2023

High body mass index changes Peri-tumor adipose tissue which in turn promotes triple negative breast cancer

Cora Elizabeth Miracle
miracle13@marshall.edu

HIGH BODY MASS INDEX CHANGES PERI-TUMOR ADIPOSE TISSUE WHICH IN TURN PROMOTES TRIPLE NEGATIVE BREAST CANCER

A dissertation submitted to
Marshall University
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
in
Biomedical Research
by
Cora Elizabeth Miracle
Approved by
Dr. Travis Salisbury, Committee Chairperson
Dr. Richard Egleton
Dr. Piyali Dasgupta
Dr. Joshua Hess
Dr. Sandrine Pierre

Marshall University
May 2023

Approval of Thesis

We, the faculty supervising the work of Cora Elizabeth Miracle, affirm that the dissertation, *High Body Mass Index changes Peri-Tumor Adipose Tissue which in turn promotes Triple Negative Breast Cancer* meets the high academic standards for original scholarship and creative work established by the Biomedical Research Program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

Dr. Travis Salisbury, Department of Toxicology Committee Chairperson Date
2/15/2023

Travis Salisbury Digitally signed by Travis Salisbury
Date: 2023.03.03 16:24:24 -05'00'

Dr. Richard Egleton Committee Member Date
2/15/2023

Richard Egleton, PhD Digitally signed by Richard Egleton, PhD
Date: 2023.03.03 17:06:51 -05'00'

Dr. Piyali Dasgupta Committee Member Date
2/15/2023

Piyali D. 3/3/2023

Dr. Joshua Hess Committee Member Date
2/15/2023

3/6/2023

Dr. Sandrine Pierre Committee Member Date
2/15/2023

Sandrine V Pierre Digitally signed by Sandrine V Pierre
Date: 2023.03.05 15:39:27 -05'00'

Dedication:

I dedicate this thesis to Lynard Elizabeth Souder and Eddie Souder.

I wish you two could be here to see this. I love you forever.

Acknowledgements

I do not know where to begin, I am so very blessed to have so many wonderful, supportive, and amazing individuals in my life. God has truly placed me in an amazing program and given me the opportunity to meet such wonderful people. I firstly would like to thank my mentor Dr. Salisbury for his constant guidance through-out my PhD. Chelsea McCallister, I would not have graduated without you, you are the best scientist and human I have ever met, thank you for all the hours you spent listening to me just talk and all the techniques you helped me learn, and to help me find things that were right in front of me. Thank you to Dr. Richard Egleton who was always in my corner and never let me waiver in my love for science and medicine, you also served as an amazing mentor without being asked. Dr. Nitin Puri, I would not be here without you, you go above and beyond for your students, and I am eternally grateful. Dr. Sundaram, thank you for always guiding me on the path to be the best physician scientist I can be.

To my family, my loving grandparents, your calls, and texts always put a smile on my face and cleared up any cloudy day I had. To my parents, the words, and thanks that I owe to you are insurmountable. You listened to me cry, you drove two and half hours to cook for me and do laundry just to relieve stress. You are the reason I am able to persevere and do what I do. Please know everything I do; I do for you. I love you. To my cousins who always text me, visited me, and told me they loved me, and forgave me for the events I missed, thank you, I love you.

“A sweet friendship refreshes the soul” and to all of you, you refreshed my soul in the times I didn’t think I had one (because I surely sold it to get this degree). Leah Ching, Jess Conatser, Jess Roth, Nadye Meinking-Colby, Kadi Harvey, Cherishma Nagisetty, Skylar Cooper, and Mary Piscura, you all supported me in my worst times. You picked me up and carried me through the long days and late nights, with dinners, movie nights, or phone calls. Thank you for

watching my heathens on my long days. Thank you for being you. Maria Ivanenkov, Marianne Sand, Ruhi Gulati, thank you for all the facetimes and girls' nights back home, thank you for believing in me when I didn't believe in myself. Becca Adams-Clark, your constant friendship, encouragement, aid, and visits helped me more than you know you are my life long best friends, I love you so much.

Ashley Cox, Ceci, and Siva, thank you for being my PhD friends and getting me through grad school.

Justin Edward, you, and your family have come into my life and given me so much support, and love, your encouragement has been far more than I could have ever imagined. You have been my rock. Thank you so much for being such an amazing human, I love you so much.

Marshall and Bella, my sweet fur babies, you will never know how much you mean to me and the happiness you bring to me. I love you both more than words can describe.

Finally, I want to thank Dr. Mark Rochman and Dr. Marc Rothenberg, it is you who is responsible for me being. Dr. Rothenberg is who inspired me to become an MD/PhD, his training and wisdom along with Dr. Mark Rochman has stayed with me and will continue to my entire career. Thank you for always believing in me. You continue to inspire me and push me to be the best scientist and physician that I can.

Table of Contents

List of Tables

List of Figures

Abbreviations

ADS – adipose derived secretome

AHR – aryl hydrocarbon receptor

AKT – protein kinase B

AMPK – AMP activated protein kinase

AT – adipose tissue

ATP – adenosine triphosphate

BCAA – branch chain amino acids

BCAT – branched chain amino acid aminotransferase

BCKDC – branched chain alpha-keto dehydrogenase

BCSC – breast cancer stem cells

BMI – body mass index

CCND1 – cyclin D 1

CDK8 – cyclin dependent kinase 8

CDKN1 – cyclin dependent kinase inhibitor 1

CRC – colorectal cancer

CSC – cancer stem cells

E4EBP – eukaryotic translation initiating factor 4E binding protein

ECM – extracellular matrix

EMT – epithelial mesenchymal transition

ERK – extracellular signal-regulated kinase

EV- extracellular vesicles

FBS – fetal bovine serum

GSK3B – glycogen synthase kinase

HER2 – human epidermal growth receptor 2

HES1 – hairy and enhancer split 1

HIF1a – hypoxia inducible factor 1 alpha

IL-6 – interleukin 6

IL-8 – interleukin 8

IRS – insulin receptor substrate

JAG1 – jagged 1

JAK – Janus Kinase

KRAS – Kirsten rat sarcoma viral oncogene homolog

LAT-1 – L-type amino acid transporter 1

LEPR – Leptin Receptor

MCP1 – monocyte chemoattractant protein 1

MCP1 – monocyte chemoattractant protein 1

miRNA – microRNA

MMP- matrix metalloproteinase

mTOR – mammalian target of rapamycin

mTORC1 – mammalian target of rapamycin complex 1

mTORC2 - mammalian target of rapamycin complex 2

MTTP – microsomal triglyceride transfer protein

NDRG1 – n-myc downregulated gene 1

NFKB – nuclear factor kappa B

NICD – notch intracellular domain

NTA – nanoparticle tracking analysis

PBS – phosphate buffered solution

PI3K – phosphoinositide 3-kinase

PKC – protein kinase C

RAG – recombination activating genes

S6K – ribosomal protein S6 kinase

shRNA – short hairpin RNA

SIN1 – stress activated map kinase interacting protein 1

SNP – single nucleotide polymorphism

STAT3 – signal transducer and activator of transcription 3

TCA – tricarboxylic acid cycle

TCDD – 2,3,7,8-tetrachlorodibenzo-p-dioxin

Th-17 – T helper 17 cells

TNBC – triple negative breast cancer

TNFa – tumor necrosis factor alpha

VEGF – vascular endothelial growth factor

WNT – wingless-related integration site

XIAP – X-linked inhibitor of apoptosis protein

Abstract

Cancer is one of the leading causes of death worldwide, responsible for over half a million deaths each year. There are multiple risk factors associated with the development of cancer. Some of these risks include genetics, smoking, and most recently, obesity (Lewandowska et al., 2019) (De Pergola & Silvestris, 2013). Research has shown that obesity is linked to the promotion of fourteen different cancers, including aggressive triple negative breast cancer (TNBC). Patients that are obese are more likely to develop cancer (Park et al., 2014). In addition, if the patient is obese at the time of a cancer diagnosis, they tend to have a larger tumor size and higher tumor grade (Neuhouser et al., 2015; Thompson et al., 2021; Yang et al., 2011). According to the world health organization, 41.9% of the world's population is obese. While the link between obesity and its impact on the clinical outcome of cancer has been made, the mechanism by which obesity promotes cancer remains understudied, especially in TNBC. TNBC is the deadliest form of breast cancer due to the limited amount of treatment options and aggressive phenotype. The role of obesity in TNBC remains understudied compared to estrogen receptor positive breast cancer. This work provides insight into the role of obesity on the pathogenesis of TNBC. In this study deidentified peritumor breast adipose tissue was obtained from breast cancer patients being treated by the Edwards Comprehensive Cancer Center. Peritumor breast adipose tissue was cultured in cell culture media for 24 hours, and media containing adipose tissue secretions was collected. Peritumor breast adipose tissue obtained from patients with BMIs > 30 were deemed high BMI adipose-derived secretome (ADS), conversely, BMI <

30 was deemed low BMI ADS. TNBC MDA-MB-231 and MDA-MB-436 cells were treated with high BMI ADS or low BMI ADS in serum free media. Cell migration and invasion assays were performed on treated TNBC cells. Images were taken at time 0, and 24 hours to assay changes in cell migration with Image J software. Treated TNBC cells were then processed for western blot analysis to determine changes in the levels and activity of signaling pathways.

This work is the first to characterize the effects of BMI on human peritumor adipose tissue on TNBC cells, demonstrating a novel new system for studying the effect of obesity on peritumor ADS regulation of TNBC cell signaling and invasiveness. In addition, we investigate the effect of BMI on the regulation of signaling and invasiveness of TNBC cells by peritumor breast adipose tissue secreted factors. We hypothesize that high BMI ADS has a stronger cancer promoting effect on TNBC cells than low BMI ADS and this could in part underly the mechanism by which obesity promotes TNBC progression.

