OBESITY-INDUCED MAST CELL INFILTRATION AND ACTIVATION IN NORMAL MAMMARY

TISSUE AND CLAUDIN-LOW BREAST TUMORS

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A thesis submitted to the University of North Carolina at Chapel Hill in partial fulfillment of the

requirements for the degree of Bachelor of Science in Public Health in the Gillings School of Global
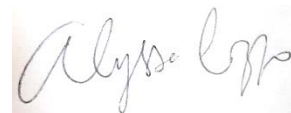
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ABSTRACT

Ottavia Zattra: Obesity-Induced Mast Cell Infiltration and Activation in Normal Mammary Tissue and Claudin-Low Breast Tumors

(Under the direction of Dr. Liza Makowski Hayes)

Obesity is among the most prevalent conditions worldwide: in the United States alone, more than 60% of the population is either overweight or obese. The condition remains a well-known modifiable risk factor for multiple diseases, including cancer. Obesity is characterized by a low-grade smoldering inflammatory state, which both induces and is sustained by many polarized, pro-inflammatory immune cells. Recent studies have also highlighted the role of the immune system in the genesis and progression of cancer, with many immune cell lineages working to promote angiogenesis, tumor growth and metastasis. One of these immune cells lineages are mast cells (MCs), a type of long-lived myeloid stem cell-derived granulocyte that are traditionally known for their role in the allergic response known as anaphylaxis, but also mediate wound healing and vessel formation. MCs play a crucial role in the tumor microenvironment, by secreting proteases, cytokines and other factors that promote angiogenesis, extracellular matrix degradation and metastasis. Previous studies have reported increased mast cell presence in visceral adipose tissue in obesity. Thus, this study investigated whether obesity promotes mast cell infiltration into and activation within normal mammary tissue and claudin-low breast tumors, an aggressive triple negative subtype of breast cancer. To test our hypotheses, we utilized C57BL/6J and FVB/NJ mouse strains. Female C57BL/6J mice were weaned onto either low fat (LFD, 10% kcal from fat) or high fat (HFD, 60% kcal from fat) diet at 3 weeks of age. At 13 weeks of age, C57BL/6J mice underwent syngeneic orthotopic transplant of M-Wnt claudin-low breast cancer cells into their mammary fat pad. In addition, female FVB/NJ mice were randomized to either LFD or HFD at 8 weeks of age; at 13 weeks of age, mice in a Diet Switch (DS) group were also transitioned from HFD to LFD to induce weight loss prior to tumor injection. FVB/NJ mice underwent syngeneic orthotopic transplant of C3-Tag-luc claudin-low breast cancer cells into their mammary fat pad at 18 weeks of age. We sought to measure mast cell infiltration and activation through histological quantification of mast cell density (cells/mm^2), and through quantification of Tryptase β-2 mRNA expression levels, respectively. Relative to tumors, higher mast cell density, as well as higher Tryptase β-2 mRNA expression, was observed in normal mammary of C57BL/6J mice. Importantly, in C57BL/6J mice, higher mRNA expression was also observed across tissues in HFD-fed as compared to LFD-fed mice by two-way ANOVA. In FVB/NJ mice, higher MC density was observed in tumors as compared to normal mammary. Furthermore, higher Tryptase expression was observed in normal mammary tissue upon conditions of obesity, and was significantly reversed with weight loss before tumor development. In summary, these results indicate that low and high fat diets have differential effects on murine models, including body weight and body composition, and that high fat diet may influence mast cell activation in normal mammary tissue, independently of mast cell density. This pilot study offers insights into a potential role of obesity in modulating activation of mast cells, emphasizing the need for future investigation into mast cells as mediators of the relationship between obesity and breast cancer risk or progression.

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STUDY AIMS AND HYPOTHESIS

Several recent studies have highlighted a strong and worrisome correlation between high body mass index (BMI) and cancer malignancy, with multiple immune cell lineages found to play a significant role in the development of both obesity and cancer. Mast cells (MCs), a type of myeloid stem cell-derived granulocyte that are classified as part of the innate immune system, are one of these lineages. In the tumor microenvironment, MCs were reported to secrete angiogenic signals, growth factors and proteases that support tumor development and promote progression and metastasis [1]. In many cancers, high MC density is indicative of adverse clinical outcomes [2]. In obesity, MC accumulation is observed within a number of body fat depots, including visceral (epididymal) and subcutaneous fat pads [3, 4], with MCs sustaining a pro-inflammatory and angiogenic extracellular milieu [4]. Importantly, to date, there have been no studies addressing the influence of obesity on mast cells in the normal breast or breast cancer, leaving a gap that needs to be studied to better understand the underlying mechanisms of obesity-associated breast cancer. Thus, the enclosed studies investigated obesity-induced mast cell infiltration of normal mammary tissue and triple negative breast tumors in two lean and obese pre-clinical murine models. We hypothesized that obesity influences mast cell infiltration into and activation within mammary adipose tissue. We further hypothesized that weight or body composition status influences tumor mast cell infiltration into and activation within claudin-low breast tumors.

To study these hypotheses, we used two orthotopic transplant models of claudin-low breast cancer [5] implanted into adult female mice of C57BL/6J and FVB/NJ backgrounds. Prior to injection of cells to create tumors, mice were exposed to low fat (LFD, 10% kcal from fat) and high fat (HFD, 60% kcal from fat) diets in order to generate lean and obese animals, respectively. A portion of the FVB/NJ obese mouse cohort underwent a diet switch (DS), wherein mice were transitioned from HFD to LFD to induce weight loss before tumor cell injection. Our study was conducted by completing the following two aims. In Aim 1, we

investigated the correlation between obesity and mast cell density and activation in mammary adipose tissue. In Aim 2, we examined the same relationship within the tumor mass itself.

SPECIFIC AIMS

Aim 1: To determine if obesity alters mast cell infiltration and activation in the mouse mammary fat pad

HYPOTHESIS: Adipose MC density will parallel weight gain and weight loss. Mice fed HFD (60% kcal from fat) will present higher MC infiltration and activation within their normal (uninjected) mammary fat pad than mice fed LFD (10% kcal from fat) or induced to lose weight by DS to LFD following a period of obesity. Higher MC infiltration will be observed as higher MC density (cells/mm^2) in HFD vs. LFD/DS normal mammary fat depots, while higher MC activation will be observed as increased mRNA expression of MC-specific Tryptase ß-2 in obese mammary tissue.

RATIONALE: Obesity has been associated with increased MC density in a number of adipose depots, including the epididymal and other visceral fat pads in male mice, and visceral and subcutaneous fat pads in humans [3, 4, 6], but MC accumulation in normal breast tissue has not been extensively studied. The breast is a major adipose depot in the female body. Previous studies by our group and others have shown that, upon conditions of obesity, this depot is subject to infiltration by immune cells, such as macrophages [7]; thus, we posit that obesity might equally induce mast cell infiltration of mammary adipose tissue. Moreover, studies have shown that MC-derived tryptases promote the development of obesity-induced fibrosis in obese adipose, a phenomenon that impairs the structure of fat depots - leading to severe tissue malfunctioning - but that can also be reversed with anti-tryptase antibody treatment [8]. Thus elevated tryptase levels in obese mammary adipose tissue will be used as a proxy measure of MC presence and activation.

EXPERIMENTAL DESIGN:

C57BL/6J mouse cohort:

- Weaned mice onto LFD and HFD at 3 weeks of age

- At 13 weeks of age, performed orthotopic transplant of claudin-low M-Wnt breast cancer cell line into 4th (abdominal) left mammary fat pad

- Monitored weekly for tumor growth by digital caliper

- Collected tumor (tumor length 1 ± 0.25 cm in any direction) and contralateral uninjected normal mammary fat pad at sacrifice

- Established a subset of two mice groups exposed to LFD (n=5) and HFD (n=5)

- Used Toluidine Blue staining to assess MC content (cells/mm^2) in normal mammary in both LFD- and HFD-fed groups

- Assessed tissue MC activation through quantitative gene expression analysis of MC-specific Tryptase β-2 mRNA (qPCR)

FVB/NJ mouse cohort:

- Randomized mice onto LFD and HFD at 8 weeks of age

- At 13 weeks, transferred mice in the DS cohort from HFD to LFD

- At 18 weeks, performed syngeneic orthotopic transplant of C3-Tag-luc claudin-low cancer cell line obtained from lean C3(1)-T_{Ag} mice into the left 4th mammary fat pad

- Monitored thrice weekly for tumor growth by digital caliper

- Collected tumor and contralateral normal mammary fat pad at sacrifice, 3 weeks after injection

- Established a subset of three mouse groups exposed to LFD (n=7), HFD (n=8) and DS (n=8)

- Used Toluidine Blue staining to assess MC content in normal mammary in LFD-fed, HFD-fed and DS groups

- Assessed tissue MC activation through quantitative gene expression analysis of MC-specific tryptase β-2 mRNA (qPCR)

Aim 2: To determine if obesity alters mast cell infiltration and activation in claudin-low breast tumors

HYPOTHESIS: Mice fed HFD group (60% kcal from fat) will present higher MC infiltration in their breast tumor mass than mice fed LFD (10% kcal from fat) or mice in the DS group (60% → 10% kcal from fat). Higher MC infiltration will be observed as higher MC density (cells/mm^2) in obese tumors, while higher MC activation will be observed as increased mRNA expression of MC-specific Tryptase β-2 in obese tumors.

RATIONALE: MCs are one of the immune cell lineages that are polarized within the tumor microenvironment toward a pro-tumoral phenotype [1]. MCs support tumor growth through the secretion of angiogenic factors and proteases, and many MC-derived inflammatory factors, such as tryptase β-2, play a critical role in the promotion of angiogenesis and metastasis [9, 10]. Obesity remains a strong risk factor in the development of breast cancer, especially triple-negative types [11-13]. As an endocrine organ, adipose tissue is increasingly being implicated in the activation process of immune cells toward a pro-inflammatory and pro-tumoral phenotype [14]. Thus, it is plausible that obesity may support MC presence or activation as a contributor to the establishment of an extracellular milieu favorable to tumor growth.

