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The Effect of Physical Activity and Energy Restriction on Tumor Blood Vessel Architecture and Hypoxia in the 4T1.2 Murine Breast Cancer Model

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ABSTRACT

The American Cancer Society estimates that there will be 287,850 new cases of invasive breast cancer diagnosed and 43,250 breast cancer deaths in the United States in 2022. The average five-year survival rate for women with invasive breast cancer localized in the breast is 98.9% but is only 28.1% for women diagnosed with metastatic breast cancer. Thus, developing strategies to prevent metastatic disease is key to reducing mortality from breast cancer. Triple-negative breast cancer (TNBC) is a highly metastatic and aggressive form of breast cancer with limited treatment options due to being negative for all three hormone receptors that are traditionally used as therapy targets. The normalization of tumor vasculature is emerging as a new target for cancer treatment.

One mechanism proposed for tumor vessel normalization is the normalization of hypoxia within the tumor microenvironment. The protein hypoxia inducible factor-1 alpha (HIF-1α) becomes activated in hypoxic cells and induces the production of over 200 proteins including vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF). VEGF reduces hypoxia by stimulating the formation of endothelial cells and capillaries that enable the tumor to receive nutrients and oxygen rich blood. Endothelial cells release PDGF which enables pericytes, cells that wrap around endothelial cells to provide structural stability, to concomitantly release VEGF. Pharmacologic anti-angiogenic therapies inhibit VEGF resulting in decreased tumor growth, normalization of the vasculature and sensitization of tumors to chemotherapy by improving oxygenation and delivery of chemotherapy. However, these results are often short-lived, and long-term clinical benefit has not been observed. Thus, there is a need to develop novel interventions. Both physical activity and energy restriction have well established antitumorigenic effects in human and animal models. Emerging data suggest that physical activity and energy restriction not only reduce tumor growth but may improve tumor vascularization and hypoxia in preclinical tumor models, however no studies have assessed the combined effect of these interventions on tumor architecture and hypoxia.

This study was a pilot study undertaken to understand the role of physical activity, energy restriction, and combination, in resulting tumor growth, blood vessel architecture, and markers of hypoxia and angiogenesis in the 4T1 murine mammary tumor model. The objectives of this

project were to 1) characterize the effects of physical activity, energy restriction, or combination, on blood vessel architecture within the tumor microenvironment 2) characterize the effects of physical activity, energy restriction, or combination, on levels of hypoxia and angiogenesis within the tumor microenvironment.

Mice (n=10-12/group) were randomized to one of four interventions: sedentary and ad libitum fed (SED+AL), physical activity and ad libitum fed (PA+AL), sedentary and 10% energy restricted (SED+ER), and physical activity and 10% energy restricted (PA+ER) for 34 weeks. All mice were injected orthotopically with 5 X104 4T1.2^{luc} cells in the 4th mammary fat pad and were sacrificed at day 27-28 post tumor implantation. Half of the mice were assigned to assess the effects of physical activity and energy restriction on the microbiome and metabolome, the other half to this dissertation study. For the vessel architecture and immunohistochemistry cohort, whole tumors were removed from each mouse at sacrifice. Half of the tumor was used to assess tumor vessel architecture using immunohistochemistry (3-5 mice/group) and the other half was used to assess markers of hypoxia and angiogenesis using qPCR (3-6 mice/group).

This was the first study to characterize the effects of the combination of physical activity and energy restriction on vessel architecture and tumor hypoxia in the 4T1.2 murine mammary tumor model. We found that sedentary and energy restricted mice as well as physical activity and energy restricted mice had significantly lowered tumor volumes at sacrifice compared to control ($p= 0.003$, Dunnett's multiple comparisons, $p<0.05$). All other outcomes were similar between groups ($p>0.05$). We observed decreased vessel density in the sedentary energy restricted group and increased vessel density in the physical activity ad libitum group. Vessel maturity was increased in sedentary energy restricted mice as well as physical activity energy restricted mice. All markers of hypoxia (HIF 1 α, VEGF-A, VEGF-C, IL-6) and angiogenesis (Ang, Pecam-1, Cdhr5) were higher for all groups in comparison to control, except for a downregulation of VEGF-C, in sedentary energy restricted mice. Overall, energy restriction, and the combination of physical activity and energy restriction, improved vessel maturity in mice, and our findings suggest this normalization was not due to the normalization of hypoxia.

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LIST OF ABBREVIATIONS

Ang- Angiogenin

AMPK- AMP-activated protein kinase

ANOVA- Analysis of variance

BRCA1/BRCA2- BReast CAncer 1/ Breast CAncer 2

CD31- Cluster of differentiation 31

CT- cycle threshold

Cdhr5- Cadhedrin-related family member 5 precursor

DFS- Disease free survival

eNOS- Endothelial nitric oxide synthase

EMT- Epithelial mesenchymal transition

GUSB- Beta-glucuronidase

HER2- Human epidermal growth factor 2

HIF-1α- Hypoxia inducible factor 1 alpha

IGF-1- Insulin growth factor-1

IL-6- Interleukin-6

Log2- Logarithm 2

Luc- Luciferase

mTOR-mammalian target of rapamycin

NK- natural killer

PA+AL- Physical activity + *ad libitum*

PA+ER- Physical activity + energy restriction

PARP inhibitor- Poly-ADP ribose inhibitor

Pecam-1-Platelet endothelial cell adhesion molecule-1

PDGF- Platelet derived growth factor

PDGFRβ- Platelet derived growth receptor beta

PDL1-Programmed death-ligand 1

SED+AL- Sedentary + *ad libitum*

SED+ER- Sedentary + energy restriction

SEER- Surveillance, Epidemiology, and End Results

SERMs- Selective estrogen receptor modulators

SEM- Standard error of the mean

TILs- Tumor Infiltrating Lymphocytes

TNBC- Triple negative breast cancer

TNM- “Tumor”, “Node”, “Metastasis”

VEGF- Vascular endothelial growth factor

VEGF-A- Vascular endothelial growth factor-a

VEGF-C- Vascular endothelial growth factor-c

VEGFR 1, 2- Vascular endothelial growth factor receptor-1

VEGFR 2- Vascular endothelial growth factor receptor-2

qPCR- Quantitative polymerase chain reaction

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LITERATURE REVIEW

Introduction

Breast cancer is the second leading cause of cancer death in women [1]. Approximately 5% of all breast cancers are due to BReast CAncer 1/ Breast CAncer 2 (BRCA1/BRCA2) germline mutations. BRCA2 carriers are more likely to develop estrogen receptor and/or progesterone receptor positive tumors whereas BRCA1 mutation carriers mainly develop triple negative breast cancer (TNBC) [2]. TNBC is negative for the estrogen, progesterone, and human epidermal growth factor receptor 2 (HER2) receptors and has the poorest prognosis and survival rates of all breast cancer subtypes [3]. The risk for developing TNBC is highest in premenopausal, younger (less than 40) [4], African American women [5]. TNBC is an aggressive subtype due to its propensity to metastasize and recur [6], as well as lack of treatment options due to limited drug targets [4].

The tumor microenvironment

The tumor microenvironment (TME) is the environment that surrounds the tumor [7]. There are several key players in the TME such as immune cells, the extracellular matrix, blood vessels and cancer cells [8]. The TME can be protumorigenic or antitumorigenic [9]. A protumorigenic TME provides optimal nutrients and growth factors for the growth of the cancer, an antitumorigenic environment is one that does not result in tumor growth and may even result in the regression and necrosis of a tumor [10]. A TME that is antitumorigenic can result in the elimination of cancer, providing an attractive target for novel cancer therapies.

Breast cancer

Prevalence and survival rates

Breast cancer is the most commonly diagnosed form of cancer in women in the United States [11]. Approximately 1 in 8 U.S. women will develop invasive breast cancer over the course of her lifetime [12]. Female breast cancer is most frequently diagnosed in postmenopausal women aged 55 to 64 [13]. In the United States, breast cancer incidence continues to rise;

however overall survival rates have improved because of improvements in screening and treatment [14]. Generally, the five year survival rate for breast cancer varies between 100% and 12% depending on the subtype and disease progression [5]. Although survival rates have improved, they remain grim for patients with metastatic disease. Most patients that die from breast cancer die from metastatic disease, or cancer that has spread from the breast to a distant organ or lymph node [15]. Breast cancer remains a major cause of mortality for American women and although survival rates are increasing, treatment options are still limited for metastatic disease, representing an important opportunity to identify new therapies.

Subtypes of breast cancer

Breast cancer is divided into four subtypes depending on the receptors present on tumor cells. Hormone receptor positive cancers can be positive for estrogen receptors, progesterone receptors or both receptors [16]. HER2 is a protein involved in cell growth, in HER2 receptor positive cancer, the HER2 receptors are either overexpressed or amplified, leading to increased cancer growth [17]. The four subtypes are: luminal estrogen receptor positive (luminal A and luminal B), protein receptor HER2 (enriched, and basal-like tumors. Luminal A breast cancer has receptors for estrogen and progesterone (HR+) and lacks receptors for the HER2 receptor (HER2 -). Luminal B breast cancer has receptors for estrogen and can be positive or negative for progesterone and HER2 receptors. HER2 breast cancers are positive for the HER2 receptor. Triple negative breast cancer lacks hormone receptors (HR-) estrogen and progesterone as well as the HER2 receptor (HER2-), hence “triple negative”[5, 18]. The breast cancer subtype determines treatment options and affects prognosis and survival [19]. Due to the lack of receptors for drug targeting, TNBC has the poorest prognosis and survival rates of all breast cancer subtypes [3].

Stages of breast cancer

Breast cancer is divided into stages to determine the treatment course. Stages include stage 0, stage 1, stage 2 (II), stage 2A (IIA), stage 2B (IIB), stage 3 (III) A, B, C, and stage 4 (IV). Stage 4 (IV) represents metastatic breast cancer, which has the poorest prognosis of all the stages [20]. The American Joint Committee on Cancer (AJCC) classifies breast cancer stage by combining the “T- tumor, N - node, M - metastasis”, (TNM) system, grading system, and

biomarker status. Biomarkers include hormone receptors (estrogen and progesterone) and HER2 receptors [20]. In 2018 the AJCC cancer staging system was revised to include other molecular hormone receptors, oncogene expression, and multigene panels [21]. Other molecular hormone receptors include CXCR4, caveolin, miRNA, and FOXP3 [22].

Molecular hormone receptors in breast cancer include estrogen receptor (ER)/progesterone receptor (PR), HER2/neu [23]. Oncogenes, or genes that are associated with mutations that can result in cancer include ErbB2, MYC, PIK3CA and tumor suppressors TP53, BRCA1/2 and PTEN [24]. The revised AJCC staging guidelines have yet to be adapted in all clinics and traditional staging utilizing the TNM system, grading system, and biomarker status staging is still the most popular method of staging for tumors.

The TNM system

The TNM system is used to classify the anatomic extent of the cancer. Tumors ("T") are classified from TX to T4 and are based on the size and shape of the tumor. Tumors that are classified as TX are not detectable, while more than 1/3 of breast tissue may be inflamed in T4 graded tumors. Lymph nodes ("N") are graded from NX to N3 and are based on the size and shape of the lymph node. Lymph nodes that are classified as NX are not detectable, while N3 cancers may have spread to the axillary lymph nodes or lymph nodes above the collar bone. Metastasis ("M") represents the spread of cancer to other parts of the body. When a cancer is classified as M0 there is no sign of metastasis. A cancer that is classified as M1 represents a cancer that has metastasized [3]. Distant metastases represents stage IV cancer [25]. Sites of distant metastasis in breast cancer include brain, bone, lungs, liver and lymph nodes [26]. Of all breast cancer subtypes, TNBC has the highest propensity to metastasize [6]. A study of 26,863 TNBC patients from the 2010-2015 Surveillance, Epidemiology and End Results (SEER) data base found that 1,330 patients (5.0%) had distant metastasis. Among those with single organ distant metastasis, lung metastasis was the most frequent (19.6%), bone metastasis was the second most frequent (19.4%), and brain was the least frequent (2.9%) [27]. Distant metastases, or stage IV cancer is seen in TNBC patients.

Grading system

The grading system utilizes cell morphology to determine how quickly the cancer will progress. Three grades are possible: low grade or well differentiated, intermediate grade or moderately differentiated, and high grade or poorly differentiated. Low grade cancer cells are more differentiated and tend to grow slowly whereas high grade cancer cells are less differentiated and tend to grow rapidly. Cancers that are poorly differentiated have the worst prognosis [20].There is heterogeneity within TNBC, but most tumors of this subtype are high-grade tumors that push the borders of the breast and have areas of tissue death [28]. High grade TNBC is quick to progress and difficult to treat [29].

Hormone and HER2 receptor positive therapy

The presence of hormone receptors serves as a drug target in cancer therapy. Endocrine therapy is used to treat hormone receptor positive breast cancers [30-32]. Approximately, 70-80% of breast cancers are hormone receptor positive [5]. Selective estrogen receptor modulators (SERMs) and aromatase inhibitors are commonly used forms of endocrine therapy [33]. Selective estrogen receptor modulators block the effects of estrogen in breast tissue and slow cancer growth in premenopausal women [34]. Aromatase inhibitors are used in postmenopausal women to inhibit estrogen production in the breast. Endocrine therapy has resulted in significant decreases in cancer related mortality [35]. Endocrine therapy cannot be utilized in TNBC because there are no hormone receptors present on the tumor cells.

The presence of the HER2 receptor is a drug target in cancer therapy. About 10-20% of breast cancers in the United States are HER2 positive [5]. Monoclonal antibody drugs bind to domain IV (Trastuzumab) or domain II (Pertuzamab) of HER2 and downregulate HER2 expression, blocking the growth signal and reducing tumor growth [36]. HER2 targeted therapy cannot be utilized in TNBC because there is no HER2 receptor in triple negative breast cancer.

Triple negative breast cancer

Prevalence and survival rates

TNBC is aggressive and difficult to treat. In the United States, TNBC accounts for 15-20% of all breast cancer subtypes [29] and is associated with the lowest 5-year survival rate of all breast cancer subtypes [37]. In 2018, TNBC made up 10% of breast cancer cases [5]. The 5-year survival rate for women with localized TNBC is 91.2% but this drops to 12.2% for TNBC that has metastasized to a distant location [5]. TNBC is an aggressive subtype due to its propensity to metastasize and recur [6]. One reason for improved survival rates for other breast cancer subtypes is the availability of drug targets; however, TNBC lacks the hormonal and molecular targets commonly used in other forms of breast cancer therapy and is considered incurable [4]. Because treatment for TNBC is difficult to treat, targeted forms of therapies are needed to improve survival.

Risk factors

There are several risk factors associated with breast cancer and TNBC. Risk factors for the development of breast cancer and TNBC include: genetics, age, ethnicity, and menopausal status [38].

Genes

Genomic mutations are a hallmark of cancer [39]. Certain gene mutations are associated with TNBC. Mutations in genes such as: BRCA1, p53, PTEN, and PALB2 increase risk [40]. Almost 70% of breast cancer in premenopausal BRCA1 carriers are triple negative [41]. The BRCA1 and BRCA2 mutation is more common in certain ethnicities such as Ashkenazi Jews [42]. PALB2 is another common germline mutation in patients with TNBC [43]. Genomic mutations in early-stage estrogen receptor positive breast cancers are rare, but very common in TNBC [44].

Age risk factors

Increased age is a risk factor for breast cancer development [45] and breast cancer screening typically starts at age 50 [46]. Breast cancer screening for women at high risk may begin as early as age 30 [47]. Female breast cancer is most frequently diagnosed in women aged 55 to 64 [4]. However, TNBC is more common in younger women (less than 40) [4]. Therefore, age does not have a linear relationship to TNBC risk as it does with other breast cancer subtypes.