Chapter 1

Literature Review

Abstract

Cancer is the second most common cause of death worldwide, responsible for over 600,000 deaths each year (Siegel et al., 2021). Breast cancer is the most common cancer in women followed by lung and then colorectal cancer (CRC) (Siegel et al., 2021). Triple-negative breast cancer is the deadliest form of breast cancer due to the limited treatments available and the increased likelihood of metastasis. In addition, colon cancer is one of the deadliest cancers, being the second most common cause of death (Frezza et al., 2006). Obese women have a 20% greater chance of developing (TNBC) compared to lean women. Epidemiologic studies on CRC reveal a 30-70% increase risk of developing colon cancer in obese patients (Bardou et al., 2013). While obesity and CRC have been linked, there is little literature published on this connection as well as a knowledge gap in the potential mechanism. The literature establishing link between obesity and breast cancer, including TNBC, is much more extensive. These results indicate a causality between obesity and cancer development. The obesity-cancer link is mediated by unique changes in the adipose tissue-derived secretome (ADS) in obesity. Potential signaling factors whose levels are differentially expressed in obese ADS compared with lean ADS that promote cancer include leptin, inflammatory cytokines, cellular matrix proteins, extracellular microvesicles (MVs) and exosomes (EXs). The signaling and cellular mechanisms induced in cancer cells by obese ADS have not been fully elucidated, however prior reports have shown a role for mTOR

hyperactivity. In this review, we will discuss the literature and recent clinical studies linking obesity to increased risk for colon and breast cancer and potential mechanisms by which obesity-associated changes in metabolism promote mTOR signaling in cancer cells.

Section 1:1 Clinical Studies

Obesity and Cancer link

One in 20 people will be diagnosed with colorectal cancer (CRC), with 90% of those cases being in those over 50 years of age. Even with the advancements in detection, CRC treatment has not advanced. CRC is one of the most difficult cancers to treat leaving it with one of the highest morbidity rates (Van Cutsem et al., 2009). CRC is the second leading cause of cancer related deaths, making it one of the deadliest cancers in the United States. In colon cancer, 75-80% cases develop sporadically, meaning they do not have a family history of CRC (Wang & Zhang, 2014). Primary risk factors for breast and colon cancer include, older age, smoking, alcohol intake, hormone replacement therapy, and obesity (Haggar & Boushey, 2009; Lukasiewicz et al., 2021).

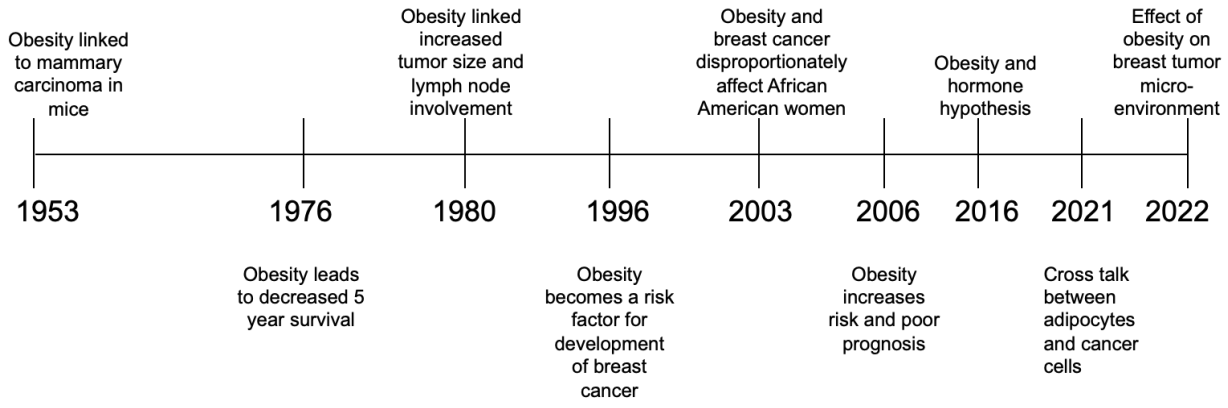
More than one-third (~40%) of the United States is obese (BMI > 30 kg/m^2), reaching epidemic proportions (Flegal et al., 2016; Hales et al., 2017; Pi-Sunyer, 2002). Obesity occurs when the energy consumed is greater than the energy expended, otherwise known as a nutrient overload. This is becoming more common in wealthy countries and urban areas of developing countries (Calle & Thun, 2004). With these numbers increasing each year, research into the effects of obesity in cancer is paramount. Current studies show obesity is linked to 14 different cancers (Xu & Mishra,

2018) (see Table 1). In addition, a recent study has shown that the incidence rates of these cancers in patients under 50 is currently on the rise (Ugai et al., 2022). In this review we will focus on breast and colon cancer because they have high incidence rates compared with other cancers and both have been linked to obesity. A case study in Israel showed obesity was associated with a 53% higher risk of colon cancer in men and a 54% higher risk in women (Levi et al., 2017). In addition, each 5kg/m^2 increase in BMI was linked to a 5% increase in risk for CRC (Clinton et al., 2020). A recent study (N=85,256 women) showed a positive association between early onset of CRC (media age of 45) with obesity when analyzed by multivariable relative risk (RR) (P. H. Liu et al., 2019). A meta-analysis of publications regarding obesity and breast cancer outcomes showed that women who were obese had ~30% increase of CRC recurrence and CRC mortality (Protani et al., 2010). Klintman showed obesity was statistically significantly linked with increased risk for developing node positive ER positive breast cancer (N = 35,412 postmenopausal women) (Klintman et al., 2022). In Appalachia, nearly 50% of women with TNBC are obese (Vona-Davis et al., 2008). Obese women have a 20% higher risk of developing TNBC compared with lean women (Sun et al., 2017). Larger breast tumors (P = 0.02), higher breast cancer T stage (P=0.001), and higher breast tumor grade (P = 0.01) is also statistically significantly associated being overweight and obesity in women (N = 183) positively associated with being overweight. Mowad et al (2013) showed obesity was significantly associated larger tumors (P = 0.02), a higher T stage (with explored the effects of obesity on 183 women with TNBC (Mowad et al., 2013). They found that patients who were overweight or obese had

larger tumors (P = 0.02), a higher T stage (P=0.001), and they also had a higher tumor grade (P = 0.01) (Mowad et al., 2013).

Figure 1

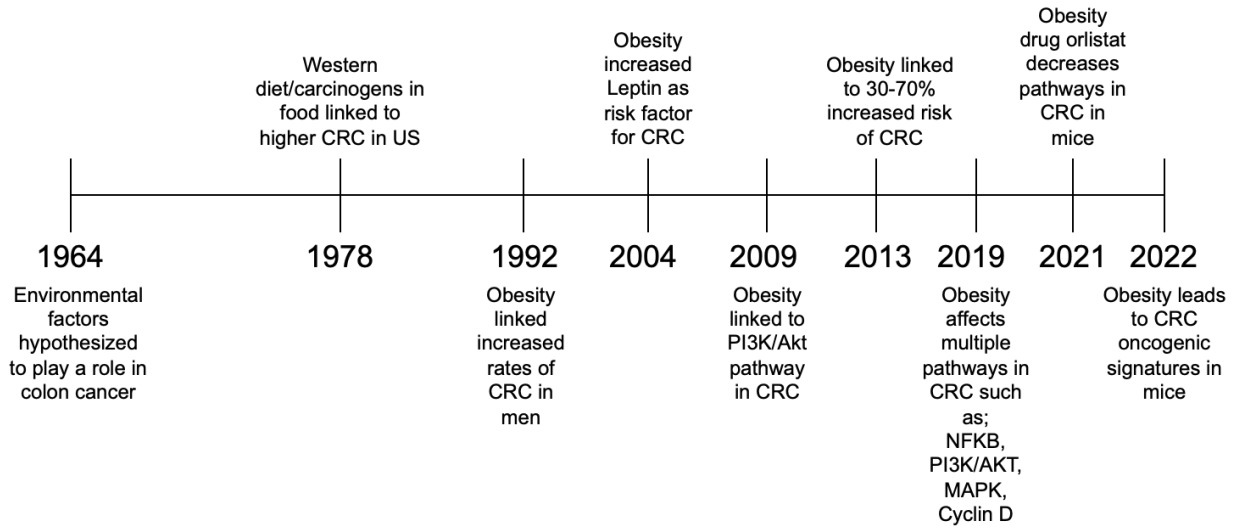
Timeline of Obesity and Breast Cancer Research



Note. Timeline indicating the first link of obesity to breast cancer (1953) and the advancements made to present day.

Figure 2

Timeline of Obesity and Colon Cancer Research



Note. Timeline indicating the first link of obesity to colon cancer (1964) and the advancements made to present day.

Table 1

Link Between Obesity and Cancer

Cancer Type	Reference:

Breast- (Calle & Thun, 2004),(Calle, 2007), (Avgerinos et al., 2019), (De Pergola & Silvestris, 2013), (Jiralerspong & Goodwin, 2016), (Wolin et al., 2010)

Endometrium - (Calle & Thun, 2004), (Avgerinos et al., 2019), (De Pergola & Silvestris, 2013), (Wolin et al., 2010)

Renal- (Calle & Thun, 2004), (Avgerinos et al., 2019), (De Pergola & Silvestris, 2013), (Wolin et al., 2010)

Esophageal- (Calle & Thun, 2004), (Avgerinos et al., 2019), (De Pergola & Silvestris, 2013), (Wolin et al., 2010)

Hepatocellular - (Avgerinos et al., 2019), (Wolin et al., 2010), (Marengo et al., 2016)

Pancreatic Adenoma- (Avgerinos et al., 2019), (Pothuraju et al., 2018)

Gastric Cardia - (Avgerinos et al., 2019) , (Li et al., 2012)

Meningioma- (Avgerinos et al., 2019), (Wolin et al., 2010)

Multiple Myeloma- (Avgerinos et al., 2019), (De Pergola & Silvestris, 2013), (Wolin et al., 2010)

Ovarian- (Avgerinos et al., 2019), (Valladares et al., 2014)

Gallbladder- (Avgerinos et al., 2019), (Wolin et al., 2010)

Thyroid- (Avgerinos et al., 2019), (De Pergola & Silvestris, 2013), (Wolin et al., 2010), (Matrone et al., 2020)

Prostate- (De Pergola & Silvestris, 2013), (Wolin et al., 2010)

Melanoma - (De Pergola & Silvestris, 2013), (Clement et al., 2017)

Leukemia- (De Pergola & Silvestris, 2013), (Wolin et al., 2010)

Lymphoma - (De Pergola & Silvestris, 2013), (Wolin et al., 2010), (Matos et al., 2016)

Colorectal - (Calle & Thun, 2004), (De Pergola & Silvestris, 2013), (Wolin et al., 2010), (Bardou et al., 2013)

Note. Papers citing clinical and mechanistic links between obesity and cancer.