EXPERIMENTAL DESIGN:

C57BL/6J and FVB/NJ mouse cohorts:

- Used Toluidine Blue staining to assess MC content (cells/mm^2) in tumors of LFD, HFD and DS groups
- Assessed tissue MC activation through quantitative gene expression analysis of MC-specific tryptase β-2 mRNA (qPCR)
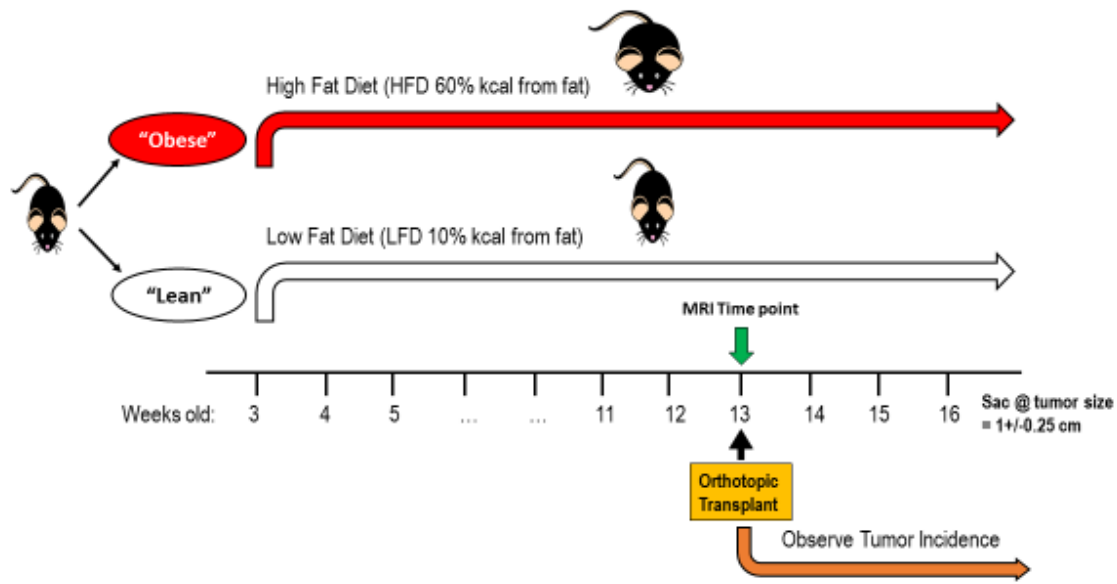
Figure 1. Diet and Orthotopic Transplant Study Design for C57BL/6J mice. Female C57BL/6J mice were weaned onto LFD (10% kcal from fat, n=5) or a micronutrient- and sucrose-matched HFD (60% kcal from fat, n=5) at 3 weeks of age. At 13 weeks of age, mice underwent syngeneic orthotopic transplant of M-Wnt cancer cell line into the left 4th mammary fat pad. Tumor growth was monitored weekly, and detected tumors were measured with calipers every two days. Mice were sacrificed at tumor diameter of 1±0.25 cm in any direction. Tumors and contralateral, uninjected mammary fat pads were collected at sacrifice.
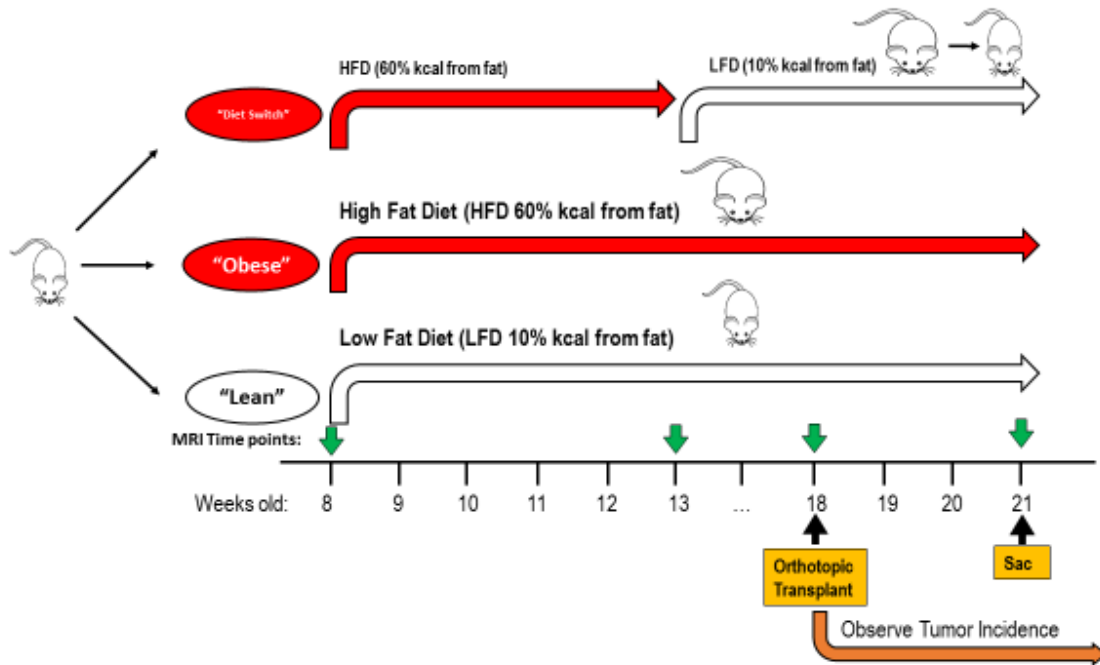
Figure 2. Diet and Orthotopic Transplant Study Design for FVB/NJ mice. Female wild-type FVB/NJ mice were started onto LFD (10% kcal from fat, n=7) and HFD (60% kcal from fat, n=8) at 8 weeks of age. At 13 weeks of age, mice in the DS cohort (n=8) were transferred from HFD to LFD to induce weight loss. At 18 weeks, mice underwent syngeneic orthotopic transplant of the claudin-low C3-Tag-luc cancer cell line into the left 4th mammary fat pad. Tumor growth was monitored thrice weekly. All mice were sacrificed 3 weeks after tumor cell injection, at 21 weeks of age. Tumor and contralateral uninjected mammary fat pads were collected at sacrifice.

INTRODUCTION

Obesity levels in the United States have reached epidemic proportions. According to the Centers for Disease Control and Prevention (CDC), in 2014 more than one-third of U.S. adults and 17% of adolescents were obese, with no U.S. state experiencing a rate below 20% [15] The rising prevalence of obesity is a major public health concern, as the condition is a renowned risk factor for a variety of chronic illnesses, most notably hypertension, cardiovascular disease, stroke, and type-2 diabetes [16].

Importantly, recent epidemiological studies have also identified obesity as a significant risk factor for multiple cancer types, including esophagus, colon and rectum, endometrial and breast malignancies [17] Indeed, excess adiposity contributes to approximately 20% of all cancers [17], with a total health burden that will soon surpass that of cigarette smoking [18]. In women, obesity is strongly associated with breast cancer, with high BMI linked to greater incidences, higher rates of lymph node positivity and poorer prognosis [11-13].

The Carolina Breast Cancer Study conducted in 2006 by Carey et al. characterized breast cancer subtypes on the basis of their molecular presentation of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2). The four main breast cancer characterizations that resulted from the study were: Luminal A (ER+, PR+, HER2-), Luminal B (ER+, PR+, HER2+), HER2-enriched (ER-, PR-, HER2+), and triple-negative (ER-, PR-, HER2-) breast cancers [19].

Among the four subtypes, triple-negative breast cancers (TNBCs) account for approximately 10-20% of invasive breast cancer incidence [20, 21], and disproportionately affect women who are of African ancestry, premenopausal and below the age of 40, have a family history of breast cancer and/or present a BRCA1 mutation [19, 20, 22]. An analysis of 2544 invasive breast cancer cases from two prospective studies also found that a TNBC diagnosis is more likely associated with overweight and obese BMIs [23]. TNBCs display a highly aggressive phenotype, with peak recurrence within 3 years of diagnosis [20], greater incidence of metastasis, poorer prognosis and lower 5-year survival rates relative to other breast

cancer subtypes [19]. Because of their molecular profile, no biologically targeted therapy is currently available for the treatment of TNBCs, a fact that makes clinical management of these cancers particularly challenging.

A new molecular class of TNBC, known as claudin-low, was recently described based on extensive genomic profiling of breast cancer patient biopsies [24, 25]. Claudin-low breast cancers are characterized by a low genetic expression of tight junction proteins claudin 3, 4 and 7, as well as low calcium-dependent cell-cell adhesion glycoprotein E-cadherin [21]. In comparison with more widely known basal-like breast cancer subtype, claudin-low tumors are slower-cycling, thus showing reduced RNA levels of proliferation marker Ki67, but present increased expression of immune response, angiogenesis, and cell migration genes [21]. In particular, due to the tendency for extensive immune response, we have utilized claudin-low breast cancers in our studies of obesity-mediated immune cell presence and activation.

