they will see the results of later on in life. Investigating how diet effects mammary gland development and differentiation could lead to some preventative treatments that may not only reverse the damage done early on in life, but possibly help to find better treatments for TNBC, which is still hard to treat. All of these studies proposed, in addition the studies discussed in previous chapters will help fill a knowledge gap and help us better understand triple negative breast cancer.

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