Section 1:2 Obesity-Associated Endocrine and Paracrine Signaling that Promotes Breast and Colon Cancer

Obesity-mediated cancer risk is hypothesized to be mediated by endocrine changes and altered paracrine interactions between adipose tissue (AT) and cancer cells. Regarding endocrine changes, the levels of leptin, estrogen, thyroid stimulating hormone, and T3 are increased in obesity (Pearce, 2012; Sidhu et al., 2000) Conversely, adiponectin and testosterone are lower in obesity (Kelly & Jones, 2015; Xu & Mishra, 2018). A study by Otake et al measured adiponectin in 124 men with colorectal adenoma, early CRC, advanced CRC, and predisposed risk. They found that low levels of adiponectin had a significant increase in the odds ratio in patients with an increased risk of colorectal adenoma, linking low levels of adiponectin to an increased risk of CRC (Otake et al., 2010). The effect of obesity on other hormones such as testosterone have been linked to prostate cancer. In a study by Garcia-Cruz et al, obesity induced low levels of testosterone in prostate cancer patients. These low levels were linked to poor prognosis (Garcia-Cruz et al., 2012). These studies demonstrate the effect obesity has on the endocrine system and how it relates to cancer.

Leptin; a Link Between Obesity and Cancer

Leptin is a 16 kDA protein encoded by the LEP gene (Munzberg & Morrison, 2015). In adults, leptin is released by adipose cells and gastric luminal cells (Cammisotto & Bendayan, 2007). Leptin is primarily secreted by white adipose tissue where it circulates in the blood freely (active form) or bound to protein (Obradovic et al., 2021). The primary function of leptin is to diminish fat storage by signaling through the Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway, an important regulator for energy homeostasis (Kelesidis et al., 2010). Leptin

induces signaling in the hypothalamus (ventral tegmental area) to reduce hunger and regulate energy balance (Zhou & Rui, 2013). The role of leptin in suppressing hunger has been confirmed in leptin deficient patients (Kleinendorst et al., 2020). Patients that express lower levels of LEPR (leptin receptor) experience hyperphagia and obesity (Kleinendorst et al., 2020). Leptin also regulates proinflammatory immune responses, angiogenesis, and lipolysis (Obradovic et al., 2021). Leptin upon binding to its cell surface leptin receptor signals through the JAK/STAT3 pathway, which primarily regulates gene expression, and the phosphatidylinositol 3-kinase-related kinase (PI3K) pathway which stimulates the phosphorylation of downstream targets that regulate proliferation and cell growth. PI3K-mediated induction of mechanistic target of rapamycin (mTOR) induces mTOR associated signaling that in turn regulates cell size, proliferation and cell survival (Park & Ahima, 2014). Leptin by stimulating signaling promotes the secretion of inflammatory cytokines such as tumor necrosis factor (TNF) alpha and interleukin 6 (IL-6), promote vascular endothelial growth factor (VEGF), hypoxia inducible factor 1a (HIF-1a), and increase expression of antiapoptotic proteins such as x-linked inhibitor of apoptosis protein (XIAP). The positive association between increased levels of leptin in obesity with increased levels of tumor necrosis factor (TNF) alpha, and interleukin-6 (IL-6) have been confirmed in studies from different laboratories (Dutta et al., 2012).

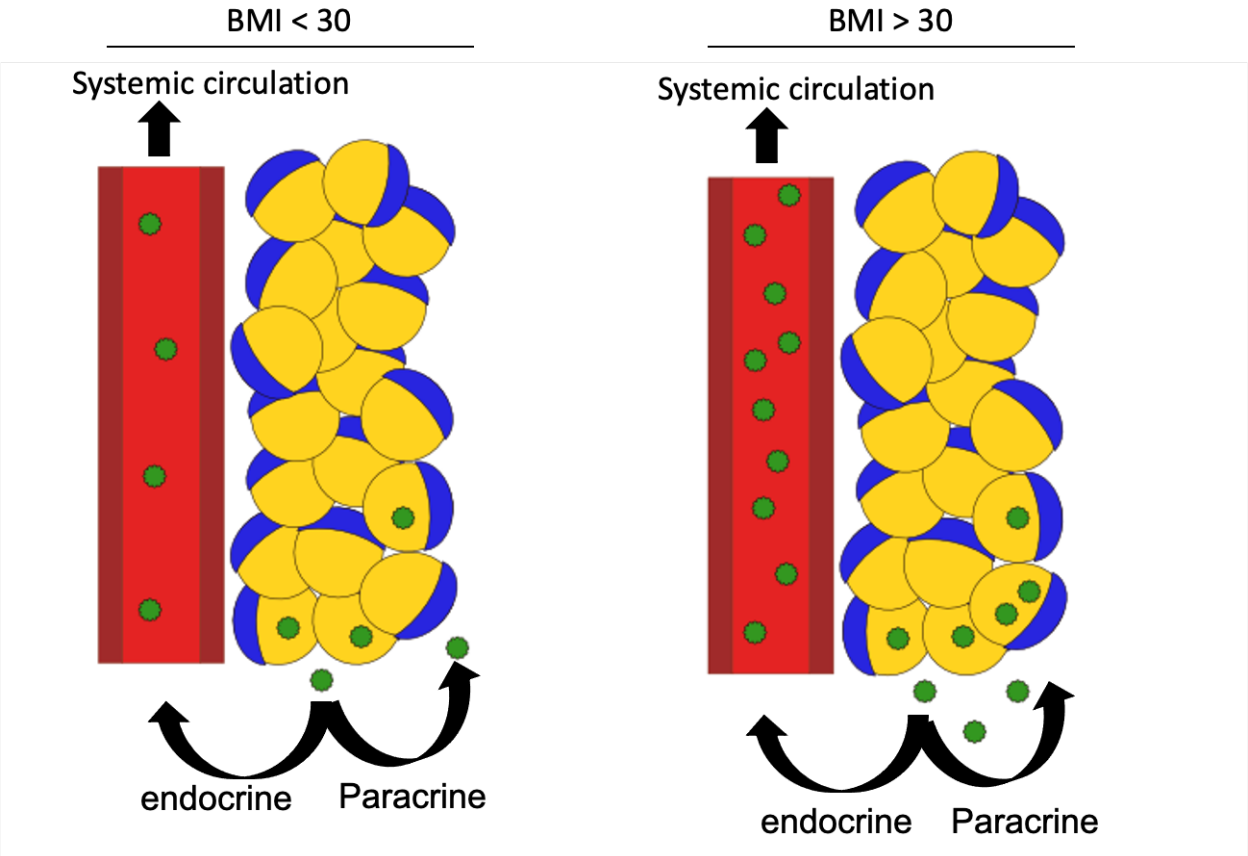
A study by Stattin et al used logistic regression analysis to link high levels of leptin with increased colon cancer risk (odds ratio 2.72) (Stattin et al., 2004). A more recent study solidified these results by demonstrating that two leptin (LEP) single nucleotide polymorphisms (SNP) significantly correlated with CRC in women (Chun et

al., 2018). Endo et al hypothesized that leptin stimulates the growth of colon cancer by inducing growth factor signaling pathways in colon cancer cells (published in 2010) (Endo et al., 2011). They showed increased colon cancer cell proliferation in obese mice compared with obese mice lacking leptin. This result linked leptin to the proliferation of colorectal cancer cells in obesity via stimulation of WNT and increased activation of STAT3 (Endo et al., 2011). Leptin could also promote cancer in obesity by contributing to chronic low-level inflammation in AT in obesity. In Landman et al, Leptin was shown to be released in response to endotoxin treatment (used to induce inflammation) in rhesus monkeys (Landman et al., 2003). The higher levels of inflammation in AT in obesity is due to adipocyte hypertrophy. The hypertrophied adipocytes secrete higher levels of chemoattractants, leading to an increase in macrophage infiltration. In addition, rapid AT expansion does not allow for proper angiogenesis, leading to hypoxia, apoptosis, and cell stress in AT (Zatterale et al., 2019). These cellular changes stimulate the release of chemoattractant molecules such as leptin and eotaxin from AT that recruit additional inflammatory cells to AT in obesity (Francisco et al., 2018). Leptin also induces signaling that promotes the differentiation of naïve T cells into proinflammatory Th1 cells in humans and mice (Perez-Perez et al., 2020; Procaccini et al., 2010). High levels of leptin have also been correlated with lower activity of T regulatory cells (Zeng & Chi, 2013). Specifically, leptin inhibits the expansion of T regulatory cells (De Rosa et al., 2007). Collectively, leptin promotes cancer progression through two mechanisms. In the direct pathway, leptin (100 ng/mL) acts directly on breast tissue (Juarez-Cruz et al., 2019). In the indirect pathway, leptin promotes low level inflammation in obesity by regulating T regulatory cells, stimulating

inflammatory cells, and promoting the synthesis and release of proinflammatory cytokines. Leptin is also able to stimulate angiogenesis, which in turn can promote growth of cancer (Bouloumie et al., 1998). In order to perform these functions, leptin acts in a paracrine and endocrine fashion (Vona-Davis & Rose, 2007) (figure 3).

Figure 3

Leptin Release from Adipose Tissue in Low BMI and High BMI Conditions



Note. Low BMI = BMI <30. High BMI = BMI >30 Leptin is a hormone released from adipose tissue and has the capability to act on nearby adipose cells (yellow) in a paracrine fashion. Leptin is also able enter circulation to elicit effects in distance tissues, acting in an endocrine fashion. In high BMI patients, leptin release is increased.