While a cancer's ability to survive and proliferate is often attributed to genetic aberrations of the cancer cell itself, studies in the last few decades have highlighted the pivotal role that tumor-driven modulations of physiological processes, both in the surrounding microenvironment and at a systemic level, have in the process of tumorigenesis. In particular, the development of malignancy is characterized by a profound suppression of immunosurveillance, with tumor cells obstructing immune system-driven antitumoral responses [26]. This process not only manifests as changes in cancer cells that make them less recognizable by the immune system, but also as the recruitment of select immune cell populations and/or their polarization toward phenotypes that favor cancer growth [26]. For example, among immune cells, immunosuppressive T-regulatory cells play a crucial role in tumor progression [27, 28], and remain the main obstacle to successful immunotherapy treatment [28]. Tumor-Associated Macrophages (TAMs) are also involved in cancer growth, working to establish a mutagenic and cancer-promoting environment through their oxidative function, favoring migration of tumor cells through ECM degradation, and promoting the formation of new blood vessels for tumor survival through the secretion of angiogenic factors [29, 30]. Similarly, Tumor-Associated Neutrophils (TANs) have been found to sustain angiogenesis

through the secretion of Vascular Endothelial Growth Factor (VEGF) and Matrix Metalloproteinase-9 (MMP9) [31-33]. Indeed, infiltration of several immune cell populations into cancers is associated with poor outcomes: infiltration of TAMs in mammary tumors is an independent prognostic factor linked to lower overall survival [29, 34, 35], tumor recruitment of MMP9^{+} TANs has been correlated with increased angiogenesis and intravasation [32], and systemic removal of Tregs was found to boost natural and vaccine-induced anti-tumoral T-cell immunity [27].
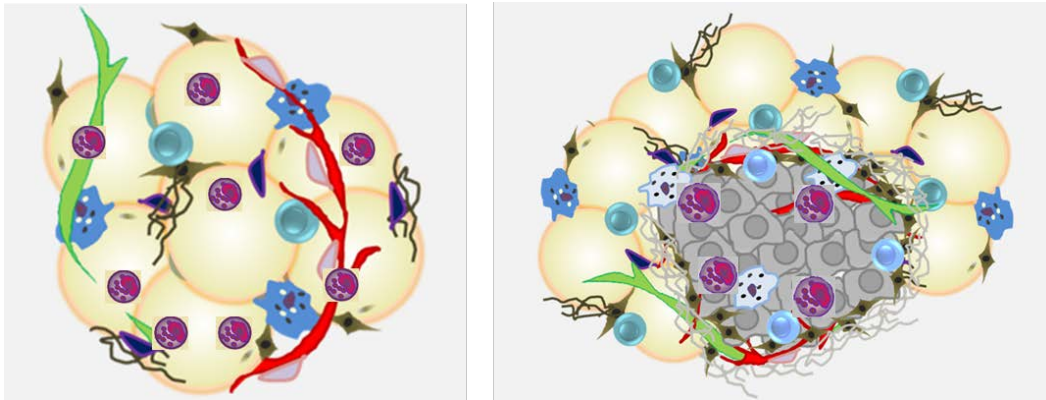

Figure 3. MC Infiltration in obese adipose tissue and tumors. (Cozzo, Fuller and Makowski, submitted)

Our current study focuses on an immune cell subset known as mast cells (MCs), which are a type of long-lived myeloid stem cell-derived granulocytes. MCs are traditionally known for their role in anaphylaxis, through their release of histamine, heparin and proteases tryptase and chymase, but have also recently been associated with processes of wound healing, immune tolerance and angiogenesis [2]. In fact, T-cell suppressing-interleukin-10, as well as potently angiogenic and vasculogenic factors VEGF, IL-6 [1] and TNFα [36] have all been detected among MC secretions. These functions make MCs perfect targets of tumor recruitment, and recent studies have confirmed that MCs play a pivotal role in the progression of malignancies such as breast cancer [1]. Once recruited at the tumor site through the exploitation of the Stem Cell Factor (SCF)/cKIT signaling pathway, MCs secrete angiogenic factors and growth factors to support vessel formation, thus often accumulating near microvessels, and tumor development. Other MC-specific secretions include proteases, such as tryptase and chymase, that activate metalloproteinases for ECM

degradation, outward tumor expansion and metastasis [1, 2]. Tryptase-containing MCs are usually found in greater numbers on the tumor invasive front and peritumoral tissue, and are associated with higher lymphangiogenic and angiogenic microvessel density [2, 37, 38]. MC infiltration remains an independent prognostic factor in many cancers, with high MC density predictive of adverse clinical outcomes in human colorectal, pancreatic and lung carcinomas, as well as murine pancreatic carcinoma and colon adenocarcinoma [2]. It is unknown to what extent MC infiltrate tumors of the breast.
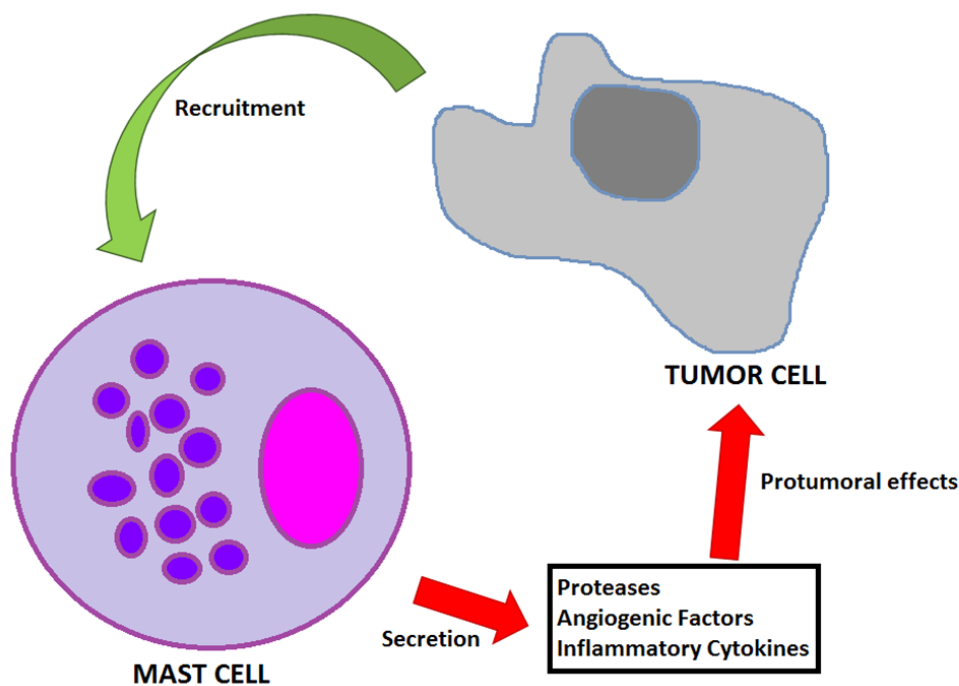
Figure 4. Interaction between a cancer cell and a mast cell in the tumor microenvironment [2]

Importantly, MC accumulation is characteristic of obesity in both mice and humans [3, 4, 6]. Several folds-increase in MC density have been reported in numerous body fat depots including white adipose and epididymal fat pad in male mice [3, 4], and white adipose tissue and subcutaneous and omental fat pads in male humans [4, 6]. As it is the case in tumors, MCs in adipose tissue were found in association with microvessels, suggesting their role in adipose angiogenic modulation [4]. In addition to MC content, obesity affects the activation status of MCs, promoting their degranulation and their high secretion levels of inflammatory cytokines IL-6, IL-1β and MCP-1 [6]. These findings, and the discovery by Ishijima et al.

that MC-deficient cKIT-knockout mice are resistant to diet-induced obesity [39], highlight the role of MCs, together with other immune cells, in the promotion of obesity and the maintenance of its characteristic smoldering inflammation in adipose depots that may contribute to oncogenesis or tumor progression.

The dual role played by MCs in the pathogenesis of cancer and obesity suggests that they may contribute to the reported association between these two epidemic conditions. This could be especially true in the case of high BMI- and high adiposity-correlated carcinomas, such as TNBCs. In both humans and mice, breast/mammary adipose is a major body fat depot, in which obesity-induced metabolic changes may also activate resident and recruited MCs toward a pro-inflammatory and pro-tumorigenic phenotype. In light of this relationship, it is imperative to elucidate the interplay between MC activation and infiltration and TNBCs in relation to weight status. Thus, we first hypothesized that obesity increases MC activation and infiltration in mammary adipose tissue. We further hypothesized that obesity promotes greater MC activation and infiltration into claudin-low breast tumors.

METHODS

Animals and Diet

This study used data from ongoing pre-clinical studies conducted by Dr. Liza Makowski's Laboratory at the Gillings School of Global Public Health within the University of North Carolina at Chapel Hill (Cozzo et al. and Fuller et al., manuscripts in preparation). Animal studies were performed with approval and in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Animals were cared for following guidelines established by the Panel on Euthanasia of the American Veterinary Medical Association, and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, which meets the National Institutes of Health standards (Guide for the Care and Use of Laboratory Animals, 85-23, 1985). The UNC animal facility providing care is AAALAC-approved. UNC also accepts as mandatory the PHS Policy on Humane Care and Use of Laboratory Animals and NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. Animal studies comply with the ARRIVE guidelines. Mice were housed in a climate controlled Department of Laboratory Animal Medicine facility with a 12 h light:dark cycle. C57BL/6J and FVB/NJ mouse strains were used in this pilot study. The *Slc2a1*/GLUT1 floxed (GLUT$^{fl/fl}$) C57BL/6J mouse strain was obtained through collaboration with Dr. Dale Abel (University of Iowa, IA). The FVB/NJ strain was obtained from Jackson Laboratories (Bar Harbor, ME). Diets were obtained from Research Diets Inc. (New Brunswick, NJ, USA), which provided 10% kcal ("LFD", D12450J) and 60% kcal ("HFD", D12492) from fat and were matched for proteins, sucrose, vitamins and minerals.

Female mice in the C57BL/6J cohort were weaned onto diet at 3 weeks of age and randomized to either LFD or HFD (n=5 on LFD, n=5 on HFD selected for analysis). Animals remained on diet until sacrifice. Female mice in the FVB/NJ cohort were randomized to diets at 8 weeks of age (n=7 on LFD, n=8 HFD selected for analysis). Additional FVB/NJ mice (n=8 selected for analysis) was started on HFD at 8 weeks of age, and were then switched to LFD at 13 weeks of age through the completion of the study.

Body Weight and Composition

C57BL/6J Cohort

Body weight was measured prior to starting animals on diet (baseline), and once weekly until sacrifice. Body composition including lean mass, fat mass, free water content and total water content of non-anesthetized mice was measured at week 13, before injection of tumors, using an EchoMRI-100 quantitative magnetic resonance whole body composition analyzer (Echo Medical Systems, Houston, TX), as previously described [40].

FVB/NJ Cohort

Body weight was measured as above. Body composition was measured at week 8 (baseline), 13 (diet switch, DS), 18 (orthotopic transplant) and 21 (sacrifice) as above [40].