Race and ethnicity

2014-2018 SEER data finds that the rate of new female breast cancer diagnosis is the highest among African Americans compared to White, Hispanic, Asian Pacific Islander, and American Indian [5]. In the United States African American women are nearly twice as likely to develop TNBC compared to Caucasian women [48]. A study utilizing 2006-2009 California Cancer Registry data found that African American women have higher rates of TNBC at all ages [49]. Utilizing 1988-2013 Cancer Genome Atlas data it was found that basal like breast cancer was more likely in African American women ($P < 0.001$) [50]. Basal like breast cancer (BLBC) is an aggressive molecular subtype of breast cancer [51]. Triple negative breast cancers are commonly basal like in nature [52]. Interestingly, African American women have a low relative incidence of BRCA1 mutations compared to Caucasian non-Ashkenazi-Jewish women [53, 54]. A version of the ANKLE1 gene that can be protective against TNBC is less likely to be found in African American women compared to Caucasian women [55]. African American women commonly have a mutation in the Duffy-null allele, resulting in an average reduced number of circulating white blood cells and conferring increased risk [55, 56].

Menopausal status and estrogen exposure

Age of menarche, age of menopause and hormone replacement therapy influence estrogen exposure in women. Age of menarche, or onset of the menstrual period, does not seem to play a role in the development of TNBC. In a pooled analysis of 554 TNBC patients it was found that decreased age of menarche is not associated with increased risk of TNBC ($p=0.33$) [57]. Age of menopause is an important risk factor for the development of TNBC. A population-based study of 2010-2014 SEER data found that women younger than 40 years had the highest

odds of diagnosis of TNBC, compared to women aged 50 – 64 years (OR = 1.95; 95%CI: 1.90, 2.01) [48]. Hormone replacement therapy use in women is a known breast cancer risk factor in cancers that are hormone receptor positive [58]. Interestingly, in an analyses of 897 breast cancer cases oral contraceptive use for more than one year was associated with a 2.5-fold increased risk for triple-negative breast cancer (95% CI, 1.4-4.3) [59]. Estrogen may play a role in the development of TNBC, but more epidemiological data is needed.

Energy balance

Obesity

Obesity is defined as having a body mass index (BMI) ≥30 kg/m^2. Obesity is associated more strongly with postmenopausal than premenopausal breast cancer. Several prospective studies and case control studies have found a strong association between obesity and the development of ER+ breast cancer [60-62]. A pooled analysis of prospective cohort studies found that there is an increased risk of ER+ breast cancer in postmenopausal women with obesity RR: 1.26 (95% CI: 1.09, 1.46) [63]. Another study found that in postmenopausal women with ER+/PR+ breast cancer, a weight gain of 10 kg or more was associated with increased breast cancer risk (RR= 1.18; 95% CI, 1.03-1.35; P = 0.002) [60]. A meta-analysis found that for each 5 kg increase in adult weight gain there was an increase in relative risk of 1.11 (95% CI = 1.08 to 1.13) for the development of postmenopausal breast cancer [71]. Excess body weight can lead to excess estrogen levels [64]. Weight loss has been shown to decrease levels of circulating estrogen in postmenopausal women [65]. Obesity has emerged as a risk factor for a poor antitumor response in cancer [66].The obese state promotes insulin insensitivity as well as a state of chronic inflammation within the individual, creating a tumor microenvironment that is more susceptible to the development of an aggressive cancer [67]. The literature strongly supports the relationship between postmenopausal obesity and weight gain and the development of ER+ breast cancer.

The relationship between obesity and TNBC is less clear [57]. A meta-analysis found that there is a positive relationship between obesity and the development of TNBC but only in premenopausal women (OR: 1.43; 95% CI: 1.23-1.65), suggesting there may be a relationship

between obesity, TNBC risk and menopausal status [68]. A systematic review of 391 articles found that there seems to be an increased risk for TNBC in premenopausal women with obesity and a decreased risk for less aggressive tumor subtypes such as the luminal A subtype [69]. A systematic review and meta-analysis of 13 studies found that overweight, as classified by a BMI of over 25 kg/m^2 was found to be associated with both shorter disease-free survival (HR = 1.26; 95%CI: 1.09–1.46) and shorter overall survival (HR = 1.29; 95%CI: 1.11-1.51) compared to normal-weight women [70]. Interestingly, a study found that the mean BMI of white women was lower in TNBC compared to estrogen receptor positive subtypes ($p < 0.008$). It was found that African American women had a significantly higher mean BMI than Caucasian women (34.4 vs 27.4; $p < 0.0001$). The relationship between obesity and triple negative is the strongest in premenopausal and African American women compared to postmenopausal Caucasian women.

Weight loss

Bariatric surgery has been cited as the most effective form of weight loss. A Swedish study of bariatric patients found that weight loss after surgery resulted in decreased cancer incidence in women but found no effect in men [71]. A retrospective study of 301 premenopausal and 399 postmenopausal women found that bariatric surgery was associated with a reduced risk of both premenopausal (HR = 0.72, 95% CI, 0.54-0.94) and postmenopausal (HR = 0.55, 95% CI, 0.42-0.72) breast cancer after 10 years of follow up. Interestingly, the effect of bariatric surgery was more pronounced among ER-negative cases in premenopausal women (HR = 0.36, 95% CI, 0.16-0.79) and in ER-positive cases in postmenopausal women (HR = 0.52, 95% CI, 0.39-0.70) [72]. Weight reduction, induced by bariatric surgery, has been associated with decreased cancer incidence in obese women.

There is a clear relationship that weight loss in subjects with obesity is beneficial in protection from the development of postmenopausal breast cancer. A retrospective cohort study found bariatric surgery was associated with a significantly lower risk of breast cancer for postmenopausal ER-positive cases (HR: 0.52, 95% CI, 0.39–0.70, $P < 0.001$) and premenopausal ER-negative cases (HR: 0.36, 95% CI, 0.16–0.79, $P = 0.01$) compared with the nonsurgical group [72]. A meta-analysis found that postmenopausal women with ER-/PR- breast cancer women with 10 kg or more of weight loss were at a reduced risk (RR, 0.43; 95% CI, 0.21-0.86; P

= .01) [73]. The relationship is less clear in premenopausal women. A study analyzing premenopausal women enrolled in the Nurse's Health Study determined that a weight loss of 5 kg or more since 18, maintained for at least four years was related to lower incidence of premenopausal breast cancer but this did not reach statistical significance (covariate-adjusted HR=0.75; 95% CI 0.52-1.09) [74]. The reason for this may be that the mechanism of action in breast cancer development may be different in premenopausal and postmenopausal women and may differ by cancer subtype. A high BMI may be protective in the development of premenopausal breast cancer, but several studies have found benefits to weight loss in both pre- and post-menopausal women, with the strongest relationship in post-menopausal women [71-74]. This highlights the importance of weight loss among obese women in general, and particularly those at risk for developing TNBC.

Physical activity

The terms "physical activity" and "exercise" are typically thought to be synonymous, but each term is nuanced. The World Health Organization (WHO), defines physical activity as "*any bodily movement produced by skeletal muscles that requires energy expenditure, including all movement during leisure time*" [75]. Exercise is defined as "*a subset of physical activity that is planned, structured, and repetitive and has as a final or an intermediate objective the improvement or maintenance of physical fitness*" [76]. Mice in our experiments have voluntary access to a running wheel, mimicking physical activity [77]. A systematic review and meta-analysis found physical activity in mice resulted in a reduction in tumor growth in 17 studies [78]. Physical activity statistically significantly decreased tumor growth rate (p=0.004) and levels of hypoxia (p=0.004) in mice inoculated with TNBC compared to sedentary controls [79]. A systematic review found that physical activity was associated with reduced all cause cancer mortality in 27 observational studies [80]. A prospective study of 1340 subjects at high risk for breast cancer looked at the effects of physical activity before, during, and after chemotherapy. Researchers found that patients meeting the requirements for physical activity, outlined by the Physical Activity Guidelines for Americans, before and 1 year after diagnosis experienced statistically significant reductions in hazards of recurrence (hazard ratio [HR] = 0.59, 95% confidence interval [CI] = 0.42 to 0.82) and mortality (HR = 0.51, 95% CI = 0.34-0.77) [81]. The American College of Sports Medicine recommends that cancer patients avoid inactivity and

concluded that exercise is safe for breast cancer patients undergoing treatment and breast cancer survivors [82]. Physical activity plays an important role in cancer prevention and is not only safe but is recommended during cancer treatment.

Physical activity plays a beneficial role in the prevention and treatment of TNBC. It was found that Ashkenazi Jewish women with BRCA1 or BRCA 2 mutations who reported being physically active as an adolescent had significantly delayed onset of TNBC [83]. In a study of 518 women with TNBC it was found that physical activity during the first 60 months postdiagnosis decreased recurrence and total disease specific mortality [84]. The prospective cohort SNPs to Risk of Metastasis analyzed the impact of physical activity on survival in triple negative breast cancer patients. Physical activity was self-reported, researchers found that median overall survival was the highest in the vigorous activity group (20.4 months (95% CI 13.1–30.9)), followed by the light group (20.2 months (95% CI 8.3–31.6)), and moderate physical activity group 15.5 months (95% CI 13.8–26.2). Physical activity has been associated with delayed onset of breast cancer, decreased recurrence, and increased survival in triple negative breast cancer.

Physical activity has been shown to delay breast cancer onset, reduce tumor growth, and decrease breast cancer mortality, showing promise as a form of prevention and adjuvant therapy.

Mechanisms underlying protective effect of physical activity

Physical activity has mechanistic effects independent of weight loss. The biological mechanisms underlying the protective effect of physical activity in cancer prevention are complex including decreased availability of nutrients such as insulin, an alteration of pathways involved in tumor metabolism, and improved immune outcomes [85].

Physical activity leads to reduced levels of glucose and glutamine available to breast tumors [86-88]. TNBC has a distinct metabolic phenotype. Compared with other breast cancer subtypes, TNBC is more glutamine dependent and overexpresses glutaminase [89]. Exercise influences metabolic reprogramming in TNBC. A study of BALBc female mice measured metabolic changes that occur in exercised mice in comparison to sedentary control. Mice were treadmill exercised for 12 weeks and then inoculated with a 4T1 TNBC cell line. Researchers found that in comparison to sedentary mice, exercised mice increased body carbohydrate

oxidation as measured by indirect calorimetry ($p < 0.0001$). Researchers also found that exercise reduced mitochondrial capacity in the tumor in comparison to sedentary mice ($p < 0.01$) [90]. It has been shown that there is aberrant activation of the PI3K/Akt/mTOR pathway in TNBC which leads to mammalian target of rapamycin (mTOR) activation. mTOR is a central regulator of cell proliferation and immune cell differentiation that regulates gene transcription and protein synthesis which plays an important role in tumor metabolism [91]. There are several pharmacologic inhibitors of mTOR, but exercise also inhibits mTOR [92]. Our lab has shown that independent of weight loss, exercise modulates the immune populations within the tumor microenvironment, leading to reduced tumor growth, metastasis, and survival in a TNBC murine model [77]. Mouse models have shown that exercise is associated with an increase in anti-tumor immune cells and a decrease in protumorigenic immune cells in the tumor microenvironment [77, 93]. Exercise improves natural killer (NK) cell and T cell recruitment and infiltration to the tumor due to shear stress and altered circulation patterns [94]. Exercise also results in the secretion of myokines after muscle contraction, indirectly stimulating immune cells [95]. Exercise improves hypoxia in mouse models, researchers hypothesize the effect to be due to increased levels of perfusion following exercise [96, 97]. Exercise is a nonpharmacological intervention that alters nutrient levels, metabolic pathways, immune cells, and levels of hypoxia which mechanistically alter the tumor microenvironment in TNBC.

Energy restriction

Energy restriction (ER) is defined as a 10- 40% reduction of average daily caloric intake [98]. ER has resulted in reduced tumor growth, angiogenesis, and metastatic burden in TNBC mouse models [77, 99, 100]. A 30% energy restriction resulted in a tumor growth delay of 56% ($p <0.001$) and a combination of energy restriction and radiotherapy resulted in a tumor growth delay of 82% ($p<0.001$) [100]. Mice on a 40% energy restricted diet displayed significantly lower intratumoral microvessel density ($P < 0.05$) [99]. Our lab has shown that a mild energy restriction of 10% has resulted in significantly less metastatic burden in the femur compared to mice that were fed *ad libitum* . Another study found that energy restriction coupled with radiation decreased metastatic burden in a triple negative breast cancer model. Energy restriction has resulted in improvements in the preclinical TNBC phenotype.

Very few human clinical trials have analyzed the effect of energy restriction on cancer outcomes during treatment [101-104]. One pilot clinical trial looked at the effects of a 25% energy restriction after lumpectomy and before, during, and after the following course of radiotherapy in 32 breast cancer patients [105]. In human intervention trials, energy restriction is commonly analyzed in combination with physical activity [106-111]. One clinical trial found that a combination of physical activity and energy restriction in stage 0-II overweight/ obese breast cancer patients resulted in weight loss and the upregulation of several genes in tumors associated with enhanced immune function and decreased insulin signaling [107]. A study found that Hispanic and African American breast cancer patients who adhered to a commercial diet and weight loss program (Curves) lost an average of 3.3% (±3.5%) of body weight compared 1.8% (±2.9%) in the control, demonstrating that this underrepresented population is able to adhere to medically recommended lifestyle interventions [110]. Energy restriction improves cancer endpoints in mice and shows promise as an adjuvant therapy for breast cancer patients.

Mechanisms underlying protective effect of energy restriction

There are several biological mechanisms underlying the cancer protective effects of energy restriction including a reduction in hormone levels, growth factors, reactive oxygen species, inflammation, and hypoxia [112, 113]. Energy restriction also induces autophagy and alters nutrient and energy levels [114]. Autophagy, the degradation, and recycling of cellular building blocks, both suppresses and promotes tumor growth [115]. Autophagy can lead to the degradation and cell death of tumor cells or can be used by tumor cells to increase the degradation of surrounding cells to promote tumor growth [116]. Energy restriction like exercise is an (AMP-activated protein kinase) AMPK activator [117]. AMPK plays a role in cellular energy homeostasis by stimulating catabolic processes such as glucose uptake and decreasing anabolic processes such as protein and lipid synthesis [118]. The diabetic drug metformin shares many of the same mechanistic alterations in cancer as caloric restriction, such as activation of AMPK, inhibition of mTOR, and downregulation of IGF [119]. Studies have found that the anti-tumor effects of metformin are much higher in immunocompetent mouse models than in immunodeficient ones, implying the interaction of the immune system is integral to antitumor effects [120]. Sudden glucose deprivation activates AMPK leading to enhanced CD8 T cell

response in vivo [121, 122]. Our lab has found that physical activity in combination with mild energy restriction (10% of total calories) increased the percentage of CD8+ T cells within the tumor microenvironment of mice inoculated with 4T1.2 mammary tumor cells, a TNBC cell line [85]. The intertwining mechanistic alterations of the tumor microenvironment explain the anti-cancer benefits of energy restriction.

Therapies

TNBC is an aggressive subtype with few options for treatment due to the lack of target receptors as well as propensity to metastasize and recur [123].

Traditional therapies

Chemotherapy, surgery, and radiation therapies are traditionally used in the treatment of breast cancer and non-metastatic TNBC. Traditional therapies fail to mechanistically target the TNBC due to a seeming lack of molecular targets [124]. If TNBC recurs and metastasizes the mortality rate within 3 months after recurrence is as high as 75% [29]. Reliance on systemic, untargeted therapies contributes to the difficulty of treating TNBC and contribute to high mortality rates associated with this subtype.

Chemotherapy

Chemotherapy is a systemic form of therapy that travels in the blood stream and affects all cancer cells. It is the only recommended systemic treatment for TNBC [113]. Chemotherapy may be given before surgery (neoadjuvant) to shrink the tumor or after surgery (adjuvant) to ensure there are no cancer cells remaining [6]. Smaller tumors typically do not require radiation and may be eradicated by chemotherapy. Chemotherapy targets cells that grow and divide rapidly, such as cancer cells. Chemotherapy also kills other rapidly dividing cells that are not cancer cells, such epithelial cells, which is why cancer patients lose hair [125]. Chemotherapy is physically debilitating for breast cancer and TNBC patients.