Based on these detrimental effects of leptin, pharmacological inhibitors that bind and antagonize the LEPR have been created. Otvos et al explored the effect of LEPR inhibitors on the progression of breast cancer in mouse xenograft models. Intraperitoneal treatment of mice with LEPR inhibitors reduced breast tumor size (Otvos et al., 2011). While these inhibitors showed promising results, there are a multitude of different LEPR expressed with different interaction sites, making pharmacological targeting difficult (Otvos et al., 2011). Otovos et al tested the effect of pure LEPR antagonists (Allo-Aca, Aca1, and D-ser) and the results showed that these LEPR blockers did not have side effects. These compounds showed great success in cancer reduction and treatment in cachexia. However, resistance was developed. With this great preclinical success, this begs to question why these inhibitors are not used more in clinic. While many studies have shown that leptin plays a role in increased invasion and migration of cancer cells, the concentrations frequently used in experiments are significantly higher (100-400ng/mL)(Bowers et al., 2018) than those within obese patient serum (30-70ng/mL)(Kazmi et al., 2013). Leptin clearly has a role in obesity and caner, but the significance of its role is under question. Obese AT secretes a multitude of other factors that have also been shown to play a significant role in the advancement of cancer, suggesting that leptin alone is not solely responsible.

Obesity-Stimulated Adipocyte Secretome

In obesity, adipose tissue (AT) is dysfunctional with increased levels expression and secretion of proinflammatory cytokines (such as IL-6, TNF-α, IL-8, monocyte chemoattractant protein-1 (MCP-1), leptin, extracellular proteins, free fatty acids, and extracellular vesicles (EVs) (Table 2) (Camino et al., 2020). The levels of circulating

tumor necrosis factor alpha (TNF alpha) and the proinflammatory cytokine interleukin 6 (IL-6) are also higher in obese compared with lean subjects (Ellulu et al., 2017). In obese patients IL-6 serum levels are 7.69 +/- 5.06 pg/mL, and serum levels in normal BMI patients is 1.28 +/- 0.85 pg/mL (Roytblat et al., 2000). A recent study explored the association between adipocyte size and the complexity of the adipocyte secretome (Skurk et al., 2007). Human adipocytes were divided into 4 fractions. The results showed that the fraction which held the larger adipocytes secreted more factors such as leptin, IL-6, IL-8, monocyte chemoattractant protein 1, and granulocyte colony-stimulating factor (Skurk et al., 2007). These findings provided further evidence that obesity influences the adipocyte secretome and that such changes are associated with adipocyte hypertrophy.

The release of EVs by AT in the tumor microenvironment could be a new mechanism linking obesity with cancer progression. EVs are lipid-bilayers that contain intracellular lipids, proteins, and nucleic acids (Doyle & Wang, 2019). EVs are formed via two pathways, direct budding from the cellular membrane or via the endosomal pathway (Clement et al., 2017). EVs have several subtypes that are differentiated by their formation, release pathways, but most of all, their size (Doyle & Wang, 2019). EV subtypes are exosomes, microvesicles, ectosomes, oncosomes, and apoptotic bodies (Doyle & Wang, 2019; Yokoi & Ochiya, 2021). Upon being released from cells, EVs induce signaling in other cells that can change cellular function (thus a new paracrine mechanism) (Kim & Kim, 2021; Robado de Lope et al., 2018). The number and composition of EVs is influenced by changes in cellular stress (such as hypoxia) in the EV releasing cell (King et al., 2012). Breast cancer cells in hypoxic conditions have

been shown to release higher numbers of EVs. These vesicles also contained higher levels of miR-210 (King et al., 2012). Thus, it is possible that obesity-stimulated adipocyte dysfunction influences the profile of EVs being released from AT. This hypothesis is supported by a report showing that obesity uniquely regulated the release of exosome-like vesicles from AT. As stated previously, exosomes are a subtype of extracellular vesicles (Yokoi & Ochiya, 2021). These exosome-like vesicles were taken up by macrophages that then induced increased secretion TNF-alpha and IL-6 (Deng et al., 2009). Prior reports have also linked adipocyte EVs with the progression of melanoma. The first report showed that adipocyte derived EVs induce signaling in human melanoma cells that stimulated cancer cell migration and invasiveness (Lazar et al., 2016). Human isolated exosomes added to melanoma cell lines had an increased migration and invasion as compared to controls. This migration was caused by exosome induce signaling in melanoma that stimulated fatty acid oxidation, which in turn promoted the increased migration (Lazar et al., 2016). This finding demonstrates the effect obesity has on AT secretions. These alterations occur throughout the body, showing that this may be a mechanism behind the link between obesity and other cancers. In addition, AT derived EVs have also been reported to stimulate angiogenesis, and immune tolerance in melanoma (Clement et al., 2017; Ekstrom et al., 2014). Interestingly, melanoma promoting AT-derived EVs were released from AT in the subdermal layer of the skin (Ekstrom et al., 2014). In addition to melanoma, AT-derived EVs have been linked to chemoresistance in CRC. In the plasma of obese CRC patients, serum exosomes express higher levels of microsomal triglyceride transfer protein (MTTP) (Zhang et al., 2022). This protein is a known inhibitor of ferroptosis. To

show this effect on CRC, the authors co-cultured adipocyte exosomes and CRC cancer cell lines. Cancer cell lines co-cultured with CRC cell lines showed decreased sensitivity via GSH to ferroptosis inducer erastin. This suggests adipocyte derived exosomes interfere with ferroptosis via MTTP over-expression (Zhang et al., 2022).

Obesity also influences AT by stimulating increases in adipocyte number (hyperplasia) and adipocyte hypertrophy (enlargement of adipocyte size) (Parlee et al., 2014). Hypertrophic adipocytes are lipid-laden, dysfunctional and undergo cell death that contributes to low grade inflammation and fibrosis in AT in obesity (Fuster et al., 2016). Adipose tissue fibrosis is due to a multitude of factors. The first being an increase in the release of collagen IV into the extracellular matrix (ECM) (Ruiz-Ojeda et al., 2019). The increase of collagen IV makes the ECM more rigid (Khan et al., 2009). Fibrosis is also shown to be due in part to hypoxia. The hypoxic condition of obese AT upregulates hypoxia-inducible factor 1 alpha (HIF1a) which in turn promotes fibrosis (Halberg et al., 2009). The higher levels AT inflammation in obesity is accompanied by higher numbers of inflammatory cells in AT in obesity (Zatterale et al., 2019). It is becoming more known that adipocytes are not the only cells within AT. Single cell RNA sequencing on mouse and human subcutaneous fat demonstrated the heterogeneity of adipose tissue such as mesenchymal stem cells, mural stem cells, adipocytes, vascular cells, lymphatic endothelial cells, macrophages, etc. (Deutsch et al., 2020; Emont et al., 2022). Macrophages are the most abundant inflammatory cell in AT in obesity—however—reports show the numbers of CD8+ T cells in AT are also increased in obesity (Zatterale et al., 2019). These cells can undergo fundamental changes due to inflammation, leading to alterations in their secretions. These obesity-driven changes in

AT lead to an obesity-specific AT derived secretome (ADS) (Clement et al., 2017; Lazar et al., 2016; Thompson et al., 2021).

Exosomes are extracellular vesicles that are composed of cytoplasm enclosed by a lipid bilayer (Schorey et al., 2015). They are 30-100nm in size and primarily responsible to cell to cell communication (Schorey et al., 2015). More recently, exosomes have been shown to not just affect nearby cells, but to be able to regulate distant microenvironments (Zhang & Yu, 2019). The role exosomes play in cancer is becoming more evident. Exosomes are frequently released by all cells in the body, however, cancer cells release more exosomes as compared to normal healthy cells (Zhang & Yu, 2019). Exosomes contain many different RNAs, miRNAs, proteins, and DNA, that can regulate the tumor microenvironment (Zhang & Yu, 2019). Further, exosomes released from cancer cells can transfer cancer promoting genes and proteins to non-cancer cells. This mechanism was demonstrated in CRC cells. Demory-Beckler et al isolated exosomes from CRC cell lines DKs-8, DLD-1, and DKO-1. These exosomes were found to carry mutant KRAS oncogene, known to promote CRC (Demory Beckler et al., 2013). Exosomes with KRAS mutations were stained with 1,1' dioctadecyl-3,3,3',3'- tetramethylindodicarbocyanine, 4-cholorbenzensulfonate salt (DiD). Exosomes with the KRAS mutation were quickly engulfed into the non-cancer cells, which could in turn induce oncogenic signaling in normal cells (Demory Beckler et al., 2013). Exosomes not only influence other cancer cells, but are able to also influence the tumor microenvironment (Zhang & Yu, 2019).

A study by Gesierich et al demonstrated the effect of exosomes on angiogenesis. D6.1A is a key protein that is stimulates the formation of new blood vessels in endothelial cells. D6.1A is a rat homolog tetraspanin, commonly secreted by tumor cells, however, Gesierich et al demonstrated that D6.1A was found to be secreted within exosomes as well, influencing the endothelial cells within the tumor microenvironment in pancreatic tumor cell line over expressing D6.1A (Gesierich et al., 2006). The NOTCH ligand delta-like 4 (Dll4) also promotes angiogenesis via overexpression by tumor cells (Sheldon et al., 2010). Sheldon et al showed that Dll4 can be incorporated into tumor released exosomes and influence endothelial cells within the tumor microenvironment to promote angiogenesis (Sheldon et al., 2010). HUVEC cells treated with exosomes containing Dll4 demonstrated higher levels vessel formation via imaging and CD31 analysis (Sheldon et al., 2010). In addition, exosomes have also been shown play a role in drug resistance by expelling cytotoxic drugs (Safaei et al., 2005). Ostrowski et al demonstrate the mechanism by which exosomes are secreted. Short hairpin-mediated RNA knock down of five Rab proteins (Rab2b, Rab9a, Rab5a, Rab27a, and Rab27n) prevented exosome release from Hela cells (Ostrowski et al., 2010). Blocking the release of exosomes can potentially serve as a therapeutic potential for cancer patients.