Injection of tumors

C57BL/6J Cohort

The murine M-Wnt claudin-low breast cancer cell line was obtained through a collaboration with Dr. Stephen Hursting (UNC-Chapel Hill, NC). Features of this cell line, including a $CD44^+/CD24^-$-enriched profile and high EMT marker expression, have been previously described [5]. Following 10 weeks on diet, at 13 weeks of age, animals underwent syngeneic orthotopic transplant of 50,000 M-Wnt cells in 100 μL phosphate-buffered saline (PBS) (Sigma Aldrich, St. Louis, MO) directly inoculated into the left 4th mammary fat pad. Tumor size was monitored weekly by electronic calipers until sacrifice at the previously defined tumor endpoint (tumor diameter 1 ± 0.25 cm in any direction), as described in previous studies [41].

FVB/NJ Cohort

A luciferase-labeled claudin-low cancer cell line generated from lean C3(1)-T_{Ag} mice (referred to as C3-Tag-luc) was obtained from Dr. William Y. Kim, MD through a collaboration with the Mouse Phase I Unit (MP1U, LCCC, Chapel Hill, NC) [42]. At 18 weeks of age, animals in all three diet groups underwent orthotopic transplant of 200,000 cells in a 1:1 mixture of high concentration Matrigel® (BD Biosciences, Bedford, MA) and Hank's Buffered Saline Solution (Sigma Aldrich, St. Louis, MO), into the left 4th mammary fat pad. Tumor length and width were measured three times weekly after tumor injection via electronic calipers until sacrifice at 21 days of tumor growth. Tumor volumes were calculated as length $\times$ width$^2 \times 0.5$, as previously described [40].

Tissue Harvest

C57BL/6J Cohort

Mice were euthanized through carbon dioxide asphyxiation and cervical dislocation. Tumors and the contralateral normal mammary fat pads were collected. A portion of each tissue was frozen at -80˚C, and another portion was stored in cassettes and formalin-fixed and paraffin embedded (FFPE) for histological analysis. Lungs, liver and spleen were also collected.

FVB/NJ Cohort

Mice were anesthetized by intraperitoneal injection of avertin (tribromoethanol/amylene hydrate, 1.25%) (Sigma Aldich, St. Louis, MO). Blood was collected via cardiac puncture, and cervical dislocation was also performed, in accordance with humane euthanasia regulations. Tumors and the contralateral normal mammary fat pads were collected. A portion of each tissue sample was frozen at -80˚C, and another portion was stored in cassettes and formalin-fixed and paraffin embedded (FFPE) for histological analysis. Lungs, liver and spleen were also collected.

Histological Analysis

    C57BL/6J Cohort

Paraffin-embedded tumor and normal mammary fat pad sections collected at 5 μm frequency were mounted onto clear-glass slides. Tumor samples in lean and obese groups were size-matched at 1±0.25 cm. Normal mammary samples in lean and obese groups were age-matched at 5±0.5 weeks after tumor cell injection. Both tumor and normal mammary slides were stained with Toluidine Blue, through the following protocol performed by the lab of Dr. Stephen Tilley (UNC-Chapel Hill, NC). Briefly, slides were dewaxed and rehydrated with 60% ethyl alcohol, before being exposed to Toluidine Blue O (C.I. 52040) for 1-2 minutes. Slides were then rinsed under water and dehydrated twice through exposure to acetone for 1-2 minutes. Once stained, slides were scanned into an Aperio Scanscope CS System (Aperio Technologies, Vista, CA. USA) at a magnification of 40x. Total tissue area was quantified in mm^2 using Aperio Imagescope software (Aperio Technologies, Vista, CA. USA). Also using Aperio Imagescope, total mast cell number in each slide was manually counted and normalized to total tissue area.

    FVB/NJ Cohort

Samples for analysis were chosen based on proximity to the median tumor volume of each respective diet group. Paraffin-embedded tumor and normal mammary fat pads were sectioned at 5 μm thickness in a serial interrupted fashion and mounted onto clear-glass slides. Slides were stained with Toluidine Blue, through the aforementioned protocol, and analyzed using Aperio as described above.

Gene Expression

Gene expression of mast cell-specific Tryptase β-2 was measured using semi-quantitative real-time PCR (qPCR). The eukaryotic ribosomal RNA 18S was used as an endogenous control to standardize gene expression levels. Tryptase β-2 mRNA expression levels were quantitated using TaqMan Assay-on-

Demand (AOD) TPSB2 (Thermo Fisher Scientific, Waltham, MA). All reactions had a volume of 10 μL. AOD reactions contained 1 μL cDNA, 3.5 μL of deionized water, 5 μL of SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5 μL 18S and 0.5 μL AOD. Reactions were run at 95°C for 10 seconds, then 60°C for 30 seconds for 40 cycles in a using an Applied Biosystems ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). Data were analyzed using QuantStudio Real-Time PCR System software (Thermo Fisher Scientific, Waltham, MA) and the comparative $\Delta\Delta C_T$ method.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Means were compared as indicated either by Student's *t*-test, One-way Analysis of Variance (ANOVA) or Two-way ANOVA, with Tukey's Post Hoc Multiple Comparisons testing, using the GraphPad Prism 7 software (GraphPad Software, Inc. La Jolla, CA). —P-values < 0.05 are considered statistically significant.

RESULTS

Body Weight and Body Composition

C57BL/6J Cohort

At 3 weeks of age, mice were weaned onto either Low Fat Diet (LFD, 10% kcal from fat, n = 5) or High Fat Diet (HFD, 60% kcal from fat, n = 5). Between week 16 and 17, mice fed HFD had significantly greater body weights than mice fed LFD (P < 0.05, **Figure 5a**). Body composition was measured by MRI at the time of tumor cell injection, and mice on HFD had significantly greater percentages of body fat than mice on LFD (P = 0.0028, **Figure 5b**).

FVB/NJ Cohort

At 8 weeks of age, mice were randomized to diet groups: either LFD (10% kcal from fat, n = 7), HFD (60% kcal from fat, n = 8), or DS (60% → 10% kcal from fat, n=8). Starting at week 10, mice fed HFD had significantly greater body weights than mice fed LFD (p < 0.01, **Figure 6a**) and the trend continued to the end of the study (weeks 11 to 21, P < 0.001). At week 13, DS mice were switched to LFD. One week after the switch, the body weights of DS group were comparable to the LFD group, and were significantly different from mice fed HFD (weeks 14-15, P < 0.01, weeks 16-21, P < 0.0001, **Figure 6a**).

Body composition followed a similar trend. At 8 weeks of age, before starting on diet, mice in all groups had the same body composition. At week 13, HFD-fed and DS mice had approximately 2-fold greater body fat percentages than mice fed LFD (P < 0.0001, **Figure 6b**). After being switched to LFD, the body composition of mice in the DS group returned to the LFD baseline: at weeks 18 and 21, the percentage body fat of mice in the DS group was found to be significantly different from the mice on HFD (P < 0.0001, **Figure 6b**).

Within the subset of mice selected for mast cell analysis in this pilot study, mice fed HFD also showed increased tumor volumes with respect to LFD-fed mice and DS mice (P < 0.01 HFD vs. LFD/DS, **Figure 7A**).
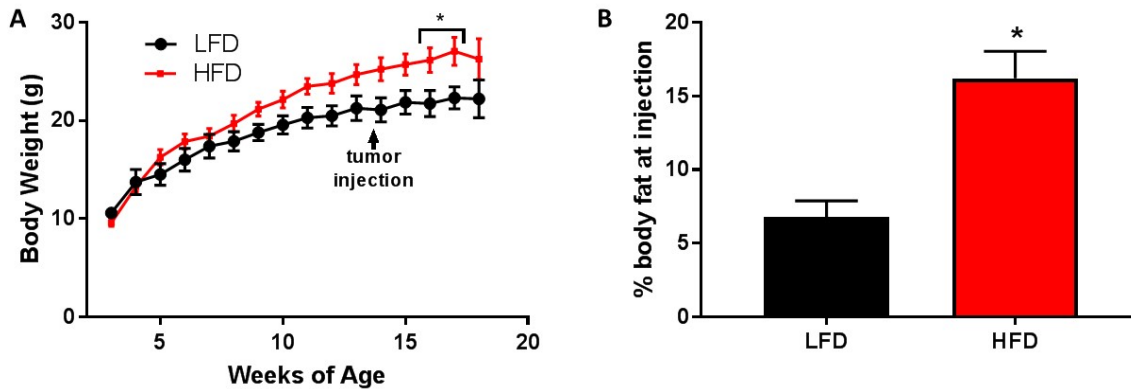
Figure 5. C57BL/6J mice on HFD experienced significant weight gain and increased percentage body fat. (A) Body weight (g) was measured weekly from 3 weeks of age until sacrifice for low fat diet (LFD, n=5) and high fat diet (HFD, n=5) groups. * P < 0.05 for weeks 16-17 HFD vs. LFD. **(B)** Body composition (g fat mass/g body weight) from MRI was obtained at week 13 upon injection of tumor cells. * P = 0.0028 HFD vs. LFD.

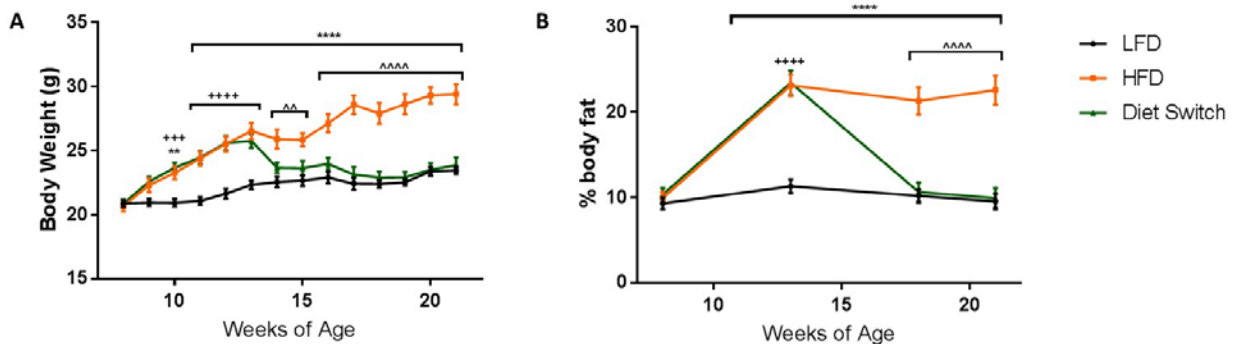**Figure 6. FVB/NJ mice fed HFD experienced significant weight gain and increased percentage body fat, while Diet Switch mice experienced body weight and fat loss and returned to LFD baseline. (A)** Body weights (g) was measured weekly from 8 weeks of age until sacrifice for LFD (n=7), HFD (n=8) and DS (n=8) groups. ** P < 0.01 for week 10, LFD vs. HFD. **** P < 0.0001 for weeks 11-21, LFD vs. HFD.