Surgery

More aggressive forms of surgery are necessary for TNBC patients. Forms of surgery that are commonly used to remove the cancer are a lumpectomy, removal of the tumor (lump), or mastectomy, removal of the breast [126]. TNBC patients in early stages, or even those at high risk for developing TNBC [127], are encouraged to undergo a mastectomy [37] in order to prevent metastases. Surgery is an invasive form of therapy that is used to prevent metastasis.

Radiation

Radiation is used as an adjuvant form of therapy after surgery in TNBC patients. The American Society of Clinical Oncology recommends radiation after mastectomy for patients with four or more positive lymph nodes or stage III cancer, but there is no recommendation for earlier stages [128]. In metastatic breast cancer, surgery and radiation are commonly used as palliative forms of care [129]. Poly- ADP ribose (PARP) inhibitors can sensitize the cancer to radiotherapy, making radiation therapy more effective [130]. Radiation is used to reduce the risk of metastasis in triple negative breast cancer patients, or as a form of palliative care.

Novel therapies

Novel therapy is more personalized than traditional therapy. Novel cancer therapy works by targeting specific characteristics of cancer cells that normal cells do not have [131]. Some novel therapies for TNBC include antibody drug conjugates and serine/threonine AKT inhibitors, immunotherapy, antiangiogenic therapy, and PARP inhibitors [4, 132].

PARP Inhibitors

PARP inhibitors prevent cancer cells from repairing damaged DNA, therefore resulting in cancer cell death [133]. The BRCA 1 and 2 genes are a target for PARP inhibitors [134]. Preclinical data has demonstrated that PARP inhibitors can sensitize TNBC to radiotherapy, making radiation therapy more effective [135, 136]. Olaparib was the first developed PARP inhibitor [137]. While Olaparib improves overall survival in ovarian cancer patients [138] , to date there has been no statistically significant improvement in overall survival for TNBC patients treated with Olaparib [139, 140]. PARP inhibitors cannot be prescribed in all patients, it is estimated that only 20–30% of TNBC patients have a proven BRCA1/2 mutation [141].

Resistance to PARP inhibitors occurs due to drug target-related resistance and restoration of BRCA [142]. PARP inhibitors are proven safe in clinical models and improve radiosensitivity in preclinical models but cannot be prescribed to all TNBC patients and those who do respond to PARP therapy often develop resistance and relapse.

Immunotherapy

Immunotherapy utilizes the immune system to kill cancer cells via activation or suppression of the immune system. There are several different forms of immunotherapy including immune checkpoint inhibitors, CAR-T cell therapy, monoclonal antibodies and immunomodulators [143]. The most successful immunotherapeutic agent to treat TNBC are immune checkpoint inhibitors [144]. There are certain markers within TNBC that make immune checkpoint inhibitors a viable treatment option [145]. Tumors with high mutational burden, or high number of genetic mutations within the tumor, have better responses to immune checkpoint inhibitors [146]. TNBC has the highest mutational burden compared to all other breast cancer subtypes [123]. TNBC presents with high levels of tumor infiltrating lymphocytes (TILs) compared to other breast cancer subtypes [147, 148]. TNBC also has high expression of PDL-1 compared to other breast cancer subtypes [149, 150]. A study analyzing The Cancer Genome Atlas data showed significantly greater expression of the PD-L1 gene in TNBC ($n = 120$) compared with non-TNBC ($n = 716$; $P < 0.001$) [149] . In July 2021 the Food and Drug Administration (FDA) approved Pembrolizumab with chemotherapy as a neoadjuvant and radiation as an adjuvant treatment [151]. Pembrolizumab is an immune checkpoint inhibitor that uses monoclonal antibodies to block the action of PD-L1 [151]. A clinical trial in high-risk early stage clinical TNBC patients found that pembrolizumab plus chemotherapy as neoadjuvant therapy resulted in a 60% pathological complete response rate and twelve-month event-free and overall survival rates ranging from 80% to 100% in the treatment arm [152]. Researchers found that high levels of PDL-1 and TILs were significantly associated with patients who saw improved outcomes [152]. TNBC is heterogeneous and patients exhibit differing levels of TILs and PDL-1 [124]. A systematic review and meta-analysis found that TNBC patients with high TIL levels showed a higher rate of pathological complete response to treatment ([OR] 2.14, 95% [CI] 1.43–3.19) and better overall survival ([HR] 0.58, 95% [CI] 0.48–0.71) compared to patients with low levels of TILs [153]. TNBC patients that express high levels of PDL-1 would

respond well to immune checkpoint inhibitors, but those with low PDL-1 expression would need to utilize an alternate form of therapy.

Antiangiogenic therapy

Antiangiogenic therapy targets aberrant angiogenesis within the tumor microenvironment. The drug bevacizumab was the first angiogenic inhibitor developed [154]. Bevacizumab is a monoclonal antibody that blocks vascular endothelial growth factor (VEGF) receptor binding [155]. In 2008 bevacizumab was approved for metastatic breast cancer under the FDA's Accelerated Approval Program. In 2011 the FDA revoked bevacizumab's approval for metastatic breast cancer after concluding the drug was not safe and effective [156]. A phase II clinical trial of 78 HER-2 negative breast cancer found that patients who received neoadjuvant bevacizumab followed by combined bevacizumab and chemotherapy saw a pathologic complete response in TNBC patients (11/21 patients or 52%) [157]. In 2013 the GeparQuinto clinical trial of 678 patients with TNBC found that neoadjuvant bevacizumab and chemotherapy resulted in a statistically significantly increased pathological complete response rate ($P = 0.003$) compared to patients receiving chemotherapy alone [158]. In 2014 results from the GeparQuinto trial demonstrated that disease free survival and overall survival were not different for patients receiving bevacizumab (HR 1.03; $P = 0.784$ for disease free survival (DFS) and HR 0.974; $P = 0.842$ for OS) compared with patients receiving chemotherapy alone [159]. Though bevacizumab has shown the ability to shrink tumors and improve pathological complete response it has not improved overall survival in TNBC patients due to the cancer's ability to eventually acquire resistance [160]. One of the mechanisms behind the failure of bevacizumab is believed to be the tumor's ability to adapt different vascularization methods [161].

In order to maintain the oxygen levels necessary for tumor survival, angiogenesis is imperative [162]. Tumor vasculature is abnormal and immature [163, 164]. The disordered blood flow of tumor vasculature results in pockets of hypoxia further stimulating the growth of more abnormal vasculature [165]. Tumor vessels are not organized into traditional venules, arterioles, and capillaries, but rather share features of all of them within their extracellular matrix [166]. Endothelial cells are a type of epithelial cell that make up the extracellular matrix of blood vessels [167]. Under hypoxic conditions signals are released from the extracellular matrix

resulting in an epithelial mesenchymal transition [168]. The epithelial mesenchymal transition (EMT) is a phenomenon observed in tumor cells where epithelial cells lose several defining characteristics such as their ability to adhere to other cells and undergo a transition that enables them to migrate from one place to another [169]. This transition allows metastasis to occur [170]. Tumor blood vessels are composed of abnormal endothelial cells and have poor pericyte coverage [171, 172]. Pericytes provide structural support for endothelial cells. Poor pericyte coverage results in less support and a "leaky" phenotype [173]. Tumor vessels have increased permeability, plasma leakage, and interstitial pressure in comparison to healthy vasculature [165]. In order to survive the hypoxic tumor microenvironment, cancer cells acquire invasive and metastatic properties as well as resistance to therapy [174]. Targeting hypoxia within the tumor microenvironment may decrease angiogenesis and metastases, leading to improved patient outcomes for patients with TNBC.

Hypoxia and angiogenesis: an overview

Hypoxia promotes vessel growth by upregulating multiple angiogenic pathways [175]. The growth of new vessels from preexisting capillaries is known as angiogenesis. Judah Folkman is known as the "father of angiogenesis", his ideas on angiogenesis were popularized in the early 1970s [176]. Angiogenesis mainly takes place during embryogenic development but physiological stimuli, such as hypoxia, reactivate angiogenesis in adulthood [175]. Vessel formation is initiated by the production of angiogenic growth factors, including VEGF, angiopoietin-1, and cytokines [177]. Angiogenic growth factors promote endothelial cell stabilization by recruitment and interaction with pericytes or smooth muscle cells [178].

Vessel branching is necessary for healthy and mature vasculature. Tip cells lead to branching vessels, stalk cells elongate the sprout, and phalanx cells line vessels [179]. Filopodia are endothelial cells located on the tip cells of angiogenic sprouts [180]. Stalk cells are endothelial cells that form the body of the sprout [181]. Phalanx cells are endothelial cells that ensure quiescence and perfusion of the new branch, they are covered by pericytes [182]. Tip cells express VEGF receptors vascular endothelial growth factor receptor 2 (VEGFR2), vascular endothelial growth factor 3 (VEGFR3), and Neuroplipin 1. VEGFR3 and VEGFR2 enables tip cell formation [183]. Loss of PECAM-1 inhibits the formation of filopodia and vessel growth [184]. Proper tip cell formation is essential to the development of vessel branching.

Hypoxia inducible factor 1-alpha (HIF-1α)

HIF-1α is a master regulator of oxygen tension and angiogenesis. During periods of hypoxia, HIF-1α is released resulting in the release of several different proteins involved in angiogenesis, including VEGF, resulting in the growth of new vasculature [185]. The HIF family includes: HIF-1α, HIF-1β, HIF-2α, HIF-2β, HIF-3α, HIF-3β [186]. Higher expression of HIF-1α has been correlated with poorer survival with breast cancer patients [187-189]. In a study of 221 breast cancer patients, it was found that patients who did not express HIF-1α (in removed tumors) had a significantly higher pathological complete response (no pathological sign of disease) than patients that exhibited HIF-1α ($P=0.027$) [188]. In a mouse xenograft study utilizing MDA-MB-231 triple negative breast cancer cells tumors that were silenced exhibited delayed onset of tumor growth and decreased growth [190]. In a mouse study of TNBC it was found that activation of HIF within a tumor led to an epithelial mesenchymal transition within epithelial cells that promotes metastasis [191]. Another mouse study also demonstrated this, finding that the increased metastatic promotion due to HIF expression was due to the migration of endothelial cells within the lymphatic vessels [192]. The presence of HIF-1α is an indicator of hypoxia and metastatic potential.

Vascular endothelial growth factor (VEGF)

VEGF protein is activated by HIF-1α. It is a marker of angiogenesis in breast cancer that is expressed in endothelial cells [193]. VEGF plays a key role in vascular permeability and the migration of endothelial cells [194] The VEGF family includes: VEGF-A, B, C, D, E and placental growth factor (PGF) [195]. VEGF is more highly expressed in patients with TNBC [196]. A retrospective study of 670 breast cancer patients, found that women with TNBC and had significantly higher VEGF levels; median value in TNBC was 8.2 pg/microg DNA compared with 2.7 pg/microg DNA in non-TNBC ($P < 0.001$) [196]. VEGF is more highly expressed in patients with larger tumors than smaller ones [197]. In a controlled trial of 41 women analyzing VEGF-A (Vascular Endothelial Growth Factor-A) levels in women with metastatic breast cancer compared to breast cancer patients with no metastases found that there was a trend for higher VEGF-A levels in patients with larger tumors compared to those with

smaller ones (p = 0.053) [197]. The presence of VEGF is an indicator of hypoxia and metastatic potential.

Vascular endothelial growth factor-a (VEGF-A)

VEGF-A plays a key role in endothelial proliferation and angiogenesis. VEGFR-1 (Vascular Endothelial Growth Factor Receptor-1) and VEGFR-2 (Vascular Endothelial Growth Factor Receptor-2) are the main receptors for VEGF-A [198]. VEGF-A is released during periods of hypoxia, inflammation, and glucose deficiency [199]. VEGF-A is integral in development as well as adult organ homeostasis [200]. PD-L1 is commonly expressed in triple negative breast tumors. In a study of tumor samples from 97 women who had undergone surgery without preoperative therapy, researchers found that VEGF-A was positively associated with PD-L1 (29.7% vs. 10.0%, p=0.014) expression in primary breast cancer. Thirty-seven (38.1%) of women had positive VEGF-A expression in the breast tumor. Among the cases with PD-L1 expression, 36.7% of VEGF-positive cases, but none of VEGF-negative cases, had low TILs in the breast tumor [201]. VEGF-A expression is positively associated with a protein that is commonly expressed in triple negative subtypes. VEGF-A is translocated to the nucleus and plays an intracrine role through VEGFR-1. Western blots from a study of metastatic MDA-MB-231 breast cancer cells, showed that targeted knockdown of VEGFR1 expression by siRNA (siVEGFR1) significantly decreased the survival of breast cancer cells, while targeted knockdown of VEGFR-2 or neurolipin 1 (NRP1) expression had no effect on the survival of these cancer cells. Researchers also discovered VEGF and VEGFR-1 were located around the nucleus and not on the surface of cells and found the same pattern in primary breast tumor, providing evidence for the intracrine expression of VEGFR-1 [202]. A study of 500 tissue microarrays of premenopausal breast cancer patients, including 96 tissue microarrays of triple negative breast cancer found that high VEGFR2 was significantly correlated to decreased breast cancer survival in TNBC patients (P = 0.03) compared to other premenopausal breast cancer patients [203]. VEGFR-2, a receptor for VEGF-A, has been correlated with decreased breast cancer survival. VEGF-A expression been associated with an increase in markers of metastatic breast cancer. Expression of VEGF-A receptors, VEGFR-1 and VEGFR-2 have been associated with decreased breast cancer survival in vitro and in vivo.

Vascular endothelial growth factor-C (VEGF-C)

VEGF-C is a key player in the growth of new blood and lymphatic vessels. [204]. VEGF receptor-2 (VEGFR-2) and VEGF receptor-3 (VEGFR-3) are VEGF-C receptors [205]. VEGF-C is expressed on endothelial cells but has also been detected in immune and tumor cells [206]. The VEGF-C receptor VEGFR-3 is required for lymphatic vessel sprouting. A study found that orthotopically implanted lung and colon tumors were smaller in severe combined immunodeficiency (SCID) mice that did not express VEGF-C compared to control ($P<0.01$). Lung metastasis was also lower in mice with downregulated VEGF-C compared to control ($P<0.05$) [207]. In a study of surgically treated Japanese female breast cancer patients it was found that VEGF-C was overexpressed in 39 of 98 breast cancer specimens (39.8%) but not in adjacent normal mammary glands and there was a significant correlation with lymphatic vessel invasion ($p = 0.0004$). The 5 -year survival rate of the VEGF-C positive group was significantly worse than that of negative group ($p = 0.0356$) [208]. The expression of VEGF-C has been associated with lymphatic spread in cancer.

Angiogenin (Ang)

In 1971 Judah Folkman proposed that tumors would not be able to grow beyond 1–2 mm^3 because tumor growth is dependent on angiogenesis [209]. Angiogenin was the first protein to be discovered to stimulate angiogenesis, or the growth of new blood vessels [210]. Angiogenin plays an important role in regulating cell proliferation, survival, migration, invasion, and differentiation [211]. The chorioallantoic membrane assay used to assess angiogenic activity, found that little as 0.5 ng can induce blood vessel formation [212]. Angiogenin is a growth factor that stimulates angiogenesis.

Cadherin related family member 5 (Cdhr5)

Cdhr5 is a transmembrane cell–cell adhesion molecule composed of five extracellular cadherin repeats [213]. The Cdhr5 complex plays a key role in the differentiation of the epithelial brush border and maintenance of vascular integrity through the control of vascular permeability and inhibition of excessive vascular growth [214]. A retrospective study of 154 hepatocellular carcinoma patients found that Cdhr5 was downregulated in hepatocellular carcinoma tissues ($P = 0.020$) compared to adjacent liver tissue and that Cdhr5 was decreased in advanced stages

(TNM III–IV) compared to those with early stages (P = 0.002)[215]. Another name for Cdhr5 is VE-cadherin. Vasculogenic mimicry is a recently discovered tumor blood supply system that takes place independently of angiogenesis or endothelial cells, overexpression of VE-cadherin has been strongly associated with vasculogenic mimicry [216]. Researchers used The Cancer Genome Atlas to determine that the short RNA molecule, miR-93 was expressed at higher levels in triple negative breast cancer and was associated with worse disease-free survival. In vitro experiments resulted in the discovery that the overexpression of miR-939 expression in exosome releasing triple negative breast cancer cells resulted in the 40-60% reduction of VE-cadherin expression, and the destruction of the barrier function of endothelial monolayers [217]. Cdhr5 plays a critical role in the regulation of cancer invasion and metastasis by its effects on the endothelium, and possibly through its effects on vasculogenic mimicry.