Exosomes clearly play a large role in the progression of cancer. This begs to question what is the role obesity plays in the formation and contents of exosomes? A high fat diet results in alterations lipid composition of exosomes (Kumar et al., 2022). Dysfunctions in adipose tissue caused by obesity also leads to dysregulated assembly and packaging of AT derived exosomes (Kwan et al., 2021). Obesity does not only affect the formation of exosomes but also affects its contents. A recent mouse study

showed that obesity alters the miRNA profile in plasma exosomes. They found exosomes have increased levels of miR-122, miR-192, miR-27a-3p, and miR-27b-3p (Castano et al., 2018). These alteration within the exosomes lead to insulin resistance in lean mice treated with obese derived exosomes (Castano et al., 2018). In addition, obese adipocytes have higher levels of miR-802-5p. Higher levels of this micro-RNA are linked to increased oxidative stress (Kwan et al., 2021). The hypoxic conditions found in obesity also play a role in the cargo of exosomes (Kwan et al., 2021; Sano et al., 2014). Sano et al demonstrated this mechanism by performing proteomic analysis on exosomes derived from adipocytes cultured in hypoxic conditions. In exosomes released from hypoxic AT, proteins such as acetyl-CoA carboxylase, glucose-6-phosphate dehydrogenase, and fatty acid synthase were increased (Sano et al., 2014). The role of obese AT derived exosomes in obesity in cancer progression needs to be studied.

The specific factors in ADS that induce signaling in cancer cells include leptin, adiponectin, pro-inflammatory cytokines (such IL-6), extracellular factor collagen IV and extracellular vesicles (EVs) (Camino et al., 2020). Overall, these studies show that obesity leads to increased exosome secretion, increased expression of proteins such as MTTP, and promote invasion and migration in cancer. However, a more recent study implicates a core pathway responsible for the increased invasion and migration, the mTOR pathway (Thompson et al., 2021)

Table 2

Components of Adipose Derived Secretome

Adipose Derived Secretome	**Reference:**
MCP1	(Nosalski & Guzik, 2017)
Activin A	(Oikonomou & Antoniades, 2019)
Resistin	(Wolf, 2004)
RANTES	(Madani et al., 2009)
Serpin E1	(Vachher et al., 2020)
IGF-1	(Lengyel et al., 2018)
Lysophosphatidic Acid	(Pages et al., 2001)
VEGF	(Schlich et al., 2013)
IL-1B	(Pardo et al., 2012)
Extracellular Vesicles	(Deng et al., 2009), (Liu et al., 2012), (Kim & Kim, 2021)

- IL-6
- TNFa
- siRNA
- microRNA
- DNA
- Lipids
- Sphingolipids
- Phosphatidylserine

Note. Proposed contents of ADS.

Section 1:3 Obesity-Associated Nutrient Changes that Stimulate mTOR Hyperactivity

1. mTOR Complex

Mechanistic target of rapamycin (mTOR) is a kinase that is a member of the phosphatidylinositol 3-kinase-related kinase family (PI3K). MTOR is a part of two separate protein complexes that are activated in two different locations in the cell. The outer lysosomal membrane is the site of mTOR complex 1 (mTORC1) activation (Betz & Hall, 2013). MTORC1 is recruited to the lysosome by its interaction with RAGA and RAGB. Once recruited to the lysosome mTORC1 kinase activity is induced by RHEB (Carosi et al., 2022). Conversely, mTOR complex 2 (mTORC2) activity localizes to the cytoplasm facing side of the plasma membrane (Zoncu et al., 2011). Active mTORC2 will also bind with ribosome (Zinzalla et al., 2011). The two mTOR complexes are composed of different regulatory and signaling proteins and thus the regulation and function of mTORC1 is different than mTORC2 (figure 4). MTORC1 is composed of mTOR, raptor, GbetaL, and deptor (Yip et al., 2010). Raptor functions as a key scaffolding protein for mTOR. As a scaffolding protein, it assists in recruitment and phosphorylation of ribosomal protein S6 kinases (S6K) and eukaryotic translation initiation factor 4E binding protein (e4EBP) (Nojima et al., 2003; Schalm & Blenis, 2002). G protein beta subunit-like (GbetaL) is a scaffolding protein, that is needed for mTOR kinase activity (Kim et al., 2003). DEP domain containing mTOR interacting protein (deptor) is a regulatory protein that inhibits the kinase activity of mTOR (Morales-Martinez et al., 2021). MTORC2 is composed mTOR, rictor, GbetaL, proline rich protein 5 (PRR5) (known to be elevated in breast and colorectal cancers), deptor,

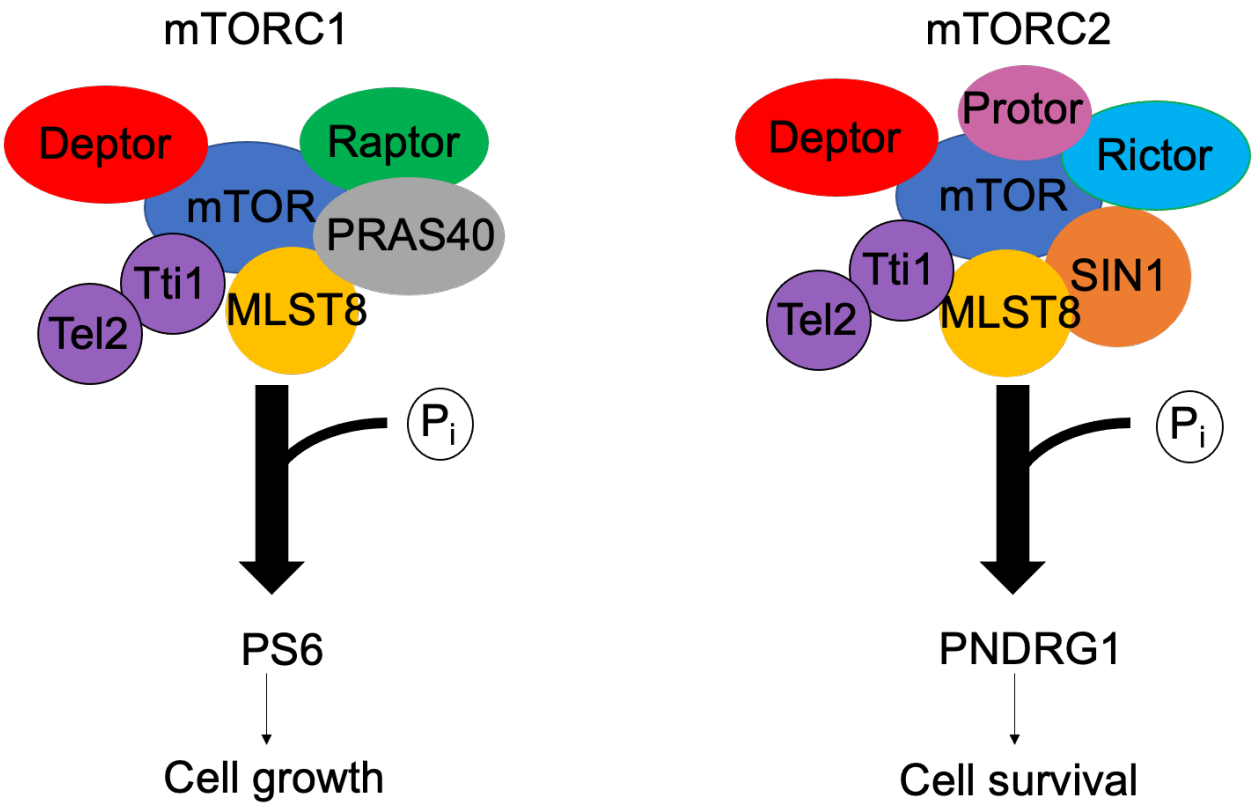
and stress activated protein kinase interacting protein 1 (SIN1) (Oh & Jacinto, 2011). In mTORC2 rictor and SIN1 plays a large role the phosphorylation of the protein kinase C (PKC) family (Cameron et al., 2011; Sarbassov et al., 2004). PKC alpha acts to regulate cellular proliferation, apoptosis, differentiation, motility, and inflammation (Nakashima, 2002). MTORC2 and mTORC1 play a dynamic and involved role in each other's regulation. For instance, mTORC2 phosphorylates AKT at serine 473 (Oh & Jacinto, 2011). Upon being phosphorylated by mTORC2, AKT will promote the activation of mTORC1 (Oh & Jacinto, 2011). AKT phosphorylates tuberous sclerosis complex 2 (TSC2) and PRAS40. TSC2 and PRAS40 are negative regulators of mTORC1. Once these proteins are phosphorylated, mTORC1 is free to act out its function as a kinase (Dan et al., 2014). Phosphorylated AKT will also phosphorylate the SIN1 component of mTORC2 activating complex 2 in a positive feedback loop (Yang et al., 2015). SIN1 acts to regulate mTOC2 in a few ways, the first being the regulation of mTORC2 formation being. The second way SIN1 regulates mTORC2 function is through the recruitment of mTORC2 substrates such as AKT. SIN1 also acts to directly receive PI3k signaling to then in turn activate mTORC2 through its pleckstrin homology domain (PH domain) (Yuan & Guan, 2015). While mTORC2 is regulated by growth factors, SIN1, and PI3K signaling, mTORC1 is primarily regulated by nutrients such as branch chained amino acids (such as leucine), glucose, and growth factors, (Oh & Jacinto, 2011; Yuan & Guan, 2015).

Under normal conditions mTORC1 regulates cell growth, protein translation, lipid synthesis, organelle biosynthesis, and autophagy (Sabatini, 2017). On the other hand, mTORC2 regulates cell shape (by regulating actin filaments), cell survival, and cell

metabolism (Oh & Jacinto, 2011). In simple terms, mTORC1, by responding to nutrients and growth factors, coordinates nutrient availability with changes in cell growth (Sabatini, 2017). The pathway by which the essential amino acid leucine stimulates mTOR has been characterized and involves two leucine sensing proteins. In the Leucyl-tRNA synthetase (LRS) pathway, leucine upon binding to LRS stimulates the hydrolysis of RagD-GTP to RagD-GDP, which in turn mediates the recruitment of mTOR to the lysosome—the site of mTORC1 activation (Zoncu et al., 2011). In the Sestrin 2 pathway, the binding of leucine to Sestrin 2 disrupts Sestrin 2 binding to GATOR2, which in allows GATOR2 to stimulate mTORC1 activity (Wolfson et al., 2016). It is postulated that higher levels of circulating leucine in obesity could mediate increases in mTORC1 hyperactivity in cancer cells and in other tissues such as adipose tissue, muscle, liver, and pancreas (Khamzina et al., 2005; Laplante & Sabatini, 2012; Saxton & Sabatini, 2017b; Shigeyama et al., 2008; Tremblay et al., 2007; Um et al., 2004; Yuan et al., 2017). While leucine is higher in obesity, it is not the only nutrient that is increased in obesity.