+++ P < 0.001 for weeks 10, LFD vs. DS. ++++ P < 0.0001 for weeks 11-13, LFD vs. DS. ^^ P < 0.01 for weeks 14-15, DS vs. HFD. ^^^^ P < 0.0001 for weeks 16-21, DS vs. HFD. **(B)** Body adiposity (% body fat) was measured at four different time points: baseline, DS, orthotopic injection, and sacrifice. **** P < 0.0001 LFD vs. HFD. ++++ P < 0.0001 LFD vs. DS. ^^^^ P < 0.0001 DS vs. HFD.
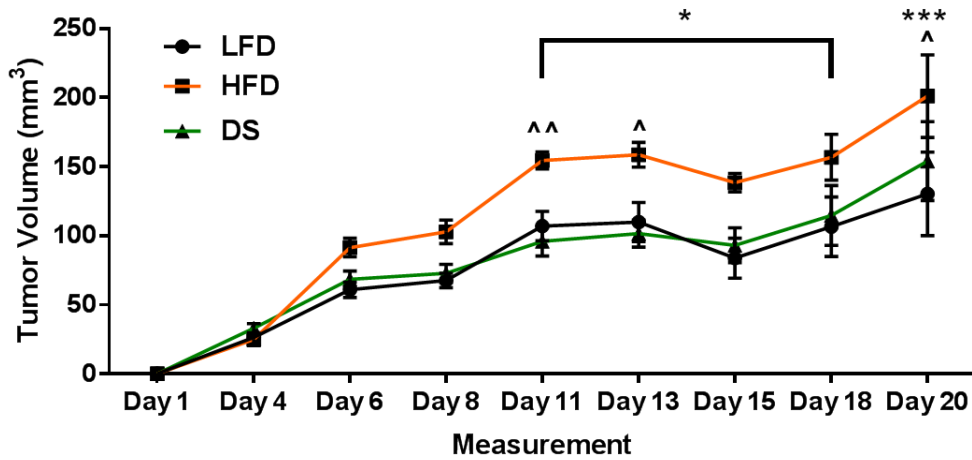

Figure 7. In the original FVB/NJ cohort, HFD exposure accelerated tumor progression. Tumor volume (mm^3) was measured from orthotopic transplant to sacrifice 3 weeks later as length × width^2 × 0.5. *P < 0.01 LFD vs. HFD. *** P = 0.001 LFD vs. HFD. ^ P < 0.05 HFD vs. DS. ^^ P = 0.009 HFD vs. DS.

Mast Cell Density

C57BL/6J

Mast cell density in both normal mammary tissue and tumors of C57BL/6J mice was reported as number of toluidine blue-positive cells per mm^2 of tissue. Mast cell density trended toward lower values in normal mammary tissue of mice on HFD as compared with mice on LFD, but the difference was not significant (**Figure 8a**). A non-significant increase was also seen in claudin-low tumors, with mice in the HFD group presenting slightly higher mast cell density than mice in the LFD group (**Figure 8b**). A comparison of mast cell densities across tissue types by two-way analysis of variance (ANOVA) indicated the presence of a tissue effect, with mast cell density of normal mammary tissue significantly greater than that of tumors (P = 0.037, **Figure 8c**). No diet effect was detected.
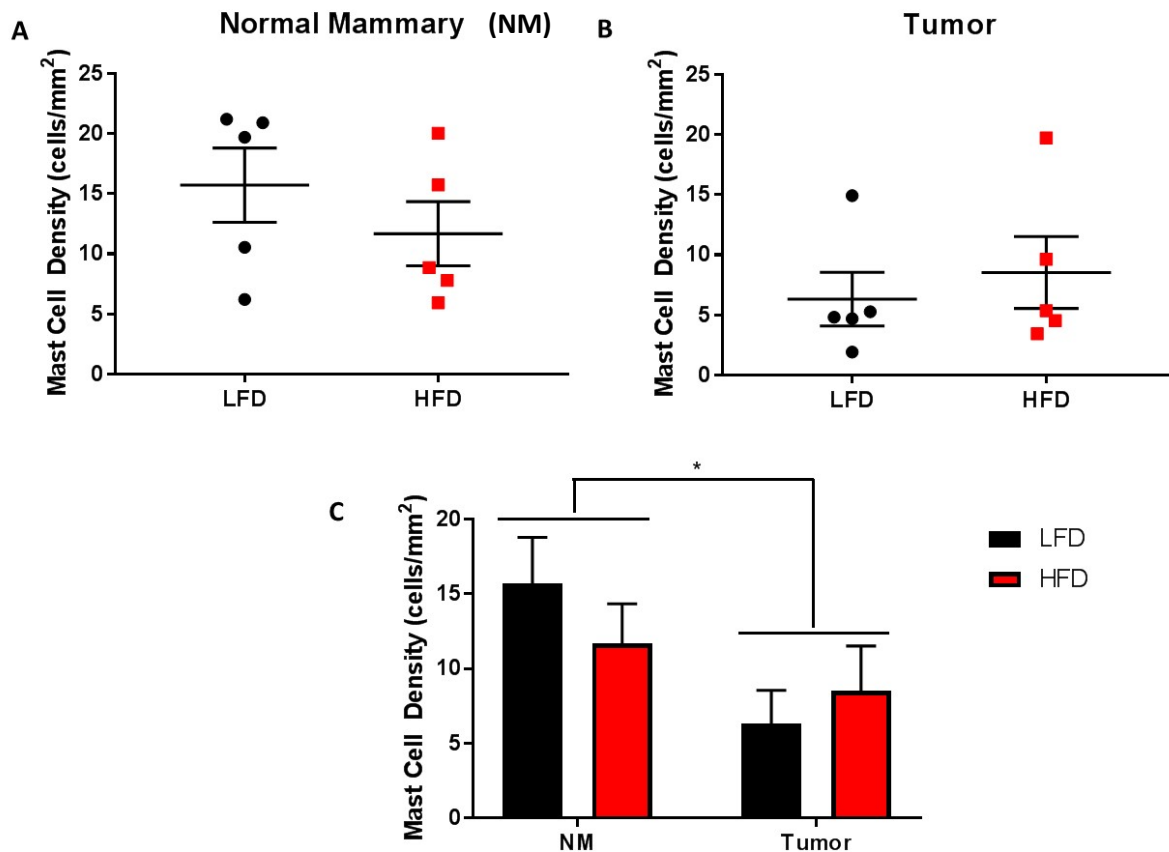
Figure 8. Comparison of Mast Cell Density between HFD and LFD groups in normal mammary and tumor tissues of C57BL/6J mice. (A) Mast cell density (cells/mm^2) in normal mammary tissue was measured. **(B)** Mast cell density (cells/mm^2) in claudin-low tumors was measured **(C)** Mast cell densities were compared across diet groups and tissue types. P = 0.037 NM vs. tumor by two-way ANOVA.