Platelet endothelial cell adhesion molecule-1 (Pecam-1)

Pecam-1, also known as cluster of differentiation 31 (CD31), is expressed on all cells in the vascular compartment. Pecam-1 supports the endothelial barrier function and is impaired during periods of vascular inflammation [218]. Pecam-1 is highly expressed on endothelial cells but is also expressed on platelets and leukocytes. Pecam-1 plays important roles in proliferation, apoptosis, signal transduction, endothelial nitric oxide synthase (eNOS) expression, and cellular immunity [219]. For vessel stabilization and integrity, endothelial cells are sufficient for small blood vessels, but larger blood vessels require endothelial cell interactions with pericytes or smooth muscle cells [220]. In blood vessels, endothelial cells exert their function through direct interactions with various sensing stimuli such as hypoxia, growth factors, cytokines and shear stress; resulting in modulation of intracellular pathways [199]. Pecam-1 plays a key role in signaling. Anti-Pecam-1 antibody inhibits angiogenesis. In a study of Pecam-1-deficient mice that were implanted with an ovarian or melanoma cell line, angiogenesis was inhibited in Pecam-1-null mice and tumor volume was significantly reduced (40 to 50%) in the Pecam-1-deficient animals [184]. A study of 114 tumor and 30 background tissues revealed a trend with elevated levels Pecam-1 in tumor samples compared with background tissue (p=0.8), and in patients with a poor prognosis using the Nottingham Prognostic Index (p= 0.3404) [221]. A study found Pecam-1 is a key regulator of angiogenesis and the absence of Pecam-1 results in the inhibition

of angiogenesis, interestingly elevated Pecam-1 expression in vivo seems to result in a poorer prognosis for cancer patients.

Desmin

Desmin is commonly used to assess pericyte coverage. Desmin is used to visualize intracellular clustered intermediate filaments, Platelet derived growth factor beta (PDGFRβ), another marker of pericyte coverage is used to determine the pericyte membrane contour [222]. Two important cells that compose the vasculature are endothelial cells and pericytes. Endothelial cells form the inner lining and pericytes provide a structural coverage for endothelial [220]. When vessels lose pericytes they become abnormal and immature [223]. Researchers characterize pericyte coverage in different subtypes of breast cancer using immunohistochemistry. Using triple negative (n=28) and luminal (n=56) breast cancer tumor samples researchers assessed micro vessel density, pericyte coverage and perivascular heterogeneity. Researchers found triple negative breast cancer patients showed significantly higher micro vessel density when compared with luminal breast cancer patients ($p < 0.0001$) [224]. The total pericyte coverage encompassing all combinations of pericyte phenotypes (PDGFRβ– desmin+, PDGFRβ+desmin– and PDGFRβ+desmin+), was significantly lower in triple negative breast cancer patients ($P < 0.0001$), indicating that a higher proportion of vessels in triple negative breast cancer are immature with diminished pericyte coverage. Our results revealed that the majority of pericytes in TNBC patients were PDGFRβ+desmin, whereas double-positive pericytes (PDGFRβ+desmin+) were the dominant pericyte population in luminal breast cancer patients [224]. Pericytes play an important role in vessel health and seem to be decreased in triple negative breast cancer patients.

Interleukin-6 (IL-6)

IL-6 is a pro inflammatory cytokine activated by M1 macrophages that stimulates the release of B lymphocytes [225]. Hypoxia has been shown to induce the expression of cytokines, such as IL-6, in preclinical models. A study looking at IL-6 expression found that hypoxia increased transcription, levels of IL-6 mRNA, and elaboration of IL-6 antigen in cultured endothelial cells and increased IL-6 transcripts in the lungs and lung vasculature of mice [226]. A preclinical study assessing the effects of intermittent energy restriction, chronic energy

restriction and *ad libitum* feeding found that mice that were intermittently or chronically energy restricted experienced significant reductions in serum IL-6 compared to ad libitum mice (24 and 3.5 fold respectively) [227] A comprehensive review of the effects of physical activity on immune outcomes found thirty-one studies investigated the effect of physical activity interventions on cancer immune outcomes using preclinical cancer models it was found that IL-6 was reduced in three studies [228-230] and unchanged in one study [231]. Hypoxia and energy restriction seems to increase the expression of IL-6 while physical activity seems to decrease the expression of IL-6.

Rationale for current study

Epidemiological, clinical, and preclinical studies have suggested beneficial effects of physical activity or energy restriction on tumor vasculature and markers of hypoxia within the tumor microenvironment [79, 232-236]. However, no study to date has characterized the **combination** of physical activity and energy restriction using a preclinical model of TNBC. Our laboratory has demonstrated that the combination of physical activity and mild energy restriction (10% reduction in total calories) results in reduced tumor growth and metastatic burden in a 4T1.2 tumor growth, a murine mammary TNBC tumor model [77]. It is unknown if the beneficial effects on tumor growth and metastases observed in the combined intervention group is mediated by the normalization of tumor vasculature and hypoxia levels. This study was undertaken to characterize markers of hypoxia, tumor vasculature, and metastatic burden in the 4T1.2 tumor model. Findings from this study will further our understanding of the role of hypoxia and angiogenesis in a triple negative breast cancer model and will provide support for future clinical trials looking at the effects of these lifestyle interventions in women.

Aims and hypotheses

The objective of this study was to evaluate the effect of physical activity, mild energy restriction and the combination of physical activity and energy restriction on hypoxia, vascularization, in a preclinical model of triple negative breast cancer. The overarching hypothesis is that physical activity and energy restriction as single intervention or in combination, will lead to decreased levels of hypoxia and vascularization, resulting in decreased tumor growth in the 4T1 murine mammary tumor model.

Aim 1. Determine if physical activity and/or energy restriction will alter vessel density and maturity.

Hypothesis 1. Physical activity and/ or energy restriction will result in decreased vessel density (evidenced by CD31) and increased vessel maturity (evidenced by desmin) in the tumor microenvironment.

Aim 2. Determine if physical activity and/or energy restriction alter gene expression of key mediators of hypoxia and angiogenesis (HIF 1 a, VEGFA, VEGF C, IL-6, Cdhr5, Ang, Pecam-1) in the tumor microenvironment.

Hypothesis 2. Physical activity and/ or energy restriction will result in decreased markers of hypoxia and angiogenesis (HIF 1 a, VEGF, VEGF C, IL-6, Cdhr5, Ang, Pecam-1) in the tumor microenvironment.

MATERIALS AND METHODS

Exploratory analysis

This thesis study (n=22) was an exploratory analysis within the 4T1.2^{luc} mammary model of the parent study (n=44). Half of the mice from the parent study were used to quantify the vessel architecture and hypoxia, and the other half (n=22) was used to quantify the microbiome and metabolome.

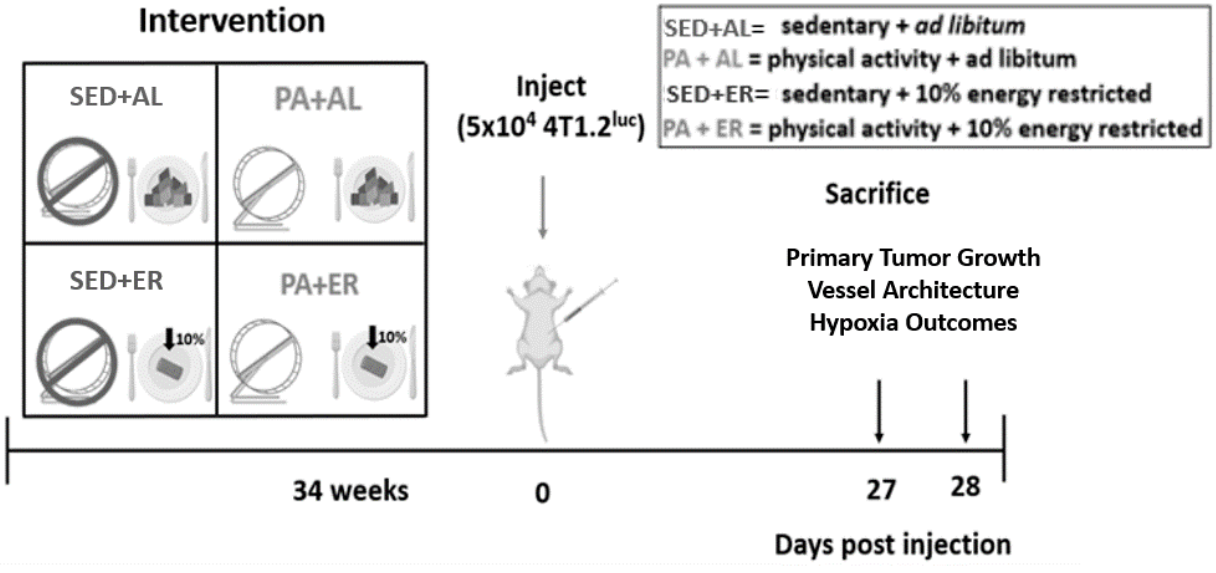


Figure 1: Experimental design. Female BALB/c mice (n=10-12/group) were randomized to one of four interventions: sedentary and ad libitum fed (SED+AL), physical activity and ad libitum fed (PA+AL), sedentary and 10% energy restricted (SED+ER), and physical activity and 10% energy restricted (PA+ER). All mice were injected orthotopically with 5 $X10^4$ 4T1.2luc cells in the 4th mammary fat pad at 34 weeks of the intervention and were sacrificed at day 27-28 post tumor implantation, interventions continued during the entirety of the study.

Tumor cell line and cell culture

The 4T1.2 cell line is a murine metastatic breast cancer line derived from a spontaneously arising mammary tumor in a BALB/cfC3H mouse [237]. When implanted orthotopically, the 4T1.2 cell line mimics the metastatic progression of human breast cancer with a tendency to metastasize to lung and bone [238]. 4T1.2 cells stably expressing luciferase (4T1.2luc) were provided by Dr. Shoukat Dedhar (Department of Cancer Genetics, BC Cancer Research Centre, Vancouver, BC, Canada), and maintained in Dulbecco's modified Eagle's medium (Life Technologies; Grand Island, NY) containing 10% fetal bovine serum (Gemini Bio-Products, Sacramento, CA), 2 mM glutamine (Mediatech; Manassas, VA), 1X nonessential amino acid (Mediatech), and 8 μg/ml puromycin (Mediatech).

Animal model

Female BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, Maine). BALB/c mice were randomized (n=11-12/group) to sedentary (SED) or activity wheel (PA) cages and fed *ad libitum* (AL) or 10% dietary energy restriction, (ER) in a 2x2 factorial design to investigate the effects of PA alone, ER alone, and the combination of PA+ER on tumor growth, tumor vessel density, pericyte coverage, and gene expression profile. After 34 weeks on the interventions, mice were orthotopically injected with $5x10^4$ luciferase-transfected 4T1.2 cells into the fourth mammary fat pad. Mice continued their respective interventions and were sacrificed on day 27 or 28 post tumor implantation. Primary tumor growth was measured two times a week using a digital caliper, and tumor volume was calculated following the equation $V=(short^2 \times long)/2$. All mice were housed at the animal facility in South Frear, Pennsylvania State University, maintained on a 12-hour light/dark cycle and fed AIN-76A diet (Research Diets, New Brunswick, NJ). The Institutional Animal Care and Use Committee of the Pennsylvania State University approved all animal experiments.

Quantification of running wheel activity

Wheel revolutions of individual mice were recorded and analyzed using Vital View software (Starr Life Sciences Corporation). Wheel revolutions were converted to total kilometers run on a weekly basis.

Energy restriction protocol

Energy restricted mice were fed 90% of control food intake (10% reduction in dietary energy intake compared with ad libitum-fed controls). Mice were weighed weekly. The body weight of ER mice was adjusted to 90% of baseline body weight by adjusting food intake. If body weight was 90% of baseline feed was maintained the same, if body weight was less than <85% feed was increased by 0.05 g and if body weight was greater than 90% feed was decreased by 0.05 g.

Fluorescent immunohistochemistry

At sacrifice half of the tumor from each mouse was placed in a Tissue-Tek intermediate cryomold (Sakura Finetek USA, Torrance, CA), embedded in Cryomatrix optimal cutting temperature (OCT) medium (Epredia, Portsmouth, NH), wrapped in aluminum foil, and stored at -80°C until sectioning. Frozen tissue sections (12 μm thick) were produced using a Leica CM3050 S cryostat (Leica Biosystems, Wetzlar, Germany). 6 slides with 2 sections per slide for a total of 12 sections per mouse were produced. Slides were stored in slide box at -80°C until staining.

CD31 and desmin staining

Slides were removed from –80°C and thawed for 20 minutes at room temperature. During thawing a border was drawn around each tissue section using an ImmEdge PAP pen (Vector Laboratories, Burlingame, CA). Slides were fixed for 20 minutes in ice cold, 1:1 solution of ACS reagent, ≥99.5% acetone (Sigma-Aldrich, Burlington, MA) and HPLC grade methanol (Sigma-Aldrich, Burlington, MA). Slides were removed from acetone/methanol solution and washed with phosphate- buffered saline (PBS) (VWR, Radnor, PA). Slides were washed five times for five minutes for a total of 25 minutes. Non-specific binding was blocked by a 30-

minute incubation with 10% donkey serum (Sigma-Aldrich, Burlington, MA) diluted in 1X PBS. Slides were incubated overnight at 4°C with primary antibodies: Rat anti-mouse CD31 antibody (BD Biosciences Pharmigen, 557355, San Diego, CA, USA), 1:100 and desmin (abcam, ab32362, Waltham, MA, USA). 1:200. All primary and secondary antibodies were diluted in the following antibody buffer: 1X PBS 1 mL composed of bovine serum albumin (Rockland, Philadelpha, PA) 10 mg and Triton X- 100- (VWR, Radnor, PA) 3 uL which was enough to cover 2 slides (4 tumor sections). The following day slides were washed with 1X PBS for five changes, each for 5 minutes for a total of 25 minutes. Slides were then incubated with fluorescently conjugated secondary antibody: DyLight 488 AffiniPure Donkey Anti-Rat IgG, which corresponds with CD31 primary antibody (Jackson ImmunoResearch 712-545-150) 1:1000 and Alexa Flour 488 Donkey Anti-Rabbit IgG, which corresponds with desmin primary antibody, (Thermo Fisher, Waltham, MA) 1:1000, (1 uL), for 1 hour room temperature, in the dark. After incubation slides were washed with 0.05 Tween-20(VWR, Radnor, PA) diluted in X1 PBS once for 5 minutes, followed by five times for five minutes for a total of 25 minutes. 30 minutes. Slides were counterstained with Flouroshield with DAPI (4′,6-diamidino-2-phenylindole) (Sigma-Aldrich, Burlington, MA) and immediately covered with a coverslip. Slides were stored in 4°C in the dark and until imaged, within the next 2-3 days. To produce images that were overlaid with CD31 and desmin slides were coimmunostained. When slides were coimmunostained, CD31 and desmin primary antibodies were applied at the same time for the overnight incubation and CD31 and desmin secondary antibodies were applied at the same time for the one-hour incubation.

Vessel density

CD31 expression was used to quantify endothelial cells. First, ImageJ Fiji software (NIH, Bethesda, MD) was used to quantify the percent of the slide containing CD31 staining and reported as percent area. Second, ImageJ software (NIH, Bethesda, MD) was used to quantify vessel branching by manual counting from four independent observers. Tumor sections were fluorescently immunostained for CD31 described in detail above. After staining, a 400x image was captured in three different areas for each tumor tissue section using a Leica DMi8 microscope (Wetzlar, Germany). The mean of the three different images for each tumor section was used to determine the mean vessel density for each mouse.