Figure 4

Composition of mTOR Complex 1 and Complex 2



Note. MTOR is formed by a series of proteins complexing together. MTORC1 structure is shown as well as the cellular read out of mTORC1 activity, phosphorylated ribosomal S6 (PS). We used phospho-S6 (S235/236) as a readout of mTORC1 activity in chapter 2. Upon activation, MTORC1 promotes cellular growth. MTORc2 read out is PNDRG1. Once stimulated, mTORc2 promotes cell survival.

Obese individuals also have higher levels of glucose. The increase in glucose could be another link between obesity and mTORC1 activity, given that glucose promotes the activation of mTORC1 (Sanguesa et al., 2019). The upregulation of mTORC1 activity in tissues could in part be mediate by obesity-associated complications like fatty liver disease, insulin resistance, type 2 diabetes, and some cancers such as postmenopausal breast and colon cancers (Calle & Kaaks, 2004; Calle & Thun, 2004; Collaborators et al., 2017; Laplante & Sabatini, 2012; Park et al., 2014; Saxton & Sabatini, 2017b; Um et al., 2004; Zoncu et al., 2011). Fatty liver disease occurs due to an imbalance in triglycerides in the liver. MTOR regulates genes that regulate lipid liver metabolism (Feng et al., 2022). Further solidifying its role in obesity is the role mTOR plays in insulin signaling. The increased consumption of nutrients in obesity leads to an over activation of mTORC1, which then causes an increase in the phosphorylation of insulin receptor substrate (IRS) leading to the development of insulin resistance (Yoon, 2017). In type two diabetes and cancer, increased nutrients result in hyper activation of mTOR. Dysregulation of this signaling pathway leads to alterations in metabolism of glucose, potentially fueling cancer (Mao & Zhang, 2018). In addition, mTORC2 role in cancer is becoming more evident (Kim et al., 2017; Masui et al., 2014). In recent studies, high expression of mTORC2 key component RICTOR, is linked to poor overall survival in several cancer types such as, bladder cancer, non-small-cell-lung cancer, and esophageal gastric cancer (D. Zhao et al., 2020).

2. Nutrient Regulation

a. Leucine Pathway

Prior reports show leucine is elevated in the plasma of humans who are obese (She et al., 2007). The exact mechanism behind why branched chain amino acids (BCAA), specifically leucine, are elevated is not fully known. One hypothesis is increased consumption of food. Another explanation is obese patients exhibit increased protein catabolism due to insulin resistance, leading to increases in serum BCAA (She et al., 2007). Obesity is also associated with a defect in the catabolism of leucine. Reduced leucine catabolism in obesity is connected to reduced expression of leucine catabolizing enzymes— branched chain amino acid aminotransferase (BCAT) (mitochondrial form) and branched chain alpha-keto acid dehydrogenase (BCKDC)(She et al., 2007). Obese rats exhibit significantly decreased levels of BCAT and BCKDC in AT and liver, and significantly higher levels of plasma BCAA. These results, however, did not hold true in muscle tissue (She et al., 2007). This study, while in rats, holds a potential mechanism for why leucine and other BCAA are increased within obesity.

Leucine is not only important for protein synthesis, but it can also regulate pathways that are relevant to obesity such as mTOR hyperactivity (Dodd & Tee, 2012). Leucine has also been linked to the release of leptin via the mTOR pathway (Lynch et al., 2006). Once stimulated by amino acids, mTOR regulates mRNA levels of leptin (Lynch et al., 2006). Rats fed a leucine deprived diet showed decreased levels of leptin (Lynch et al., 2006). This suggests that leucine stimulates leptin expression and release into the plasma. This novel link between obesity and reduced expression of leucine metabolizing enzymes was discovered in initially in obese Zucker rats (She et al., 2007). Similarly, a genomics study conducted in obese mice and humans showed that obesity is associated with decreased expression of BCAA catabolic pathway proteins (M. Zhou

et al., 2019). Our group and others have shown that increased leucine uptake by cells promotes the activity of mTORC1 (Dodd & Tee, 2012; Lynch et al., 2003; Thompson et al., 2021). We have recently provided mechanistic evidence that this aspect of leucine signaling is upregulated in cancer cells in response to obese ADS (Thompson et al., 2021). Estrogen receptor positive breast cancer cells were treated with lean (BMI <30) and obese (BMI > 30) ADS. Leucine uptake assays showed that obese ADS induced greater leucine uptake by breast cancer cells than lean ADS (Thompson et al., 2021). In addition, it was shown that the levels of LAT1 remained unchanged in response to treatment with lean or obese adipose derived secretome. LAT1 kinetic studies showed that obese ADS acted on breast cancer cells to increase the affinity of LAT1 for leucine, which in turn stimulated greater leucine uptake by breast cancer cells treated with obese compared with lean ADS (Thompson et al., 2021). Thus, obesity not only increases the levels of leucine in plasma, but it also stimulates cancer cells to absorb more leucine, resulting in mTOR hyperactivity. A recent paper published in nature gave insights into how leucine, and other branch chained amino acids such as arginine stimulate mTORC1. The findings suggest that leucine and arginine interact with different proteins that control the translocation of mTORC1 to the surface of the lysosome, the site of mTORC1 activation (Vellai, 2021). Leucine inhibits SAR1B and SESTRIN 2, while arginine inhibits CASTOR1, which confers RAG proteins that ability to recruit mTORC1 to the surface of the lysosome, which is the trigger for mTORC1 activation. In addition to leucine and arginine, there are 10 additional amino acids that have been linked to the activation of mTORC1 (Takahara et al., 2020). Of these 10 amino acids,

the majority of them are transported by the L-Type amino acid transporter 1 (LAT1), which links this amino acid transporter to mTORC1 activity (Zhao et al., 2015).

b. Leucine Uptake by Cells via L-Type Amino Acid Transporter 1 (LAT1)

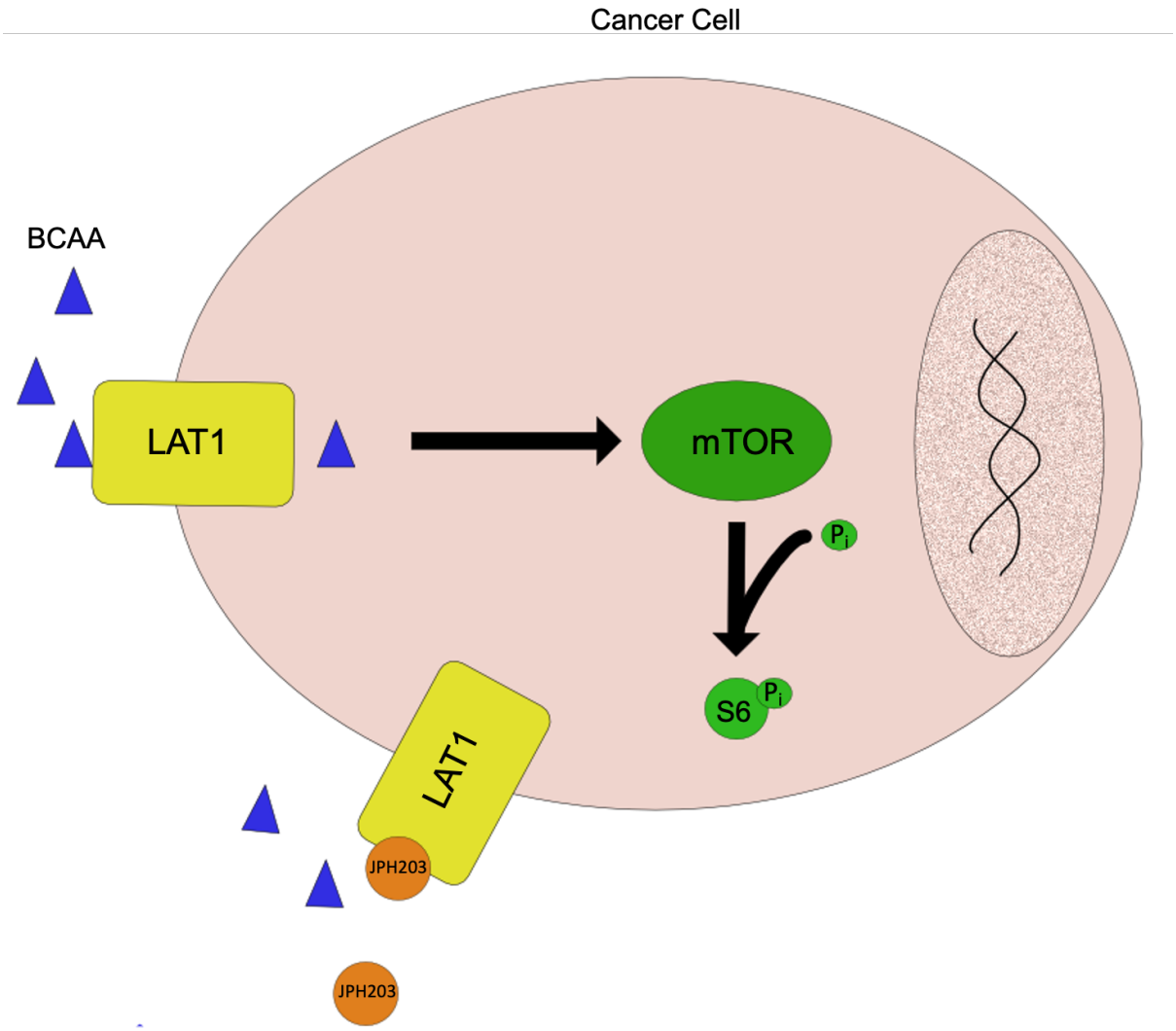
The high affinity leucine transporter, LAT1, is the primary leucine transporter expressed by cancer cells. LAT1 has been shown to have increased expression in breast cancer, non-small cell lung cancer, biliary tract cancer, pancreatic cancer, renal cancer, and prostate cancer (Higuchi et al., 2019). We and others have shown LAT1 is regulated by transcriptional and posttranscriptional mechanisms. The aryl hydrocarbon receptor (AHR), MYC, and the Notch pathways stimulate increases in LAT1 transcription in different cancer cell types (Salisbury & Arthur, 2018). An elegant study by Tomblin et al showed that TCDD, a known ligand of AHR, stimulated increases in LAT1 mRNA (Tomblin et al., 2016). The increase was suppressed by the AHR antagonist CH-223191 (Tomblin et al., 2016). Within the LAT1 gene is an AHR response element, explaining its regulation (Tomblin et al., 2016). In addition to this pathway, LAT1 expression is activated by MYC through binding to E box elements on the LAT1 gene (Yue et al., 2017). Regarding posttranslational regulation, LAT1 protein is stabilized upon its binding to its chaperon protein, CD98 (Salisbury & Arthur, 2018). The binding of LAT1 to CD98 is mediated by disulfide bonds between LAT1 and CD98 (Scalise et al., 2018). CD98 also mediates translocation of LAT1 to the cell membrane where exchanges with amino acids to promote mTOR activity (Salisbury & Arthur, 2018; Wang & Holst, 2015). LAT1 mediates leucine uptake by the intestinal epithelium, placenta, and the blood brain barrier (Singh & Ecker, 2018). In addition to leucine, LAT1 mediates the uptake of phenylalanine, tyrosine, L-DOPA, histidine, methionine, and