FVB/NJ

Mast cell density in both normal mammary tissue and tumors of FVB/NJ mice was reported as number of toluidine blue-positive cells per mm^2 of tissue. Mast cell density trended toward elevated in normal mammary tissue of mice in the DS as compared with mice on HFD or LFD, but the difference was not significant (**Figure 9a**). A similar trend was also seen in claudin-low tumors (**Figure 9b**). A comparison of mast cell densities across tissue types by two-way ANOVA indicated the presence of a tissue effect, with mast cell density of tumors significantly greater than that of normal mammary (P = 0.039, **Figure 9c**), as well as a diet effect (P = 0.033, **Figure 9c**).
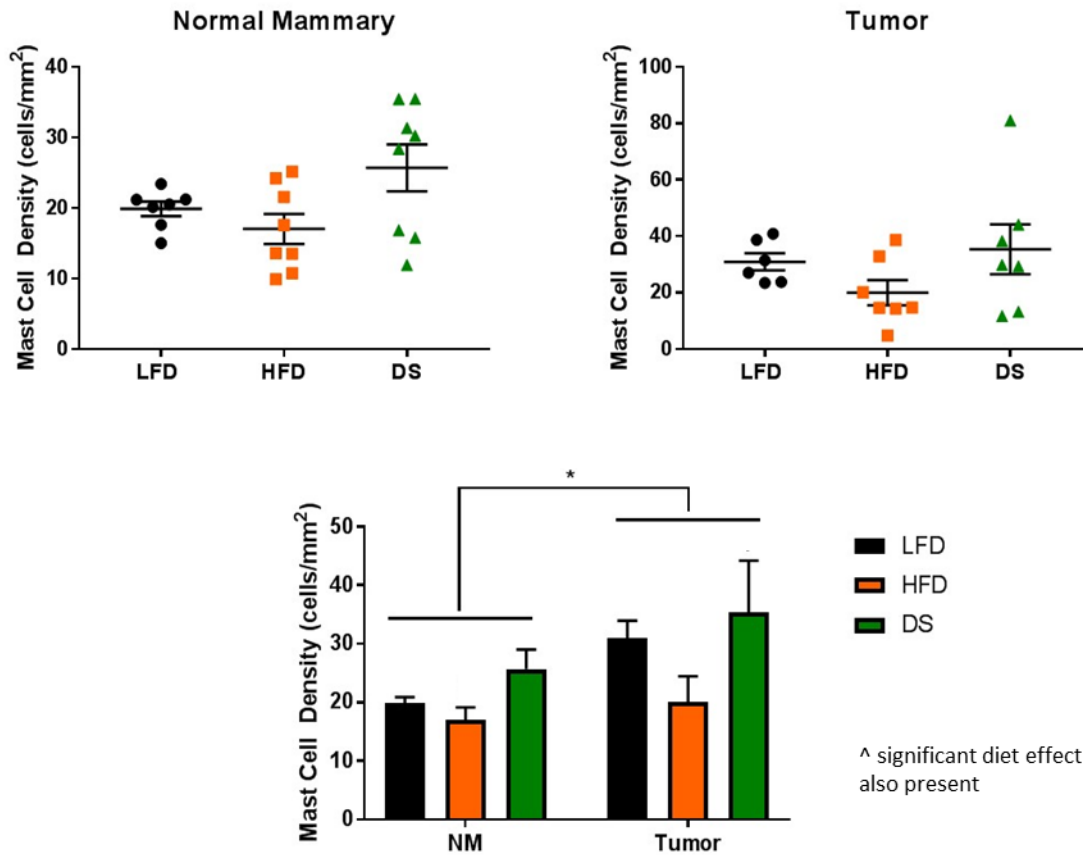
Figure 9. Comparison of Mast Cell Density between HFD, LFD and DS groups in normal mammary and tumor tissues of FVB/NJ mice. (A) Mast cell density (cells/mm^2) in normal mammary tissue was non-significant by diet (P = 0.054 by one-way ANOVA). **(B)** Mast cell density (cells/mm^2) in claudin-low tumors was measured. **(C)** Mast cell densities were compared across diet groups and tissue types. *P = 0.039 NM vs. tumor by two-way ANOVA. P = 0.033 for diet effect by two-way ANOVA.
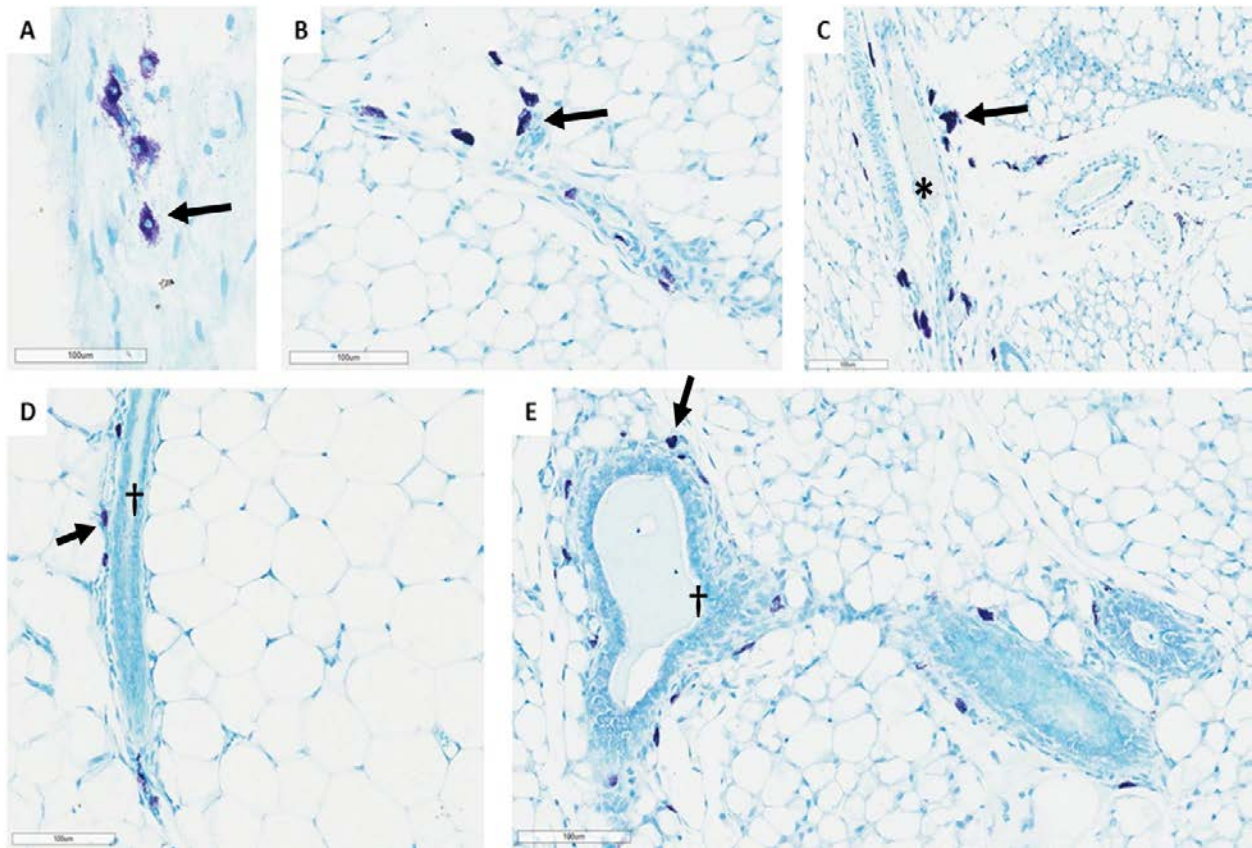
Figure 10. Representative photomicrographs of Toluidine Blue-stained Mast Cells in Normal Mammary Tissue. (A-B) Detail of toluidine blue-positive MCs (scale bar = 100 μm) in Normal Mammary adipose tissue of C57BL/6J mouse, highlighting degranulation (arrows). **(C)** Toluidine blue-positive MCs (scale bar = 100 μm) associated with blood vessels (*) and lymphatic vessels. **(D-E)** Toluidine blue-positive MCs (scale bar = 100 μm) associated with mammary ducts (†).
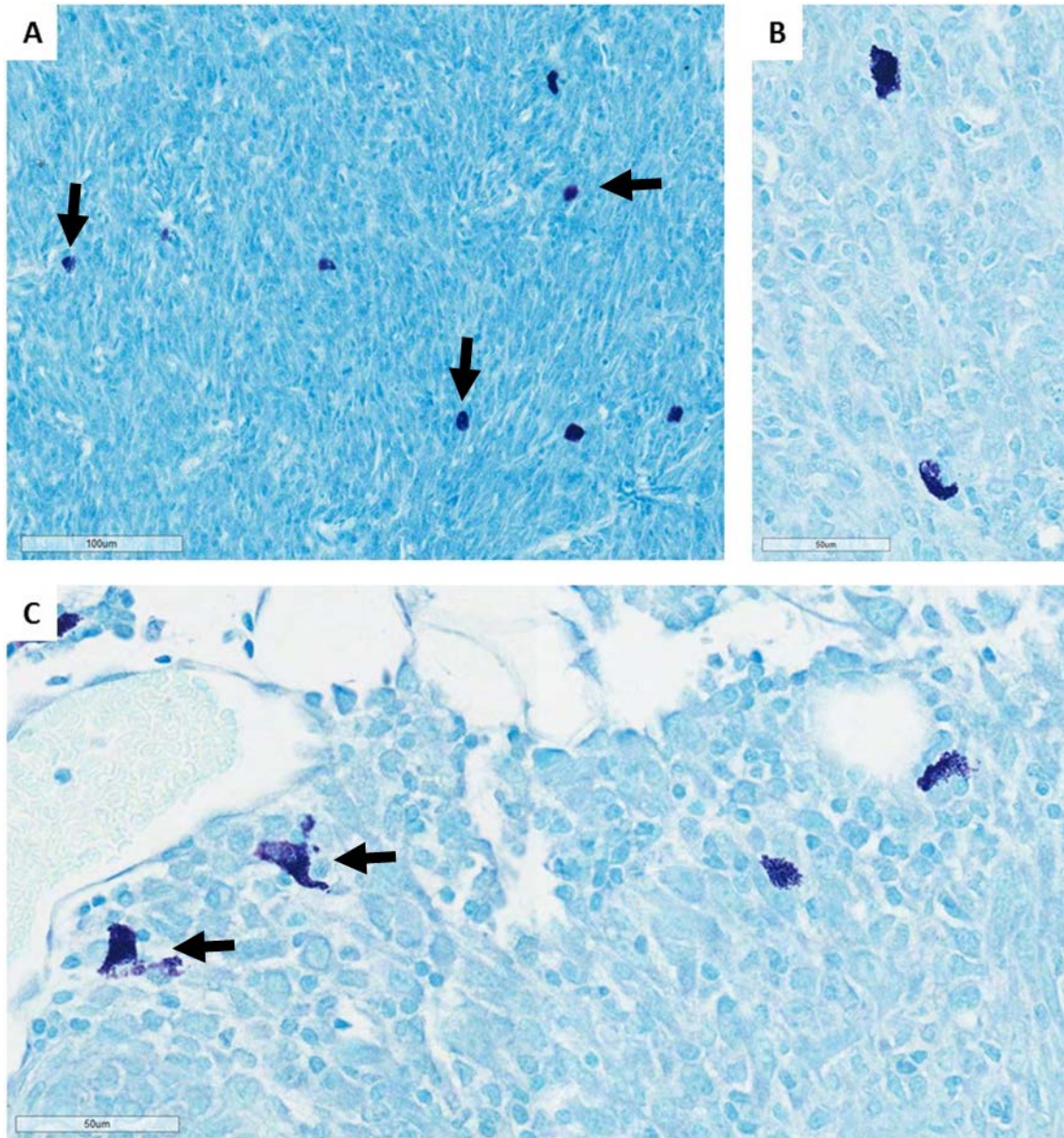
Figure 11. Representative photomicrographs of Toluidine Blue-stained Mast Cells in Claudin-Low Breast Tumors. (A) Pattern of MC infiltration in Claudin-Low tumors (scale bar = 100 μm). **(B-C)** Detail of toluidine blue-positive MCs (scale bar = 50 μm), including degranulation.