Pericyte coverage and vascular maturity

Pericyte coverage was quantified by desmin expression, which is expressed on pericytes, this was performed two ways. First, ImageJ Fiji software (NIH, Bethesda, MD) was used to quantify the percent of the slide containing desmin staining and reported as percent area. Second, ImageJ software (NIH, Bethesda, MD) was used to quantify pericytes by manual counting from four independent observers. Tumor sections were fluorescently immunostained for desmin described in detail above. After staining, a 400x image was captured in three different areas for each tumor tissue section using Leica DMi8 microscope (Wetzlar, Germany). The mean of the three different images for each tumor section was used to determine the mean vessel maturity for each mouse.

ImageJ Fiji vessel density and pericyte coverage (% area)

In ImageJ Fiji (NIH, Bethesda, MD) the vessel analysis plugin was used to quantify vessel density and pericyte coverage, quantified by percent area. First images were uploaded to ImageJ Fiji. All images were then converted to 8 bit. The skeletonize command was then selected under the Plugin toolbar. The skeleton image has a pixel value of 255 at the skeleton and 0 at the background (black pixels). Next, regions of interest (ROI) were drawn around the perimeter of each image. Vessel density was selected under the plugin bar and percent area was calculated. This number is reported as % area in ImageJ Fiji. This process was repeated for each of the three images for each tumor section. % area values were added to an excel sheet and the mean value of the three different images were reported as the vessel density percent area, or pericyte coverage percent area for each mouse.

ImageJ vessel branching and pericyte counts

In ImageJ an overlaid graph was used to count vessel branching and pericytes. First images were uploaded to ImageJ. Images were then overlaid with a graph by selecting the Analyze, then Tools, then Grid command. Grid lines were cyan blue and were 30 inches^2. All grids were centered on the image. Counters were instructed to count all vessel branching (where one vessel diverges to two)/ pericytes, count all green, even dots, count all connected green vessels/ pericytes within each grid section, as one, count within each box (connected blood

vessels/ pericytes between boxes are new blood vessels/ pericytes in each box), and to not count blood vessels/ pericytes in outer grid sections or any blood vessels/ pericytes on outer edges if more than 50% is on the outer edge. This process was repeated for each of the three images for each tumor section. Vessel branch counts and pericyte counts were added to an excel sheet and the mean value of the three different images were reported as the pericyte count for each mouse. This process was repeated by four separate people. The mean of the four counters was reported as the vessel branch or pericyte count for each group.

Gene expression in the tumor microenvironment

At sacrifice half of the tumor from each mouse was incubated in RNAlater (Sigma-Aldrich, Burlington, MA) overnight at 4°C, and stored at -80°C until homogenization. Tumor tissues were homogenized using a mortar, pestle, and liquid nitrogen. RNA was extracted using RNeasy Mini Plus Kit (Qiagen). RNA was quantified using Nanodrop ND1000 spectrophotometer (Nanodrop Products; Wilmington, DE). 260/280 between 2.0 and 2.2 were considered pure. RNA samples were reverse transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was stored at -80 until analysis**.** cDNA was subject to PCR reactions using the SYBR™ system and PowerTrack SYBR Green Master Mix (ThermoFisher, Waltham, MA) performed on a StepOne Plus (Applied Biosystems; Foster City, CA) real time PCR instrument cycled according to conditions: 95°C for 2 minutes for enzyme activation; 95°C for 15 seconds for denaturation; 60 °C for 60 seconds to anneal and extend on a StepOnePlus (Applied Biosystems; Foster City, CA). The expression of markers of hypoxia and angiogenesis, HIF 1 alpha, VEGF a, VEGF c, IL6, Cdhr5, Ang and Pecam 1 were measured. Levels of target genes were normalized to Gusb (beta-glucuronidase) housekeeping gene, and relative gene expression between groups were determined using the 2-ΔΔCt method.

Statistical analyses

All data were assessed for normality and equal variances, and either parametric or nonparametric analyses were used to detect differences between groups. Differences in body weight, food intake, running wheel activity, primary tumor volume over time, vascular density, pericyte coverage, and gene expression data between the four treatment groups were examined using two-way ANOVA or Kruskal-Wallis test, followed by Dunnett's multiple

comparison test. All data are presented as the mean plus or minus the standard error of the mean. All analyses were conducted using GraphPad Prism 9.4.0 (GraphPad Software, San Diego, CA) and statistical significance was accepted at the $p \leq 0.05$ level.

RESULTS

Body weight in 4T1.2^{luc} mice

Body weights in mice randomized to the four treatment groups did not differ significantly at baseline (Figure 2A) (one-way ANOVA, F (3, 44) = 0.1536, p=0.927); n=11-12/group. Over time there was a significant difference in weekly body weight in mice in SED+ER, PA+ER, and PA+AL groups compared to SED+AL group (Figure 2B) (two-way ANOVA, time x treatment, F (114, 1613) =8.927, p< 0.001, Dunnett's multiple comparisons, p>0.001); n=11-12/group. There was not a significant difference in weekly body weight in mice in SED+ER group compared to PA+ER group (Dunnett's multiple comparisons, p>0.05); n=11-12/group. Over time there was a significant difference in body weight as a percentage of baseline in mice in both SED+ER and PA+ER groups compared to SED+AL group (data not shown) (two-way ANOVA, time x treatment, F (114, 1613) =9.282, p <0.001, Dunnett's multiple comparisons, p< 0.001); n=11-12/group.

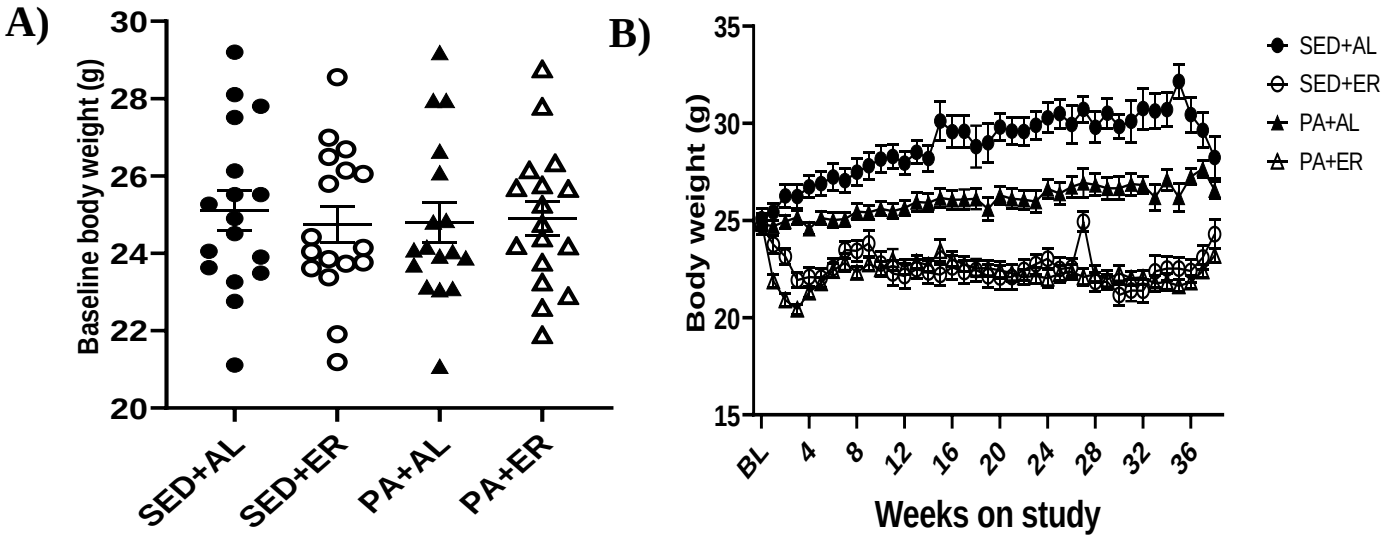


Figure 2: Body weight during the study was significantly reduced in the PA+AL, SED+ER and PA+ER mice compared to control mice (SED+AL). (2A) Baseline body weight in mice was not significantly different at baseline (one-way ANOVA, F (3, 44) = 0.1536, p=0.927);

n=11-12/group. (2B) Body weights of mice in ER groups were significantly reduced during the study compared to (PA+AL) and control mice (SED+ AL) (two-way ANOVA, time x treatment, F (114, 1613) =8.927, p< 0.001) (Dunnett's multiple comparisons, p>0.001); n=11-12/group. Data is reported as mean± SEM.

Food Intake in 4T1.2^{luc} mice

Over time there was a significant difference in food intake between SED+ER and PA+ER mice compared to SED+AL group (Figure 3A) with the energy restricted mice consuming less food. From week 18-27 there was a significant difference in food intake between SED+ER and PA+ER groups (two-way ANOVA, time x treatment, F (111, 1578) = 3.464, p<0.001, Dunnett's multiple comparisons, p<0.05; n= 11-12/ group) with PA mice consuming less food.

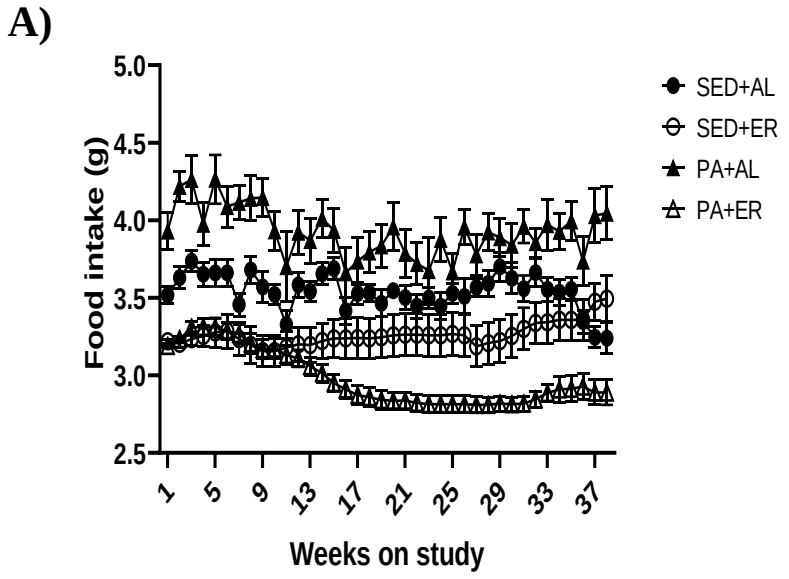


Figure 3: Food intake was significantly lower in SED+ER and PA+ER mice compared to control mice (SED+ AL) (3A) Weekly food intake data in SED+ER and PA+ER mice was significantly lower compared to control (two-way ANOVA, time x treatment, F (111, 1578) = 3.464, p<0.001, Dunnett's multiple comparisons, p<0.05); n= 11-12/group. Data is reported as mean± SEM.

Running wheel activity in 4T1.2^{luc} mice

Over time there was not a significant difference in weekly running wheel activity between PA+AL mice and PA+ER mice (Figure 4A) (two-way ANOVA, time x treatment, F (37,911) = 1.021, p=0.435); n=11-12/group

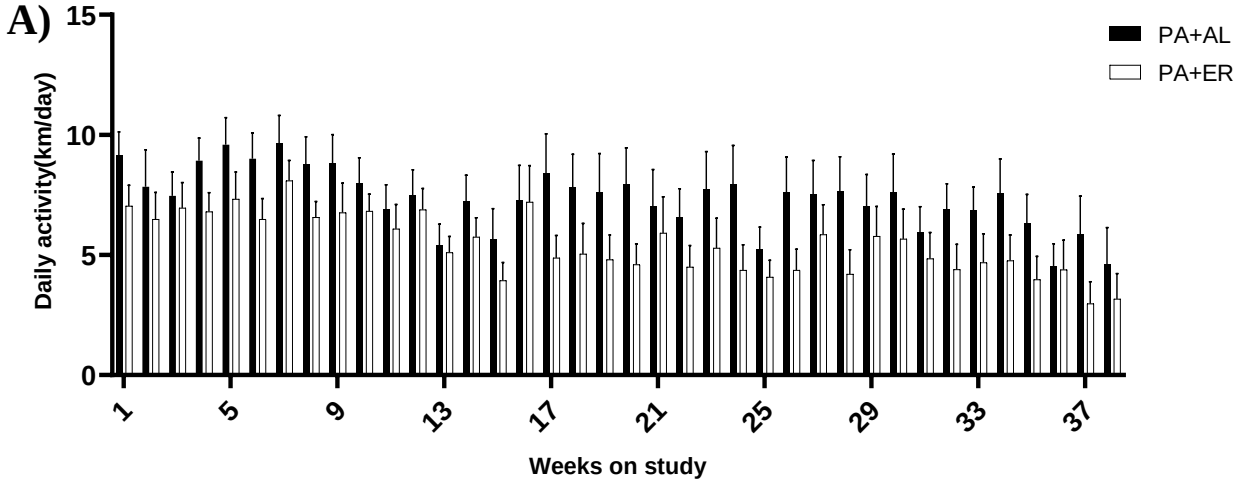


Figure 4: Running wheel activity was not significantly different between *ad libitum* and energy restricted mice (4A) Running wheel activity over the course of the study in *ad libitum* (black bars) and energy restricted (white bars) did not differ significantly between PA+AL and PA+ER mice (two-way ANOVA, time x treatment, F (37,911) = 1.021, p=0.435); n= 11-12/ group. Data is reported as mean ± SEM.

Tumor growth in 4T1.2^{luc} tumor-bearing mice

Mice in all four treatment groups developed tumors (n=11-12/group). There was a significant time x treatment interaction in primary tumor growth over the course of the study (Figure 5A) (two-way ANOVA, F (21, 305) = 3.700, p<0.001); n=11-12/group. Mice in the SED+ER and PA+ER groups had lower tumor volumes than the SED+AL group beginning at day 16 post tumor implantation (Dunnett's multiple comparisons, p<0.001 for both groups). At sacrifice, final tumor volume was significantly lower in SED+ER and PA+ER groups compared to SED+AL (Figure 5B) (one-way ANOVA, F (3, 29) = 5.945, p= 0.0027, Dunnet's multiple comparisons, p<0.05); n=11-12/group

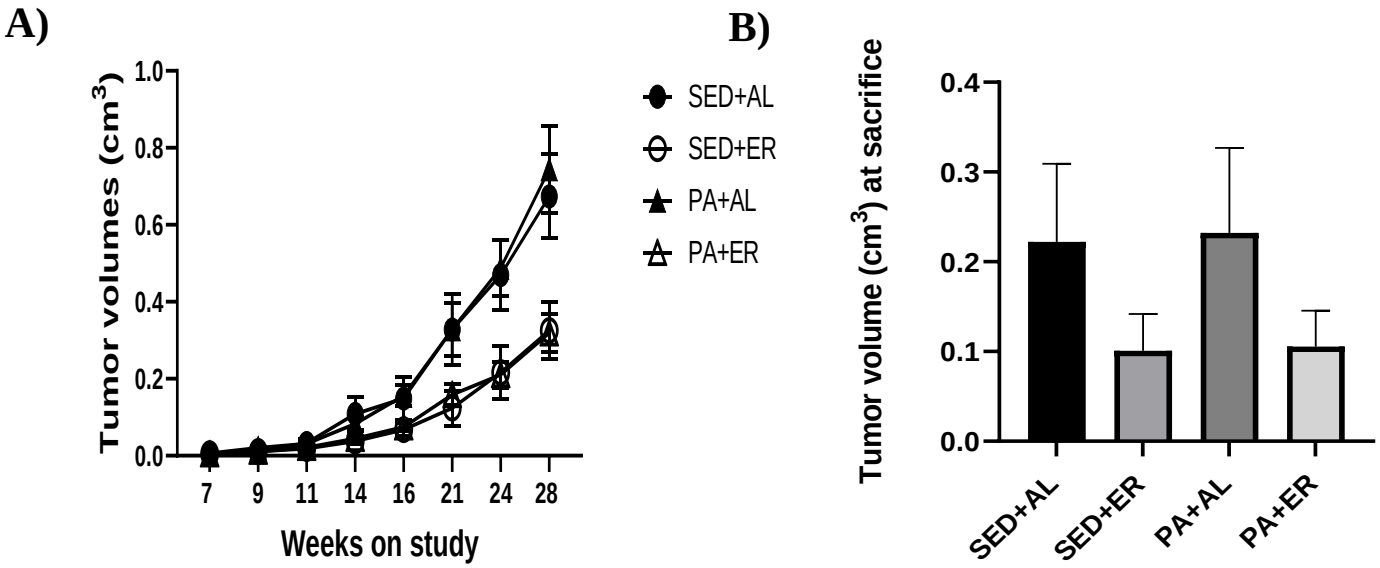


Figure 5: The energy restriction alone and combined with physical activity delayed primary tumor growth (5A) Primary tumor growth over time was significantly reduced in SED+ER mice and PA+ER mice compared to control mice (SED+AL) (two-way ANOVA, F (21, 305) = 3.700, p<0.0001). n= 7-9/ group (5B) Tumor volume at sacrifice was significantly reduced in SED+ER mice and PA+ER mice compared to control mice (SED+AL) (one-way ANOVA, F (3, 29) = 5.945, p= 0.0027, Dunnett's post hoc test, P<0.05) n= 11-12/group). Data are reported as mean ± SEM.