tryptophan (Salisbury & Arthur, 2018). Of these, methionine is known to stimulate mTOR (Takahara et al., 2020). LAT1 is overexpressed in many cancers (Hafliger & Charles, 2019). These cancers include; bladder cancer (Xie et al., 2013), bone cancer (Koshi et al., 2015), gliomas (Haining et al., 2012), liver cancer (Ohkame et al., 2001), pancreatic cancer (Kaira et al., 2012), esophageal cancer (Kobayashi et al., 2005), gastric carcinoma (Ichinoe et al., 2015), head and neck cancer (Toyoda et al., 2014), kidney cancer (Betsunoh et al., 2013), melanoma (Shimizu et al., 2015), prostate cancer (Segawa et al., 2013), thyroid cancer (Hafliger et al., 2018), breast cancer (Furuya et al., 2012), and colorectal cancer (Hayase et al., 2017). LAT1 overexpression in cancer is postulated to contribute to the progression and survival of cancer cells by mediating the uptake essential amino acids that in turn support cancer cell metabolism (Sato et al., 2021), protein synthesis (Kanai, 2022) and mTOR signaling (Rosilio et al., 2015). LAT1-via leucine has also been shown to promote cancer drug resistance (Sato et al., 2021). This is also true for the estrogen receptor modulator, tamoxifen (Saito et al., 2019; Sato et al., 2021). Resistance to tamoxifen is thought to be due to adaption to nutrient stress via LAT1 (Saito et al., 2019). The exact mechanism by which LAT1 mediates chemoresistance is not fully known, however, it is hypothesized that LAT1 mediates the absorption of chemotherapy drugs into the cancer cells (J. Zhang et al., 2020). LAT1 overexpression in cancer is postulated to mediate the high metabolic demands of cancer and to provide amino acids for the synthesis of proteins and to support mTOR hyperactivity (Singh & Ecker, 2018). Recent studies show high expression of LAT1 is observed in 72.4% of CRC patients and this high expression was associated with increased depth of invasion and venous invasion (Hayase et al., 2017). While the

mechanism is not known, it could be due to LAT1 stimulation of the mTOR pathway, which in turn decreases the expression of miR-144. A decrease in the expression of miR-144 is association with metastasis and venous invasion in CRC (Iwaya et al., 2012). Another study demonstrated that breast cancer cells treated with leucine have increased protein levels of AKT and mTOR signaling (Singh et al., 2011). With this information, LAT1 has become a significant target for anti-cancer therapy. To this end, JPH203 is highly selective LAT1 antagonist that has been tested in a phase I cancer trial (Okano et al., 2020)(figure 5). The maximum tolerated dose within patients is 60 mg/m^2 however, grade 3 liver dysfunction is seen in one patient at this dose. Disease control was seen at doses between 12 – 25 mg/m^2 (Okano et al., 2020). Other than high dose liver dysfunction, JPH203 is well tolerated, phase II clinical trials a 25 mg/m^2 dose will be recommended.

Figure 5

Mechanism of Action of LAT1 inhibitor JPH203



Note. Branch chained amino acids (BCAA) enter the cancer cell through the L-type amino acid transporter 1 (LAT1). Upon entry, BCAA act to stimulate the mTOR pathway leading to downstream effects such as phosphorylation of ribosomal protein S6. In the presence of JPH203 a competitive antagonist, BCAA acids entry into the cell is reduced, thus leading to reductions in mTOR signaling.

c. Glutamine Pathway

The amino acid glutamine plays an important role in cancer because it contributes to the synthesis of proteins and nucleic acids in rapidly proliferating cells (Choi & Park, 2018). Glutamine is unique because it becomes a condition essential amino acid in cancer cells. Further, in cancer cells glutamine is converted to alpha ketoglutarate to replenish for the TCA cycle, which in turn sustains ATP in rapidly dividing cells (Duran & Hall, 2012). Prior reports also show that glutamine acts through two pathways to support mTOR hyperactivity in cancer cells. In one pathway, glutamine increases mTORC1 activity by promoting cellular uptake of leucine by cancer cells through L-Type Amino Acid Transporter 1 (LAT1) (Hayase et al., 2017). Mechanistically, LAT1 functions as an amino acid exchanger, that exports glutamine out of the cell as it transports leucine into the cell (Saito & Soga, 2021). Interestingly, cancer cells that overexpress glutamine transporters showed increased leucine uptake—and this was mechanistically linked to intracellular glutamine driving LAT1-mediated amino acid exchange of intracellular glutamine for extracellular leucine (Saito & Soga, 2021). Recent papers however show glutamine itself can activate mTOR—in the absence of extracellular leucine (Jewell et al., 2015). Interestingly, intracellular leucine and glutamine induce mTOR through different signaling pathways. As noted above, leucine works through RAG GTPases to stimulate the recruitment of mTOR to the outer membrane of the lysosome (Takahara et al., 2020). However, glutamine induction of mTORC1 is RAG GTPase independent (Meng et al., 2020). Glutamine stimulated mTORC1 translocation to the lysosome in RagA and RagB knock out cells via v-ATPase (Jewell et al., 2015). In HEK293 cells, authors reduced the expression of v-

ATPase via short interfering RNA against v-ATPase. When v-ATPase is reduced, glutamine is no longer able to activate mTORC1 (Jewell et al., 2015). ARF1 plays a role in the trafficking of mTORC1. Treatment of HEK293 cells with ARF1 inhibitor brefeldin A (BFA), inhibited glutamine stimulation of mTORC1 (Jewell et al., 2015). This suggests ARF1 is also linked to glutamine stimulation of mTORC1.

Amino acids are not the only nutrients that play a role in mTOR stimulation (Sanguesa et al., 2019). Glucose has recently been shown to play a role in the stimulation of this pathway. High glucose uptake is exhibited by cancer cells due to their high energy needs, thus supporting increase in cell growth, survival, and metastasis (Duan et al., 2014). Glycolysis is the primary mechanism for energy in cancer cells, making glucose the primary metabolic source for cancer growth (Duan et al., 2014). Early studies involving glucose and cancer demonstrated glucose plays a large role in the motility of cancer cells (Beckner et al., 1990). In melanoma cells treated with glucose free media, migration was decreased by 75%. In addition, hyperglycemia has been linked to increased mortality of many cancers such as bladder, pancreatic, endometrial, breast, and CRC (Li et al., 2019). In a recent study by Han et al, endometrial cell cancer lines were treated with low (1mM), normal glucose (5mM) and high glucose (25mM) (Han et al., 2015). Results indicated that high levels of glucose stimulated cell growth as well as phosphorylation of the mTOR readout ribosomal protein S6 (Han et al., 2015). Another paper showed in rat pancreatic islet cells that glucose can lead to mTOR activation (Gleason et al., 2007).

The mTOR signaling pathway integrates multiple signaling cascades such as PI3K/AKT, TSC1/TSC2/RHEB, LKBL/AMPK, and VAM6/Rag GTPases (Zou et al., 2020). In addition, mTOR controls cancer cell metabolism by altering the expression and activity of various key metabolic enzymes (Mossmann et al., 2018). For example, mTORC1 will stimulate the phosphorylation of S6K1 and 4EBP, these two proteins in turn phosphorylate and activate several substrates to then promote mRNA translation (Saxton & Sabatini, 2017a). For instance, through this pathway, mTORC1 stimulates cell survival and increases cellular proliferation by regulating FOXO1/3a transcription factors, and GSK3 beta (Saxton & Sabatini, 2017a). In Roulin et al, mTOR knockdown in colon cancer cell lines HT29 and LS174T demonstrated decreased proliferation via ^{3}H-Thymidine incorporation (Roulin et al., 2010). The PI3K/PTEN/Akt/mTORC1 pathway also increases the expression of the matric metallopeptidase 9 enzyme (MMP-9) in human hepatocellular carcinoma (Chen et al., 2009). MMP-9 is an enzyme that plays a crucial role cancer cell invasiveness (Zou et al., 2020). MMP-9 is a zinc dependent metalloenzyme that degrades extracellular matrix components, allowing for invasion of cancer cells (Mondal et al., 2020). In addition to mTORC1, mTORC2-mediated stimulation of AGC kinase family members (Protein kinase A, PKG, PKC) via phosphorylation has been linked to cancer progression (Oh & Jacinto, 2011). Considering this, mTOR inhibitors that target mTORC1 and mTORC2 have become an ever-growing field of study.