Tryptase ß-2 Gene Expression

C57BL/6J

A nonsignificant trend toward increased Tryptase ß-2 expression in HFD-fed mice was observed in both normal mammary tissue and claudin-low tumors (**Figure 12a-b**), with borderline significance in normal mammary (P = 0.055, **Figure 12a**). However, a comparison of gene expression levels across diet and tissue types by two-way ANOVA indicated the presence of both a tissue effect, with Tryptase ß-2 expression significantly higher overall in normal mammary tissue than in tumors (P = 0.026, **Figure 12c**), and a diet effect, with Tryptase ß-2 expression higher overall in mice fed HFD than LFD (P = 0.035, **Figure 12c**).

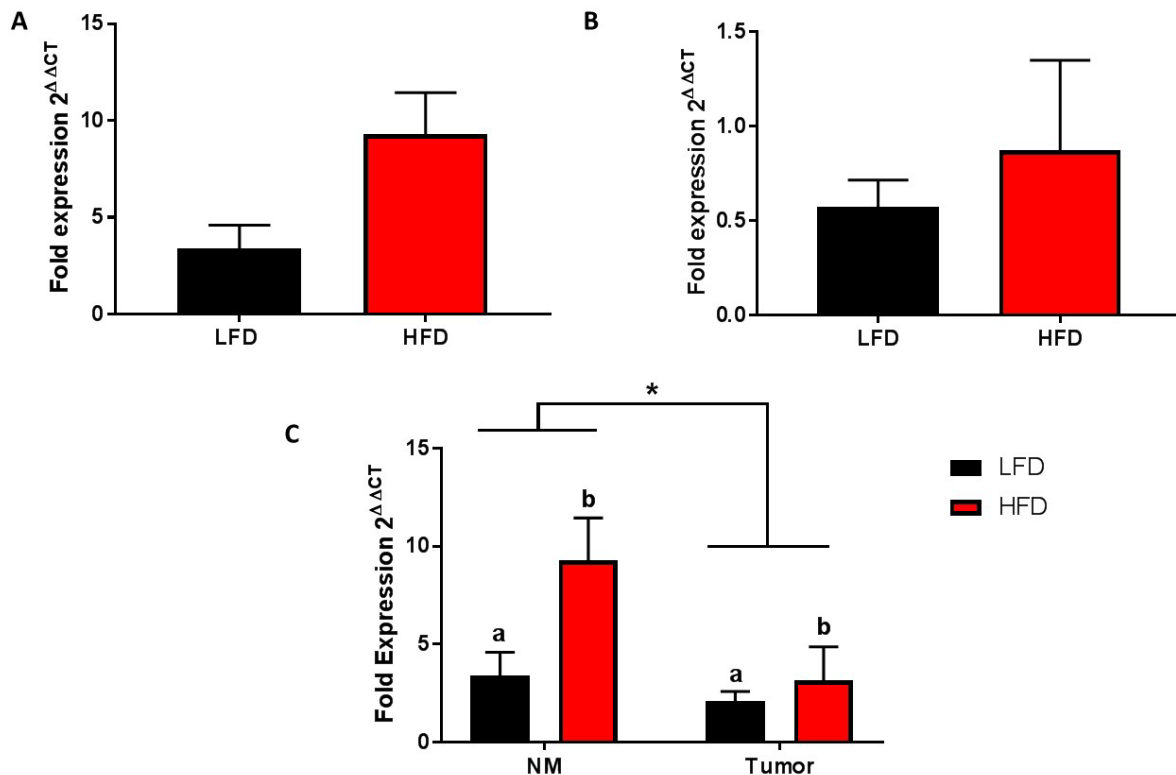**Figure 12. Comparison of Tryptase ß-2 expression between LFD and HFD groups in normal mammary and tumor tissue of C57BL/6J mice. (A)** Tryptase ß-2 expression was measured by qPCR in LFD-fed and HFD-fed mice. **(B)** Tryptase ß-2 expression in LFD-fed and HFD-fed mice did not significantly differ (P = 0.56 by Student's t-test). **(C)** Tryptase ß-2 fold expression levels were compared across diet groups and tissue types. P = 0.035 a (LFD) vs b (HFD). * P = 0.026 NM vs. tumor by two-way ANOVA.

FVB/NJ

Expression levels of mast-cell specific Tryptase ß-2 were significantly elevated in HFD-fed mice as compared to DS mice (*P = 0.017, **Figure 13a**), with a trend toward increased expression relative to LFD-fed mice (P=0.063). Tryptase ß-2 expression levels in tumors did not significantly differ by diet (**Figure 13b**). However, a comparison of gene expression levels across diet and tissue types indicated the presence of both a tissue effect and a diet effect. A Tukey's Post Hoc multiple comparisons analysis following two-way ANOVA revealed a significant difference between the HFD and LFD groups (P = 0.025, **Figure 13c**), and between HFD and DS groups (P = 0.0024, **Figure 13c**) in normal mammary.
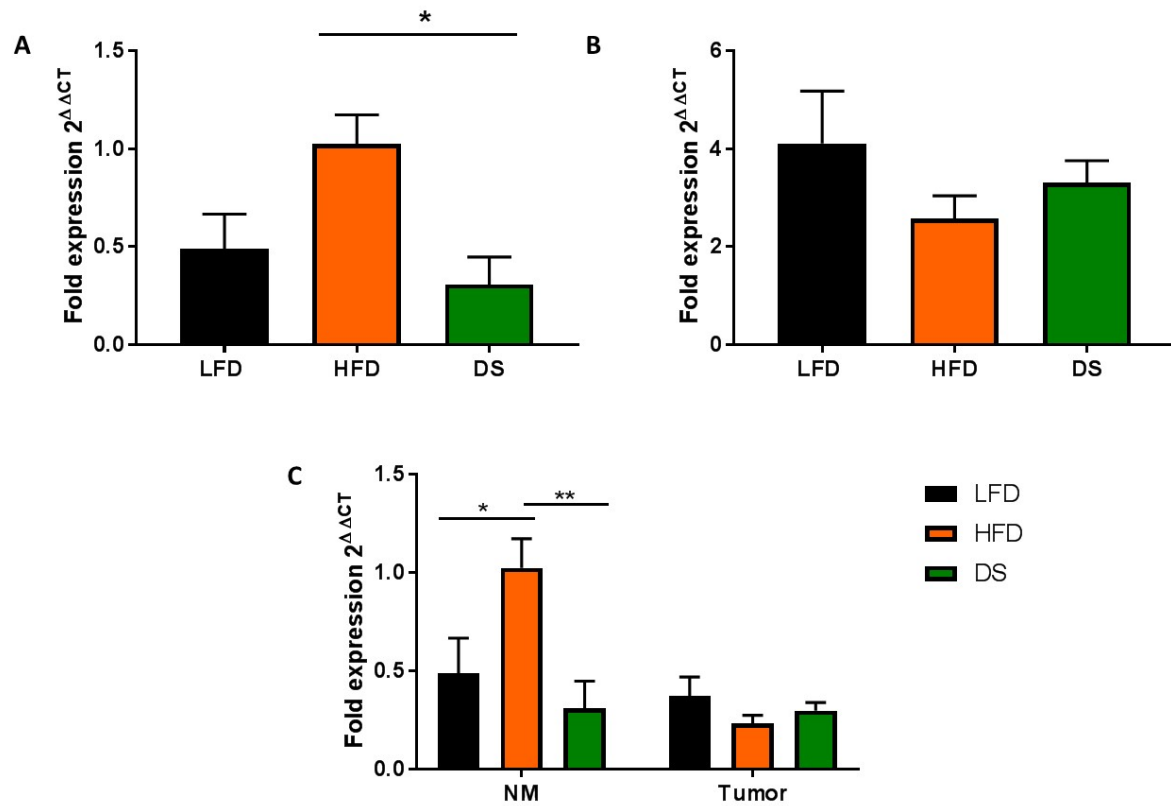
Figure 13. Comparison of Tryptase ß-2 expression between LFD, HFD and DS groups in normal mammary and tumor tissue of female FVB/NJ mice. (A) Tryptase ß-2 expression was significantly greater in normal mammary tissue of mice fed HFD vs. DS. (*$P = 0.017$ by one-way ANOVA). **(B)** No significant difference between Tryptase ß-2 expression of HFD, LFD and DS groups was observed in tumors. **(C)** Tryptase ß-2 expression levels were compared across diet groups and tissue types. *$P = 0.025$ NM HFD vs. LFD. **$P = 0.0024$ NM HFD vs. DS by two-way ANOVA.

DISCUSSION

This pilot study aimed at examining the effects of obesity on MC infiltration and activation within normal mammary tissue and claudin-low breast tumors in mice. Obesity, an epidemic condition in the United States, is a risk factor for a number of diseases, including triple negative breast cancer. The study design first tested the hypothesis of increased mast cell density and/or activation in obesity using the FVB/NJ mouse strain. Female mice fed HFD did present greater weight gain and percent body adiposity than mice fed LFD. However, despite being maintained on diets for several weeks, differences in adiposity between LFD-fed and HFD-fed C57BL/6J mice in this study were not as stark as expected. Perhaps for this reason, there were no statistically significant differences in MC density, a measure of MC infiltration, observed between HFD-fed and LFD-fed mice in tissue-specific analyses using Student's t-tests. On the other hand, a comparison across tissue and diet types using two-way ANOVA revealed an overall significant tissue effect, with normal mammary fat pads containing greater MC counts than tumors. MC activation was measured through the quantification of MC-specific protease tryptase β-2 (**Appendix A**). Remarkably, although obesity did not significantly affect MC density, obesity-induced alterations in the mammary fat pad and tumor microenvironments appear to have promoted a change toward an activated phenotype in the MCs present in these tissues. While no significant difference in tryptase mRNA levels was observed between HFD-fed and LFD-fed mice in tissue specific analyses, a two-way ANOVA showed that overall, HFD-fed mice presented higher tryptase mRNA levels than LFD-fed mice, regardless of tissue type. The analysis also revealed the presence of a significant tissue effect, with normal mammary tissue presenting higher tryptase β-2 levels than tumors, thus mirroring trends in MC density.