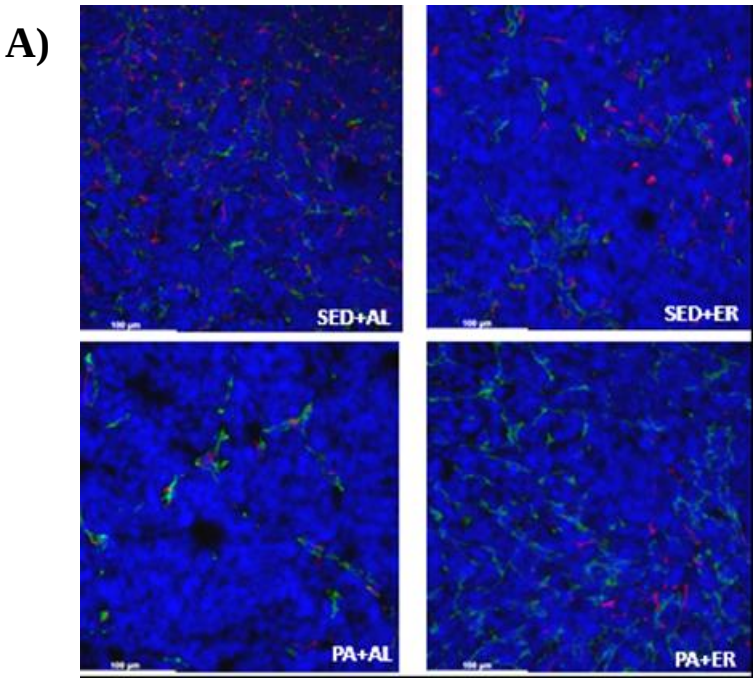
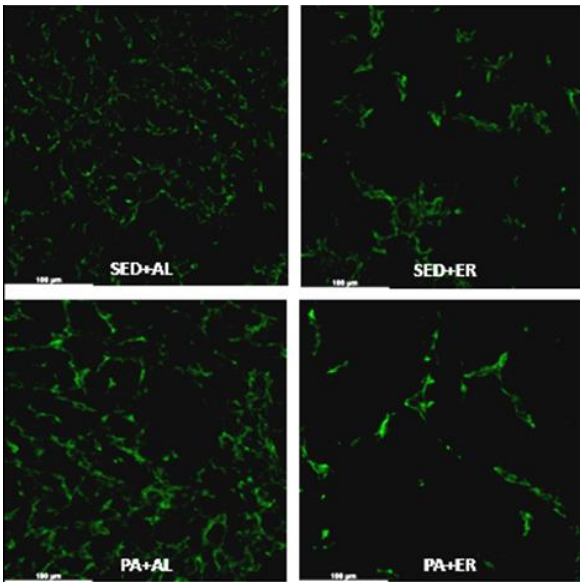


Figure 6: Effects of PA and ER on vessel architecture in 4T1.2^{luc} tumors (6A) Data shown are slides from one representative mouse in each treatment group. Blood vessels are represented by CD31 expression (green) and pericytes are represented by desmin (red) staining, tumor nuclei are represented by 4',6-diamidino-2-phenylindole (DAPI) staining (blue background).

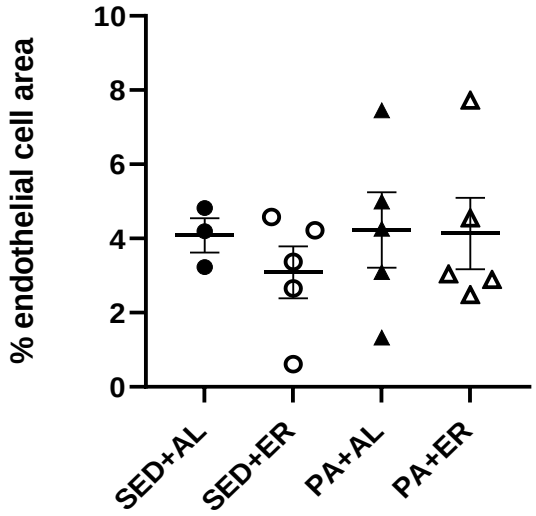
Vascular density

Expression of CD31 (green) on endothelial cells (Figure 7A) was used to identify blood vessels in tumors of mice in each of the treatment groups. In the PA+AL group there was higher quantity of CD31 staining (5.54%) compared to the SED+AL group (3.09%). However, this increase was not observed in the PA+ER group (4.14%). Both the SED+ER and PA+ER groups had lower CD31 staining compared to the SED+AL and PA+AL, respectively. However, none of these changes in the percent of CD31 staining reached statistical significance (Figure 7B) (one-way ANOVA, F (3, 14) = 0.3877, p=0.764); n=3-5/ group. There was no significant effect of physical activity or energy restriction on the ratio of vascular density to tumor volumes (Figure 7C) (Kruskal Wallis, KW= 0.654, p= 0.901). Tumor volumes and vascular density were not correlated (Figure 7D) (Spearman correlation, r=0.383, 95% CI (-0.1171 to 0.7280), p= 0.117); n=3-5/group.

A)



B)



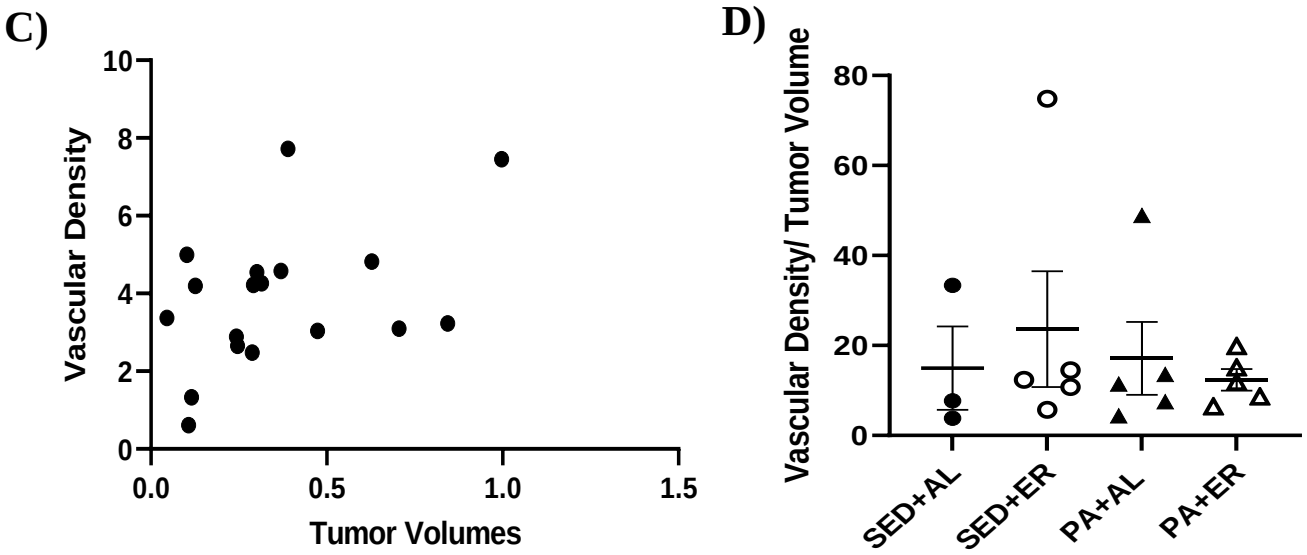
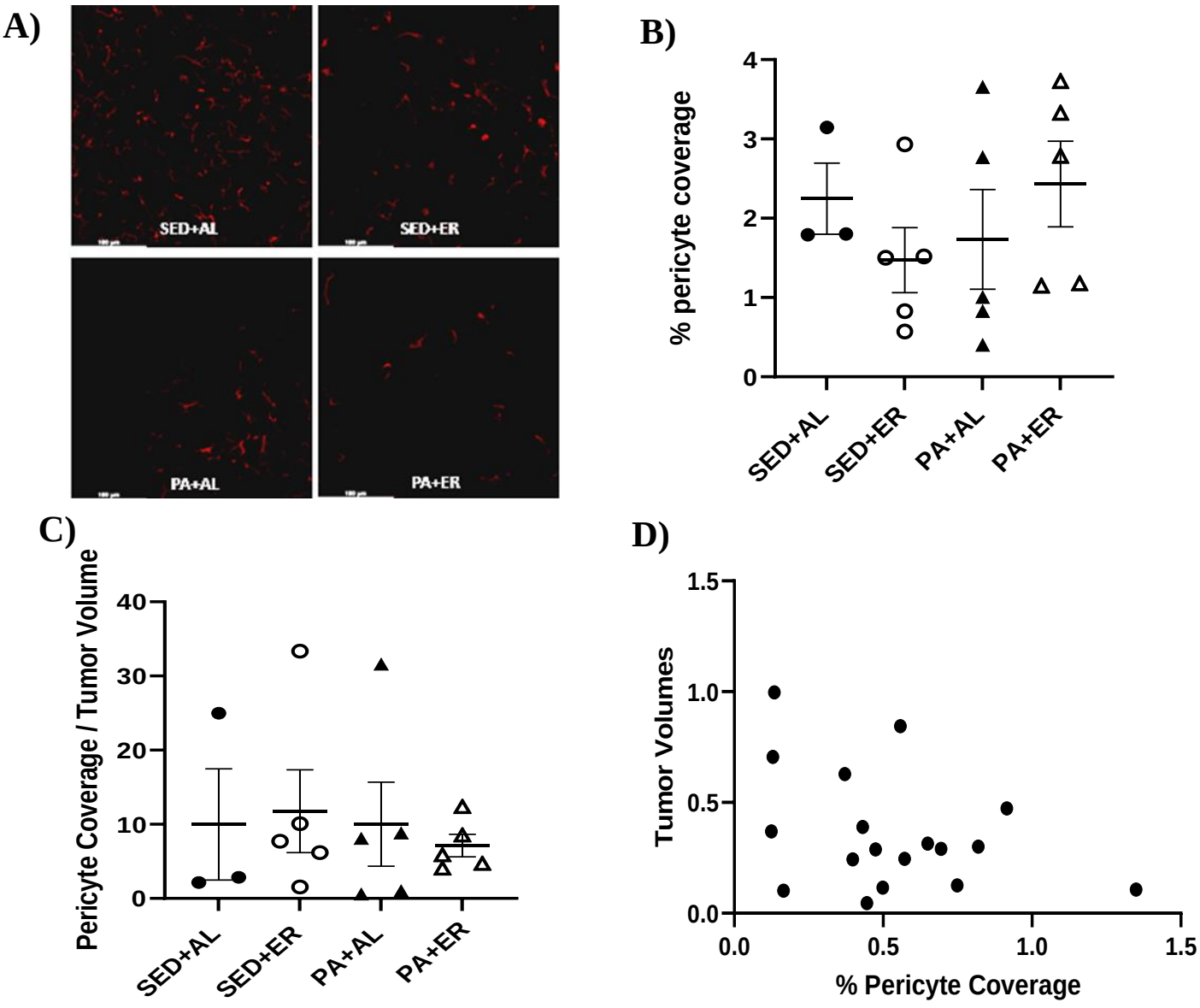


Figure 7: No difference in vascular density among four groups (7A) One representative image of CD31 staining from a mouse in each treatment group. (7B) Vascular density, quantified by % pixel area of CD31 positive endothelial cells in Fiji ImageJ, was not significantly different between groups (one-way ANOVA, F (3, 14) = 0.3877, p=0.764); n=3-5 mice/group. (7C) There was no significant effect of physical activity or energy restriction on the ratio of vascular density to tumor volumes (Kruskal Wallis, KW= 0.654, p= 0.901); n=3-5/group (7D) Vascular density did not correlate with tumor volume in 4T1.2luc tumor-bearing mice (Spearman correlation, r=0.383, 95% CI (-0.1171 to 0.7280), p= 0.117); n= 3-5/group. Data reported as mean ± SEM

Pericyte coverage and vascular maturity

Expression of desmin (red) (Figure 8A) was used to identify pericytes in tumors of mice from each of the treatment groups. The quantity of desmin staining was the lowest in SED+ER mice (1.47%) and the highest in PA+ER mice (2.43%). The quantity of desmin staining was lowered in PA+AL mice (1.73%) compared to control (2.24%). However, none of these changes in the percent of desmin staining reached statistical significance (Figure 8B) (one-way ANOVA, F (3,14) =0.7186, p=0.5572); n=3-5/group. There is no significant effect of physical activity or energy restriction on the ratio of pericyte coverage to tumor volumes (Figure 8C) (Kruskal-Wallis, KW=0.5275, p=0.926). Tumor volumes and pericyte coverage were not correlated (Figure 8D) (Spearman correlation, r=-0.01135, 95% confidence interval (-0.4872 to 0.4697, p= 0.964). Vascular maturity (desmin positive cells/CD31 positive cells) was increased in SED+ER

(0.64%) and PA+ER (0.61%) groups and decreased in PA+AL (0.32%) group compared to control (0.56%). Changes in vascular maturity did not reach statistical significance (Figure 8E) (one way ANOVA, F (3,14) = 1.105, p= 0.380); n=11-12 mice/group.



E)

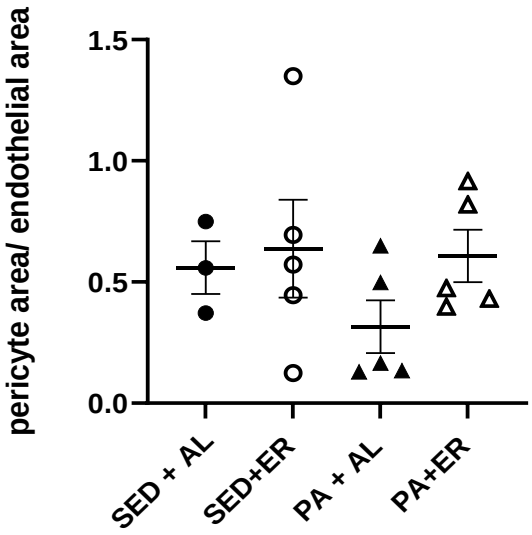


Figure 8: No difference in pericyte coverage or vascular maturity among four groups(8A) One mouse from each group was used to visualize the pericyte coverage in 4T1.2 luc tumor bearing mice. (8B) Pericyte coverage, quantified by % pixel area of desmin positive pericyte cells in Fiji ImageJ, was not significantly different between groups (one-way ANOVA, F (3,14) =0.7186, p=0.557); n=3-5/group. (8C) There was no significant effect of physical activity or energy restriction on the ratio of pericyte coverage to tumor volumes (Kruskal-Wallis, KW=0.5275, p=0.926); n=3-5/group (8D) Pericyte coverage did not correlate with tumor volume in $4T1.2^{luc}$ tumor-bearing mice (Spearman correlation, r=-0.01135, 95% confidence interval (-0.4872 to 0.4697, p= 0.964); n= 3-5/group. (8E) Vascular maturity (pericyte/ desmin area/endothelial/ CD31 area) in mice) was not significantly different between mice. (one way ANOVA, F (3,14) = 1.105, p= 0.380); n=3-5/group. Data reported as mean ± SEM.

Vessel branching and pericyte coverage counts

Vessel branching was manually counted and the average of four independent counts was used to quantify vessel branching pericyte number and vascular maturity (Figure 9A, 9B, 9C). Average vessel branch counts were the lowest in SED+ER (102 vessel branch points) and PA+ER (103 vessel branch points) groups and decreased slightly in PA+AL group (129 vessel branch points) compared to control SED+AL group (133 branch points). Differences in vessel branch counts between groups did not reach significance (Figure 9A) (one-way ANOVA, F (3,14) = 0.1074, p=0.107); n=3-5/group. Average pericyte counts were decreased in SED+ER (58 pericytes), PA+ER (59 pericytes) and PA+AL (55 pericytes) groups compared to SED+AL (65 pericytes) control group. Reductions in pericytes between groups were not significant (Figure

9B) (one-way ANOVA, F (3,14) =0.4370, p=0.4370); n=3-5 mice/group. Based on counts, vascular maturity (pericyte count/vessel branch count) was increased in SED+ER (57%) and PA+ER (57%) groups and decreased in PA+AL (43%) group compared to control SED+AL (49%) group. Differences in vascular maturity were not significantly different (Figure 9C) (Kruskal-Wallis, KW=6.282, p=0.0880); n=3-5/group

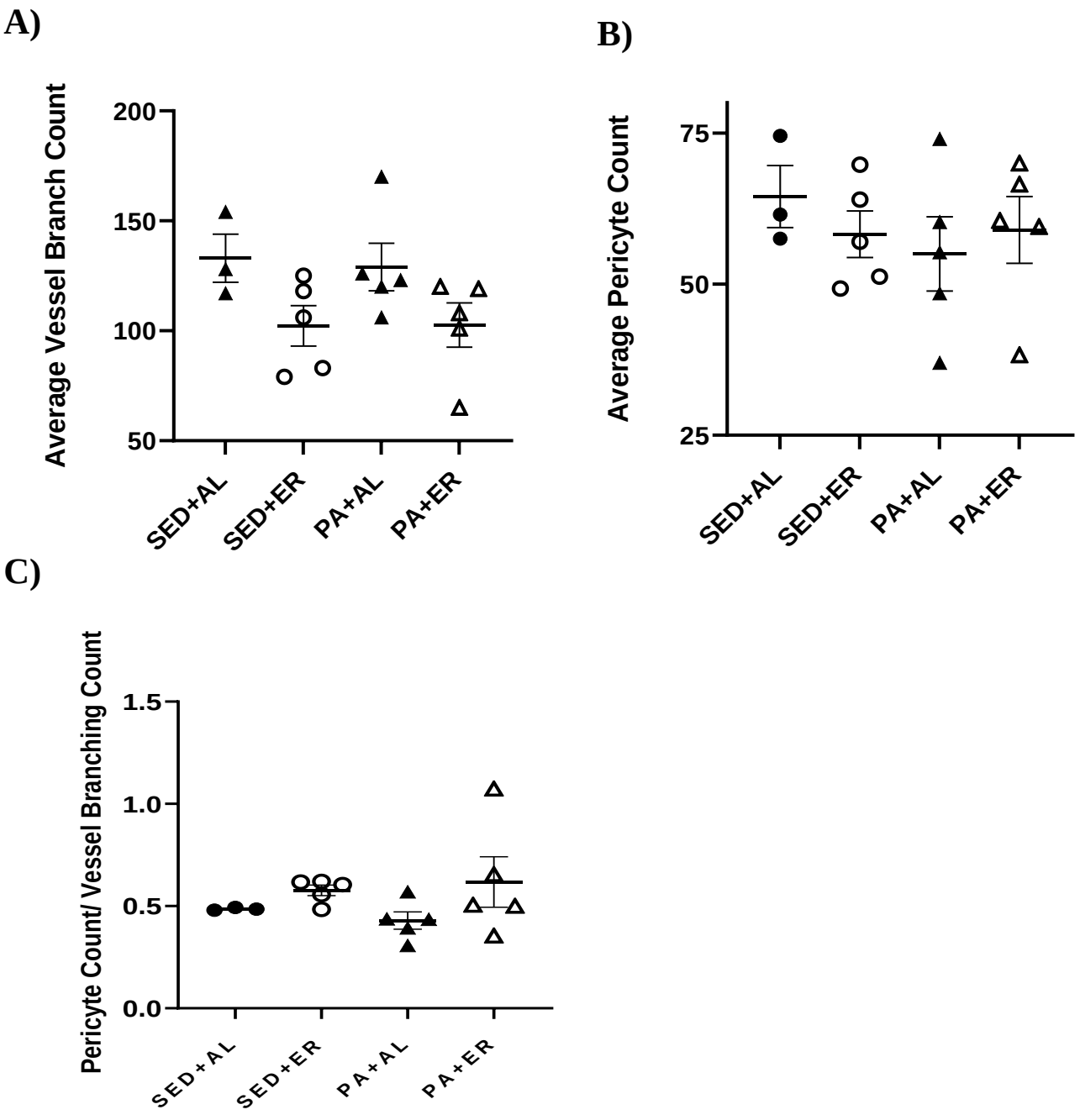
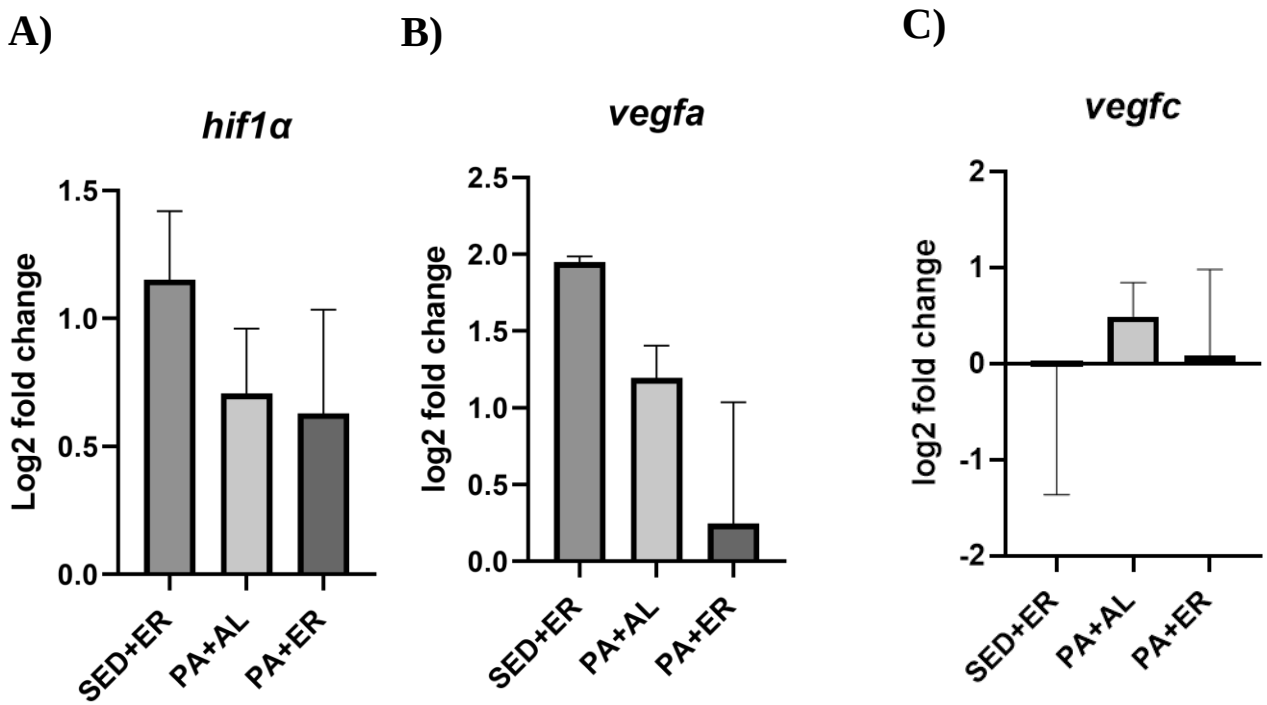


Figure 9: No difference in vessel branching, pericytes, or vascular maturity based off counts from among four independent counters (9A) Vessel branch counts were not significantly different between groups (one-way ANOVA, F (3,14) = 0.1074, p=0.107); n=3-5/group (9B) Pericyte counts were not significantly different between groups (one-way ANOVA, F (3,14) =0.4370, p=0.4370); n=3-5/group (9C) Vessel maturity (pericyte count/vessel branch count) was not significantly different between groups (Kruskal-Wallis, KW=6.282, p=0.0880); n=3-5/group. Data reported as mean ± SEM.

Markers of hypoxia

The fold change of *hif1-α* was slightly increased in all groups compared to SED+AL control; however, this increase was not significantly different among the groups (Figure 10A) (one-way ANOVA, F (3,3) = 1.508, p= 0.372); n =3-6/group. The fold change of *vegf-a* was increased in all groups compared to SED+AL control; however, this increase was not significantly different among treatment groups (Figure 10B) (one-way ANOVA, F (3,3) = 2.991, p=0.196); n=3-6/ group. The fold change for *vegf-a* expression was the lowest in PA+ER mice. The fold change of *vegf-c* was only minimally altered by any of the treatment groups compared to the SED+AL group and these differences were not significant among treatment groups (Figure 10C) (one-way ANOVA, F (3,3) = 0.06026, p=0.974); n=3-6/ group. The fold change of *il-6* was increased in all groups in comparison to SED+AL control, but changes were not significantly different among group (Figure 10D) (one-way ANOVA, F (3,3) = 0.1156, p=0.945); n=3-6/group



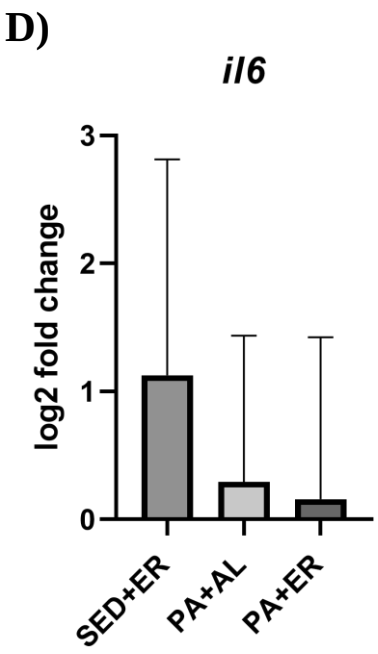


Figure 10: No difference in Log2 fold gene expression of 4 markers of hypoxia determined by qPCR (10A) There was no significant difference in Log2 fold gene expression of HIF 1 alpha compared to SED+AL control (one-way ANOVA, F (3,3) = 1.508, p= 0.372); n=3-6/group. (10B) There was no significant difference in Log2 fold gene expression of VEGF-A compared to SED+AL control (one-way ANOVA, F (3,3) = 2.991, p=0.196); n=3-6/ group. (10C) There was no significant difference in Log2 fold gene expression of VEGF-C compared to SED+AL control (one-way ANOVA, F (3,3) = 0.06026, p=0.974); n=3-6/ group (10D) There was no significant difference in Log2 fold gene expression of IL-6 compared to SED+AL control (one-way ANOVA, F (3,3) = 0.1156, p=0.945); n=3-6/group. Data reported as mean ± SEM.

Markers of angiogenesis

The fold change of *ang* was increased in all groups compared to the SED+AL group but these differences were not significant among treatment groups (Figure 11A) (one-way ANOVA, F (3,3) = 0.4599, p=0.730); n=3-6/group. The fold change of *pecam-1* was increased in all treatment groups in comparison to control, but there was no difference among treatment groups (Figure 11B) (one-way ANOVA, F (3,3) = 0.2690, p=0.845); n=3-6/group. There was a modest increase in the fold change of cdhr5 that did not differ significantly among treatment groups (Figure 11C) (one-way ANOVA, F (3,3) = 0.6235, p =0.623); n=3-6/group.

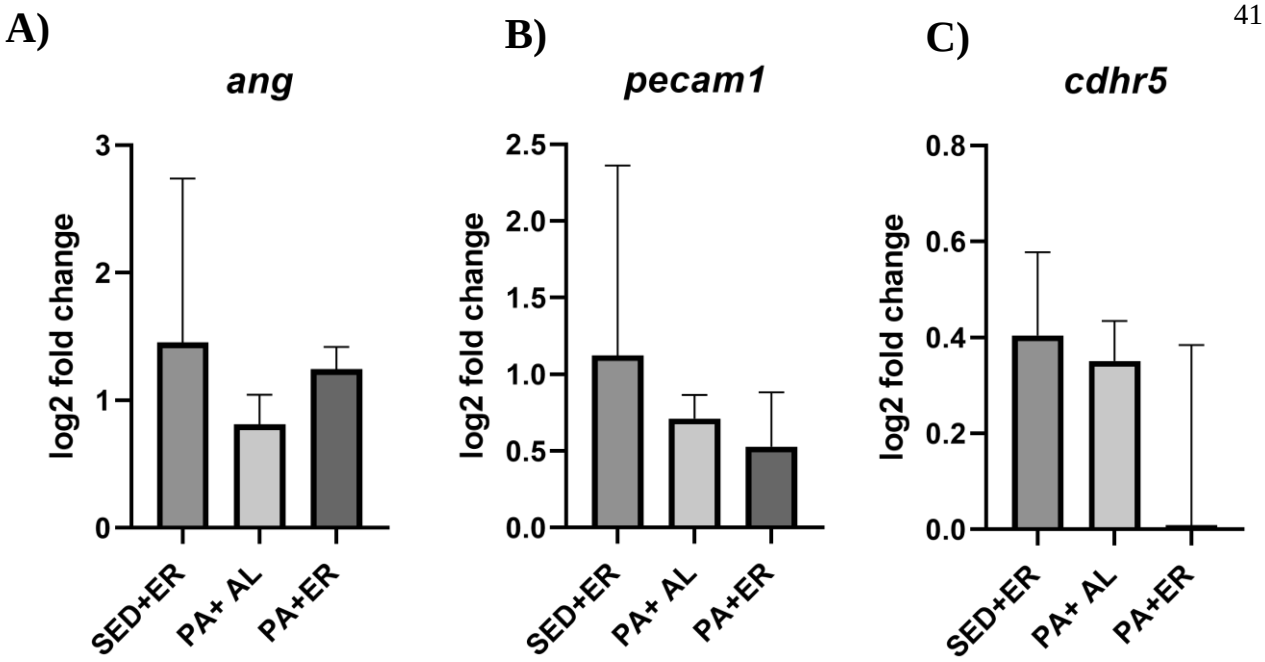


Figure 11: No difference in Log2 fold gene expression of 3 markers of angiogenesis determined by qPCR (11A) There was no significant difference in Log2 fold gene expression of Ang compared to SED+AL control (one-way ANOVA, F (3,3) = 0.4599, p=0.730); n=3-6/group. (11B) There was no significant difference in Log2 fold gene expression of Pecam-1 compared to SED+AL control (one-way ANOVA, F (3,3) = 0.2690, p=0.845); n=3-6/group (11C) There was no significant difference in Log2 fold gene expression of Cdhr5 compared to SED+AL control (one-way ANOVA, F (3,3) = 0.6235, p =0.623); n=3-6/group. Data reported as mean ± SEM.

DISCUSSION

In this study of a 4T1.2^{luc} triple negative model of breast cancer in BALBc mice, we found a trend for an increase in vessel maturity (pericyte coverage/ endothelial cell coverage) in SED+ER and PA+ER groups in comparison to control, by means of ImageJ analysis (p= 0.380) as well as manual counting (p=0.0880), this trend was not influenced by a decreased fold change in any of the groups in comparison to control for markers of hypoxia of angiogenesis (p>0.05).

Energy restriction and physical activity

Preclinical models provide evidence for the beneficial effects of energy restriction on cancer outcomes. Energy restriction has resulted in reduced tumor growth and metastatic burden in preclinical models of TNBC [77, 99, 100, 235, 239]. Physical activity in mice has resulted in a reduction in tumor growth in 17 studies [78].Preclinical models are also used as an aid to help

researchers elucidate mechanisms underlying these effects. The current study utilized a 2 x 2 factorial model to explore the independent and combined effects of energy restriction and physical activity on cancer outcomes in a TNBC model. Energy restricted mice were fed standard A1N76A mouse chow and were mildly energy restricted 10% before and after orthotopic inoculation of 4T1.2^{luc} tumor cell line. Physically active mice were given access to running wheels before and after orthotopic inoculation of 4T1.2^{luc} tumor cell line.