The results from this portion of the study deviated from our hypothesis, as we did not see changes in obesity-induced MC infiltration in either normal mammary fat pads or tumors of C57BL/6J mice. A significant departure from previous literature was our observation that the traditionally obesogenic C57BL/6J mice did not gain as much weight and adiposity when fed HFD as expected. Such finding may be ascribed to the effects of age at diet start on weight gain, as evidence suggests that post-weaning HFD

start at 6-10 weeks of age is more successful at inducing maximum weight gain in mice than weaning onto diet [43]. Moreover, the data set utilized in this analysis was limited in size and presented high variability. Based on preliminary data, we were able to conduct a statistical power analysis revealing that, given the standard deviations reported, to detect a difference in mean MC density by diet in tumors, an N of 80 for each diet group would have been required to achieve 80% power with a Type I error rate (α) of 0.05. To observe a difference between diet groups in normal mammary tissue, using the same statistical parameters, an N of 46 per diet group would have been required.

The results on MC activation from our C57BL/6J mice remained encouraging, so we decided to test our hypothesis on a different mouse strain. We repeated our analysis using an FVB/NJ cohort. As for the BL/6J cohort, female mice fed HFD presented greater weight gain and body percent adiposity than mice fed LFD. The DS was successful at reversing both weight and adiposity gain, as mice in this group first gained as much weight and body fat as HFD-fed mice, then returned to the LFD-fed mice baseline upon transition from HFD to LFD. Moreover, HFD exposure accelerated tumor progression, measured as change in tumor volume from injection to sacrifice, an observation that corroborates previous findings from our lab on the effects of obesity on tumor growth [40, 44]. In FVB/NJ mice, tissue specific analyses of MC density by one-way ANOVA also did not reveal any significant difference between diet groups; however, a comparison across tissue and diet types by two-way ANOVA showed both significant tissue and significant diet effects. Interestingly, the tissue effect was reversed with respect to the BL/6 mice, as tumors were found to have higher MC density than normal mammary. Because the FVB/NJ arm of the study had a greater N and thus greater statistical power, it is plausible that these results, as opposed to the C57BL/6J ones, more accurately reflect mast cell infiltration in normal mammary versus tumors. As for the diet effect, the statistical analysis did not yield clear results regarding which diet group presented a significantly different MC density. Interestingly, the DS group presented greater variability than the LFD and HFD groups in both tissue types, with standard deviations of 9.38 in normal mammary and 23.41 in tumors which were much greater than the other diet groups. Importantly, tryptase β-2 mRNA levels were not significantly

different between diet groups in claudin-low tumors of FVB/NJ mice, but they were in normal mammary fat pads. Indeed, normal mammary tissue of HFD-fed mice had significantly higher tryptase mRNA levels than both LFD-fed mice and mice in the DS group. In addition to the observed difference between HFD and LFD groups, the similarities in protease expression between the LFD and DS were particularly insightful, as our results suggest that weight loss may promote a reversal in the activation state of fat pad-infiltrating mast cells.

Findings from the FVB/NJ cohort of our study were very promising, as they largely corroborated our results from the BL/6J cohort, which showed that obesity influences MC activation in normal mammary tissue. However, we were not able to confirm our hypothesis in claudin-low tumors, as no increase in MC activation or density was observed upon HFD exposure. These results could be due to a number of factors, including our limited sample size with high variability that did not allow us to detect subtle differences between diet groups. Moreover, the orthotopic transplant model of cancer used in this pilot study included an intrinsic limitation in that it bypasses early carcinogenesis stages, such as pre-neoplastic lesions formation, and that it does not allow for the establishment of a strong tumor microenvironment. It might be that early stages of tumor development are critical in the recruitment and activation of MCs, which may thus play a more important role in the etiology of breast cancer, rather than its progression.

Conclusion

The body of literature on mast cell infiltration of fat depots and tumors suggests that, upon conditions of obesity, many body adipose depots, such as visceral and subcutaneous, and a number of cancers, namely pancreatic, colorectal and lung carcinomas, experience elevated MC infiltration and activation [2]. Although our study was not able to confirm increased MC infiltration and activation in claudin-low breast tumors, our findings suggested an obesity-induced MC activation effect in normal mammary tissue. Increased tryptase β-2 levels upon HFD exposure were observed independently of MC density, suggesting that HFD might activate MCs toward a pro-inflammatory phenotype, independently from MC counts. Importantly, these results were reproducible in two mouse strains and two cancer cell lines.

Moreover, the increased MC activation in normal mammary tissue upon HFD exposure occurred in concomitance with an increase in tumor progression given the same dietary conditions, suggesting that MC activation in obese normal mammary tissue may have implications for potentially influencing tumor progression. In a similar fashion, DS was successful at reversing both tryptase β-2 levels and tumor progression back to the LFD-baseline. These results corroborated findings from previous studies by our group, whereby we showed that diet switch-induced weight loss prior to tumor latency can reverse tumor progression and improve TNBC-promoting metabolic risk factors [40]. In humans, low fat dietary interventions in post-menopausal women with a primary early stage breast cancer diagnosis were also shown to improve relapse-free survival, although these results were not seen in post-menopausal women without prior breast cancer [45, 46].

In order to examine the implications of MC-dependent modulation of obesity and cancer, future studies could confirm the degree of MC infiltration and activation observed upon HFD exposure through a quantification of MC density through more sensitive cell detection methods, such as flow cytometry, and through the measurement of other MC-specific markers of activation, such as the protease chymase [2]. Moreover, because of the intrinsic limitations of the orthotopic transplant model of breast cancer, as

mentioned in the discussion above, MC infiltration and activation should be studied in spontaneous models of breast cancer, such as the C3(1)-T$_{ag}$ mouse strain, in order to examine the effects of early carcinogenesis stages in the recruitment and activation of MCs. Finally, an interesting extension of the current study would explore location-specific MC infiltration and activation, as the literature suggests that peritumoral MC density, as opposed to intratumoral, is associated with worse prognosis, but the evidence is inconclusive [2].

In all, our study highlights the need to expand the body of research on the role of immune cells, especially mast cells, in the development and maintenance of breast malignancies in the context of obesity. Our pilot study adds mast cells to a growing list of pro-inflammatory and pro-tumorigenic immune cell lineages that may be influenced by the obese mammary milieu, and thus underscore the importance of further research regarding mast cells in the pathogenesis of both obesity and claudin-low breast cancer.

APPENDICES

Appendix A: In silico analysis of TPSB2 specificity to Mast Cells (from biogps.com)
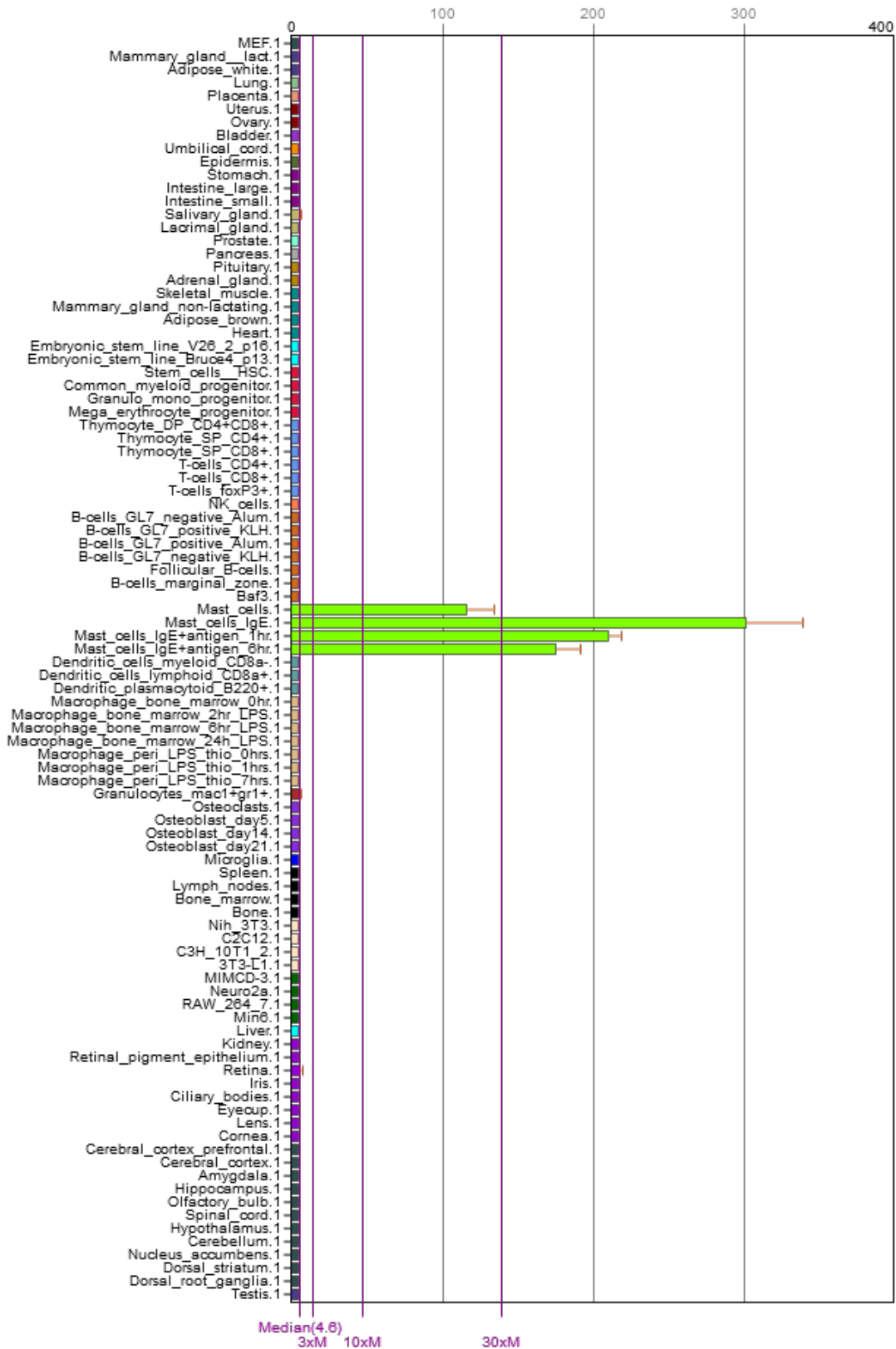
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