Over the course of the study, body weights of mice were significantly lower in ER mice compared to PA mice. PA+ER mice had the lowest food intake over the course of the study. From week 18-27 there was a significant decrease in food intake between PA+ER and SED+ER groups but the body weight of PA+ER and SED+ER mice did not differ significantly. Running wheel activity did not differ significantly between ad libitum and energy restricted mice over the course of the study. The decrease in food intake but maintenance of body weight may be due to energy restricted mice exhibiting similar physical activity patterns as mice with access to a running wheel during this time, as sedentary mice were not monitored for physical activity.

The current study found that SED+ER and PA+ER had mice had significantly smaller tumor volumes in comparison to SED+AL control providing evidence for the beneficial effects of energy restriction and the combination of physical activity and energy restriction on tumor reduction. Overall, mean tumor volume was the smallest in the PA+ER group (0.319 cm^3) but this was only marginally smaller than the mean tumor volume for the SED+ER group (0.325 cm^3). Typically studies from our lab find reductions in tumor volume to be the most significant in the PA+ER group, highlighting the benefits of the combination of physical activity and energy restriction. Results from this study may be due to the age of the mice. From our lab, Turbitt et al found no differences in energy restriction induced body weight, the amount of PA, or in primary tumor growth and metastatic outcomes between a cohort of 10-week-old and 38-week-old BALBc mice inoculated with 4T1.2^{luc} cells [1]. While Turbitt found no difference in tumor growth in the two different age cohorts, mice in this study were slightly older, at 52-weeks-old at the time of sacrifice, the extended amount of time on the energy restriction intervention may have influenced tumor volumes. A recent study found daily caloric restriction of 20% to be more effective than a 4:10 cycle (four days of 50%:70%:70%:70% energy restricted followed by 10 days *ad libitum* diet) fasting mimicking diet in reducing tumor growth and metastatic burden in

BALB c mice inoculated with a TNBC cell line [239]. This study highlights that duration of energy restriction may play a key role in the effects on cancer outcomes.

In summary, single intervention energy restriction as well as combined intervention energy restriction and physical activity resulted in significantly reduced tumor volumes compared to control, highlighting the benefits of energy restriction on cancer outcomes in the preclinical 4T1.2^{luc} model, and giving rise to the need for future clinical trials. The clinical trial CAREFOR: Precision Medicine Driving Precision Nutrition for the Treatment of NeoAdjuvant Breast Cancer (NCT02827370) began in 2016 and has a projected end date of 2022. Researchers plan to assess the effects of dietary energy restriction on pathological complete response when combined with neo-adjuvant chemotherapy. Future clinical trials are needed to further elucidate the role of energy restriction and physical activity in breast cancer care and treatment.

Vessel architecture

Previous findings from preclinical breast cancer models indicate that energy restriction decreases tumoral microvessel density [99, 235] and physical activity increases tumoral microvessel density [79, 232]. The main cohort of mice was split into two pilot studies, one to analyze the impact of the physical activity and energy restriction alone and combined on vessel architecture and hypoxia, the other to analyze the impact of physical activity and energy restriction on the tumor microbiome and metabolome. For the whole cohort of mice (n= 8-9/ group), there was a statistically significant decrease in the tumor volumes of SED+ER and PA+ER mice in comparison to control SED+AL mice. Tumor volumes were not significantly different in the smaller immunohistochemistry cohort (3-5 mice/ group), but the trends remained the same with SED+ER and PA+ER mice having the smallest mean tumor volumes. There were no significant findings from the current pilot study, but the trends follow what has been published previously in the literature. The percent endothelial cell area was quantified by positive CD31 staining in ImageJ Fiji software and was used to quantify microvessel density. Sedentary, energy restricted mice had the lowest mean percent endothelial cell area and physically active *ad libitum* mice had the largest mean percent endothelial cell area of all groups. Our study was the first to look at the combined effects of physical activity and energy restriction on microvessel density in 4T1.2^{luc} mice. The percent endothelial cell area for PA+ER mice seemed to mimic the increase in percent endothelial cell area of PA+AL mice instead of the

decrease in percent endothelial cell area seen in SED+ER mice. These results suggest that the combination of physical activity and energy restriction results in increased microvessel density in the 4T1.2 tumor microenvironment.

We found that vascular density and tumor volumes were not correlated. This is surprising considering the role of vessel density and tumor volumes reported previously in the literature [240]. The same images quantified by ImageJ for percent endothelial cell area were used to quantify vessel branching by four individual counters. Vessel branch counts, or the point where one vessel becomes two, was the lowest in SED+ER mice and was the highest for SED+AL and PA+AL mice. Vessel branch counts seemed to mimic the decrease seen in SED+ER mice. This is interesting since vessel branching is controlled by a feedback loop between vegf-a and Delta-like four (Dll4). Hypoxic tumor cells release VEGF, which activates the branching process [183]. *vegf-a* expression was the highest in SED+ER mice but the lowest in PA+ER mice, suggesting the increased expression of delta-like-four in SED+ER mice.

There are few published studies on the effects of energy restriction and physical activity on pericyte coverage and vessel maturity. One study using a triple negative mouse model found that physical activity increased pericyte coverage and vascular maturity compared to sedentary mice [79], another study using an Ewing sarcoma mouse model found an increase in pericytes in treadmill exercised mice compared to sedentary mice [241]. A mouse model of astrocytoma found an increase in vessel maturity in brain tissue in mice that were energy restricted 30% compared to *ad libitum* mice [242]. Mean percentage pericyte coverage was decreased in SED+ER and PA+AL mice and slightly increased in PA+ER mice in our study. Percent pericyte coverage was not correlated with tumor volumes. Vascular maturity was increased in SED+ER and PA+ER mice and decreased in PA+AL mice, compared to control. The reason for the decrease in the percent of pericyte coverage and vascular maturity when there was an increase in the literature may be since desmin and CD31 antibodies were not colocalized in this study. The same images quantified by ImageJ for % pericyte area were used to quantify pericyte counts by four individual counters. Mean pericyte counts were decreased for all groups and the lowest for PA+AL mice compared to control. For both ImageJ and counting analysis we found that SED+AL and PA+ER groups had the highest mean pericyte amount, but the PA+ER group had the highest mean pericyte percentage and the SED+AL group had the highest mean pericyte

count. The reason for the discrepancy between counts and ImageJ analysis may be due to the subjectivity of counting.

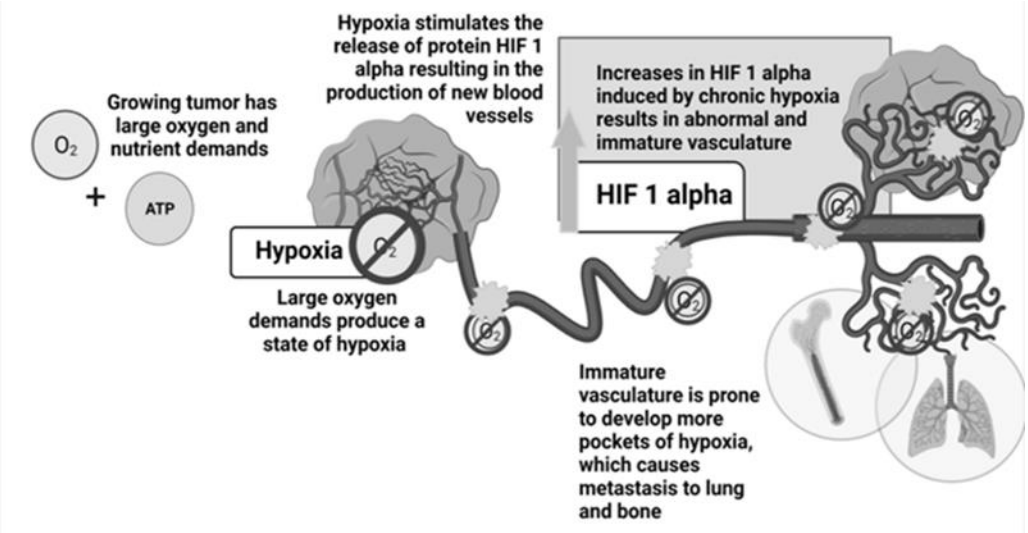


Figure 12: The role of hypoxia in the tumor microenvironment (12A) High levels of hypoxia, due to high oxygen demands of the tumor, result in abnormal and immature vasculature which increase the chances of eventual metastasis to lung and bone.

Markers of hypoxia and angiogenesis

We originally hypothesized that physical activity and/ or energy restriction would result in decreased markers of hypoxia and angiogenesis in the tumor microenvironment, normalizing levels of hypoxia and resulting in normal vasculature. Apart from the downregulation of *vegf-c* in SED+ER mice, we reported opposite effects. There was a modest increase in all markers of hypoxia (*hif1a*, *vegfa*, and *il-6*) and angiogenesis (*ang*, *pecam-1*, *cdhr5*) across all groups in comparison to SED+AL control. Hypoxia and angiogenesis were not significantly altered by our inventions. Previous preclinical studies have found that physical activity results in increased VEGF expression in the mouse tumor microenvironment [79, 232], which may be attributed to the increase in microvessel density observed in these studies. *vegf-c* expression was lower in SED+ER mice. No studies of energy restricted mice have seen an increase in VEGF in the tumor microenvironment. In mammary tumor models, 40% dietary energy restriction significantly decreased serum VEGF-A [99] and decrease Flk-1 expression, a receptor of VEGF, in the tumor microenvironment [235] compared to ad libitum fed controls. One study using human endothelial cells (HUVEC) treated with sera derived from monkeys on long-term (over 10 years) energy restriction did find an increase in the expression of VEGF [243].

One proposed mechanism explaining the increase in markers of hypoxia could be an improvement in markers of inflammation which has been previously reported in our lab [77]. Other mechanisms explaining improvements in vessel architecture other than improvements in hypoxia could be an increase in autophagy [115], activation of AMPK [244], inhibition of mTOR [245], or downregulation of IGF-1 [113]. In summary, there was an increase in markers of hypoxia and angiogenesis across all groups, indicating the mechanism behind vessel normalization may not be due to hypoxia.

Strengths

This study has strengths. One strength was the use of fluorescent immunohistochemistry to assess pericyte and endothelial cell populations using desmin and CD31 antibody detection, which has been used previously in the literature. The protocol for the immunohistochemistry for this study was obtained from the author of a study assessing the effects of physical activity on vessel architecture and hypoxia in a 4T1.2 model, immunohistochemistry images were similar. We used two analysis methods to quantify blood vessels and pericytes, ImageJ analysis along with manual counting. For counting, four individual counters were used instead of just the primary researcher. We used qPCR instead of gel electrophoresis to quantify gene expression which is a more accurate representation of gene expression. Another strength was mouse data was primarily collected by one to two students over the course of the study, instead of several. Another strength of this study is that this study design has been previously implemented in our lab where similar findings were observed, in that intervention groups had decreased tumor volumes in comparison to control.

Limitations

This study has limitations. One limitation was sample size. The parent study included 11-12 mice/group to begin, and statistical significance was reached with 7-9 mice/group for tumor volumes. According to a power analysis, 25 mice per group will be required to detect a significant difference in mean vessel density between the SED-AL and SED-ER groups with 80% power, two-sided type 1 error rate of 5%. In terms of microvessel density and pericyte coverage, a limitation of our study was that blood vessels and pericytes were not colocalized. Due to several limitations, our results should be interpreted with caution. This experiment should

be repeated in a larger cohort (25 mice/group) of mice that were inoculated with tumor after 8 weeks of interventions, with colocalization of CD31 and desmin for immunohistochemistry staining.

Conclusion

Findings from this study indicate that both physical activity and the combination of physical activity and energy restriction result in significantly reduced tumor volumes. No other findings were significant, but there were several trends. Energy restriction may decrease vascular density while physical activity may increase vascular density. There was a trend for a decrease in percent endothelial cell area, and vessel branch counts in SED+ER mice and an increase in percent endothelial cell area, and vessel branch counts in PA+AL mice. The combination of physical activity and energy restriction is less clear as there was a trend for increased percent endothelial cell area in PA+ER mice which mimicked the effects seen in PA+AL mice, but there was a trend for decreased vessel branch counts in PA+ER mice which mimicked the effects seen in SED+ER mice. Energy restriction increases mean vascular maturity in both SED+ER and PA+ER mice, compared to control. PA+ER mice have the highest mean percent pericyte coverage and SED+ER mice have the lowest mean percent pericyte coverage. SED+ER mice also have the smallest percentage of vessels. The vascular maturity (% pericyte cell coverage/% endothelial cell coverage) was increased in both SED+ER AND PA+ER mice, compared to control. Our findings also indicate that normalization in vessel architecture is accompanied by an increase in the expression of markers of hypoxia and angiogenesis.

In conclusion, our results indicate that there is improved vessel maturity in SED+ER and PA+ER groups, alongside an upregulation of markers of hypoxia and angiogenesis in this cohort of 4T1.2^{luc} mice, indicating that the trend for improved vasculature in SED+ER and PA+ER mice may be due to another mechanism other than the normalization of hypoxia within the tumor microenvironment. Future studies are needed to assess the effects of the combination of physical activity and energy restriction of tumor vessel architecture and hypoxia.

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ACADEMIC VITA

Abriana Cain

EDUCATION

The National Institutes of Health Dietetic Internship

Expected: August 2023

The Pennsylvania State University, Schreyer Honors College, University Park, PA

B.S. Nutritional Sciences, Nutrition and Dietetics option, M.S. Nutritional Sciences

WORK EXPERIENCE

The Pennsylvania State University- *Research Assistant,* University Park, PA

August 2019-July 2022

- Completed thesis work in Dr. Connie Rogers' lab which focuses on analyzing the inflammatory effects of exercise and energy restriction in a murine breast cancer model
- Paid for research work from May 2022-July 2022

Mount Nittany Medical Center- *Dietary Aide,* State College, PA

February 2020- May 2022

- Built and delivered patient trays for 200 bed facility

Taproot Kitchen-*Nonprofit employee,* State College, PA

August 2020- December 2020

- Launched a collaboration between Taproot and the Nutrition department at Penn State to provide Taproot with volunteers and nutrition students with relevant experience

Chambersburg Hospital- *Catering Associate,* Chambersburg PA

June 2017-August 2019

- Gained over two years and 2000+ hours of nutrition related experience

The Pennsylvania State University- *Peer Tutor,* Mont Alto, PA

August 2017- May 2018

- Tutor at Mont Alto campus in NUTR 251 course as well as BIO 141 course

VOLUNTEER EXPERIENCE

The Gleaning Project Volunteer Chambersburg, PA

May 2018- August 2019

- Developed over 100 "recipe cards" that were distributed along with all donated produce items. Recipe cards were in both English and Spanish

PRESENTATIONS

Undergraduate Research Exhibition- April 8, 2022

Presented a poster titled "*The effect of physical activity and energy restriction on tumor hypoxia and blood vessel normalization in a triple negative breast cancer model*". Won Gerard A. Hauser award for best poster overall.

The International Conference for Undergraduate Research- September 27, 2021

Abstract selected for oral presentation. Talk titled "*The combination of physical activity and energy restriction reduces HIF-1 alpha gene expression in the tumor microenvironment in the 4T1.2 breast cancer model*"

Huck Life Sciences Symposium- May 18, 2021

Abstract selected for oral presentation. Talk titled "*The combination of physical activity and energy restriction reduces HIF-1 alpha gene expression in the tumor microenvironment in the 4T1.2 breast cancer model*"

Spend a Summer Day PSU Panelist- July 28, 2020

Chosen to represent the nutritional sciences major for the college of HHD. Gave a talk about current college experience to 70 prospective students and parents, answered questions at the end of the session.

ACTIVITIES

The Academy of Nutrition and Dietetics, Student Member August 2019- present