Section 1:4 Pharmacological mTOR Inhibitors

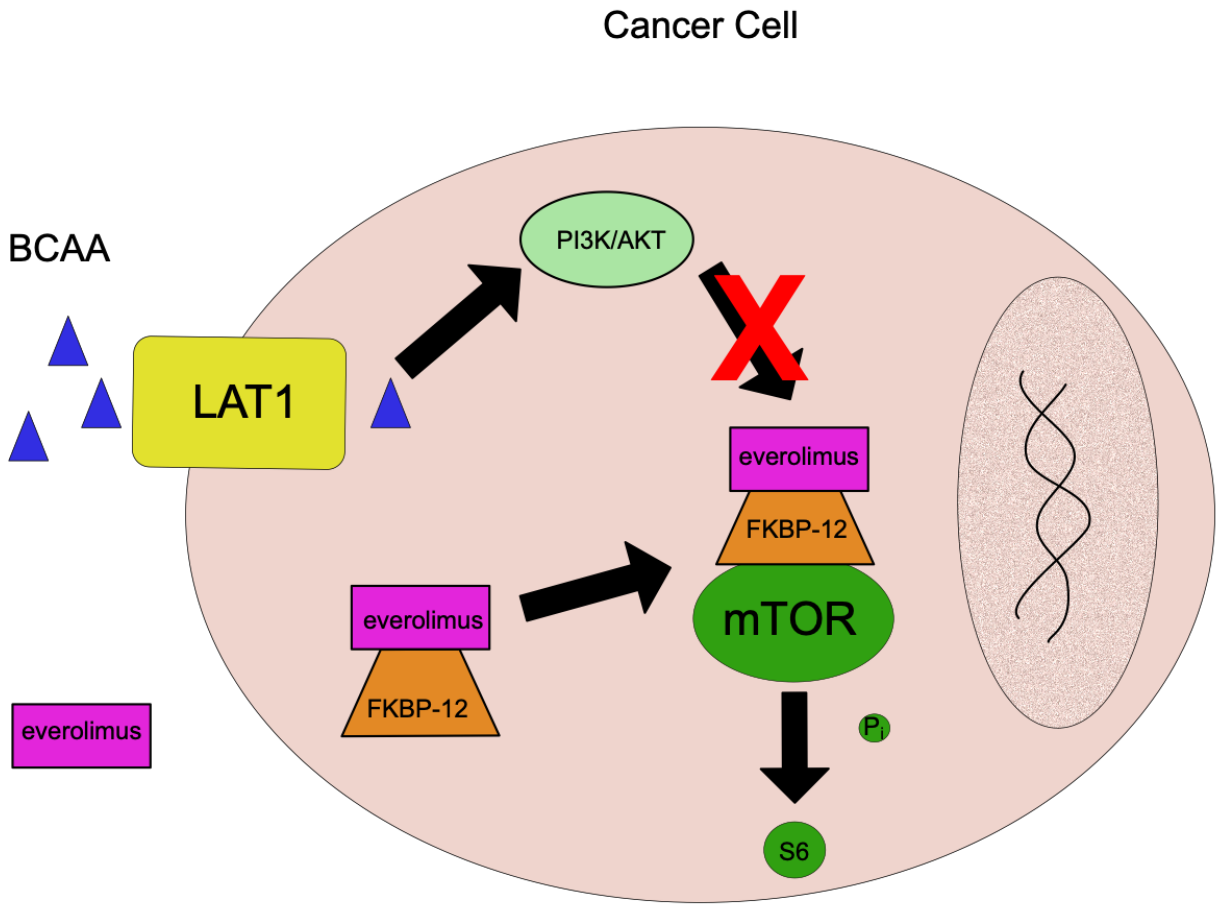
The critical role of mTOR within cancer progression had provided the premise for the development and production of mTOR inhibitors such as everolimus, and

sapanisertib. Everolimus is also approved as prophylaxis therapy to prevent renal transplant rejection (McMahon et al., 2011). Upon entering the cell, everolimus binds to the intracellular protein FKBP-12, and this drug-FKB12 complex in turn binds to and inhibits mTORC1 (Houghton, 2010) (figure 4). Everolimus is FDA approved for the treatment of advanced clear cell renal cell carcinoma (Coppin, 2010). In colon cancer, patients treated with bevacizumab and everolimus showed reduced VEGF and mTOR activity (Altomare et al., 2011). This combined therapy was tolerable, however side effects included: hypertension, fistula/abscess, mucositis, azotemia, and rhabdomyolysis (Altomare et al., 2011). More serious side effects included; gastrointestinal hemorrhage, bowel perforation, prolonged QT interval, and thrombocytopenia (Houghton, 2010). Sapanisertib inhibits TORC1 and TORC2 and has been reported to induce tumor cell apoptosis (Voss et al., 2020). Sapanisertib has been in phase 1 clinical studies for treatment of renal cancer, endometrial cancer and bladder cancer (Voss et al., 2020). Unfortunately, sapanisertib has side effects such as blood creatinine elevation, mucosal inflammation, thrombocytopenia, nausea, and vomiting, making this drug difficult to tolerate (Voss et al., 2020). Resistance to mTORC1 inhibitors is associated with the loss of negative feedback loops, which in turn increases the phosphorylation and activation of AKT (Rozengurt et al., 2014). Further, mTORC1 inhibitors only partially inhibit mTOR pathways—thus residual mTOR activity can also contribute to low drug efficacy (Zou et al., 2020). Normal cells are also dependent on mTOR activity and thus off target effects are also possible when treating patients with everolimus (Xie et al., 2016). Second generation mTOR inhibitors were created with the hopes to inhibit mTOR more strongly, and to also inhibit mTOR in complex 2. The

difference between first and second generation mTOR inhibitors is that the first generation inhibitors inhibit the FRB domain of mTOR while second generation inhibit mTOR kinase domain (Zhou & Huang, 2012). Many of these drugs, such as dactolisib, are still in clinical studies, however, they are showing serious side effects in rodent models. These side effects include, elevated blood glucose, and elevated liver enzymes (Netland et al., 2016). Sole mTORC2 inhibitors do not exist, however, inhibition of mTORC2 signaling suppressed the growth of PTEN null prostate cancer in mice (Guertin et al., 2009). MTOR inhibitors in combination with drugs such as tamoxifen and fulvestrant have shown contradicting effects in clinical trials. In some trials these combinations improve survival (Schmid et al., 2019). However, other studies show no therapeutic benefit of adding mTOR inhibitors (Yi et al., 2019). These findings indicate that further research needs to be done into this pathway to identify new upstream regulators and downstream effectors of the mTORC1 and mTORC2 pathways.

Figure 6

Mechanism of Action of mTOR Inhibitor Everolimus



Note. Everolimus enters the cancer cells and binds to FKBP-12. Branch chained amino acids (BCAA) enter the cancer cell through the L-type amino acid transporter 1 (LAT1). Upon entry, BCAA act to stimulate the mTOR pathway through PI3K/AKT. PI3K is unable to phosphorylate and activate mTOR due to the binding of the everolimus FKBP-12 complex, preventing phosphorylation of downstream proteins such as ribosomal protein S6.

Effect of Exercise on Cancer

One of the most recommended treatments in cancer with no side-effects is exercise. In healthy adults, physical activity was associated with lower risk of developing multiple cancers. This cancers include, esophageal cancer, liver cancer, lung cancer, kidney cancer, gastric cardia cancer, endometrial cancer, myeloid leukemia, myeloma, head and neck cancer, CRC, and breast cancer (Moore et al., 2016). Exercise also helps prevent the recurrence of certain cancers like breast (Holmes et al., 2005), prostate (Kenfield et al., 2011), and CRC (Meyerhardt et al., 2006). In terms of adipose tissue, exercise has a fundamental effect on its composition. Exercise has been shown to decrease AT mass, reduce adipose hypertrophy, and reduce AT inflammation (Honkala et al., 2020). This was shown in a study by Honkala et al. This group showed that in patients with insulin resistance, exercise increased glucose uptake in adipose tissue and a decreased expression of CD36 (Honkala et al., 2020). CD36 is known to mediate fatty acid induced metastasis of gastric cancer (Pan et al., 2019). This suggests that exercise makes fundamental changes to AT and thus can change the secretions of AT. This mechanism is seen in a paper written by Jones et al. Female mice were fed a high fat diet and inoculated with MDA-MB-231 breast cancer cells (Jones et al., 2010). Half of the mice had to exercise. Results from this study indicated that mice that performed exercise had high intertumoral hypoxia as measured by HIF-1(a marker of hypoxia (Jones et al., 2010). In addition, results showed alterations in the tumor microenvironment, making tumor tissue appear more like healthy tissue (Jones et al., 2010). This was due to increased vascularization within the tumor (Jones et al.,

2010). This study linked exercise to favorable alterations in the tumor microenvironment. Two studies by Tartibian et al in patients looked at the benefit of 25-30 minutes of exercise in healthy patients (Tartibian et al., 2015; Tartibian et al., 2011). They found that patients who exercised every day had decreased blood levels of inflammatory markers IL-6, TNF-alpha, and CRP (Tartibian et al., 2015; Tartibian et al., 2011). Contrary to these results, a similar test in breast cancer patients showed that exercise had limited effect on inflammatory cytokines and only saw a decrease in IL-6 (Jones et al., 2013). This study, however, did not invoke a time or type of exercise program in their study, the Tartibian study did. This could be a possible explanation for the different findings.

Exercise not only regulates the tumor microenvironment and the expression of inflammatory markers, but it also plays a role in mTOR signaling. In Bae et al, induced obesity in Sprague-Dawley rats via a high fat diet. Rats were then succumbed to a dietary change and moderate intensity treadmill training. Results showed that dietary changes and exercise significantly decreased levels of mTORC1 activity (Bae et al., 2016). In addition, another study demonstrated that obese Sprague-Dawley rats that underwent 4 weeks of exercise training were able to significantly reduce mTOR signaling (Rivas et al., 2009). Long term exercise suppressed the activation of mTOR (Agostini et al., 2018). The mechanism of this is through the activation of adenosine monophosphate-activated protein kinase (Agostini et al., 2018). Due to the cellular stress that occurs during exercise, AMPK becomes phosphorylated. Phosphorylation of AMPK in turn inhibits mTOR (Agostini et al., 2018). The tumor microenvironment consists of fibroblasts, blood vessels, immune cells, and extra-cellular matrix (Meurette

& Mehlen, 2018). Based on the results of these studies, inhibition of mTOR with exercise can prevent cancer cell proliferation and progression with no side effects.

Section 1:5 Other Signaling Pathways, Beyond mTOR, that Might Link Obesity With Cancer Progression

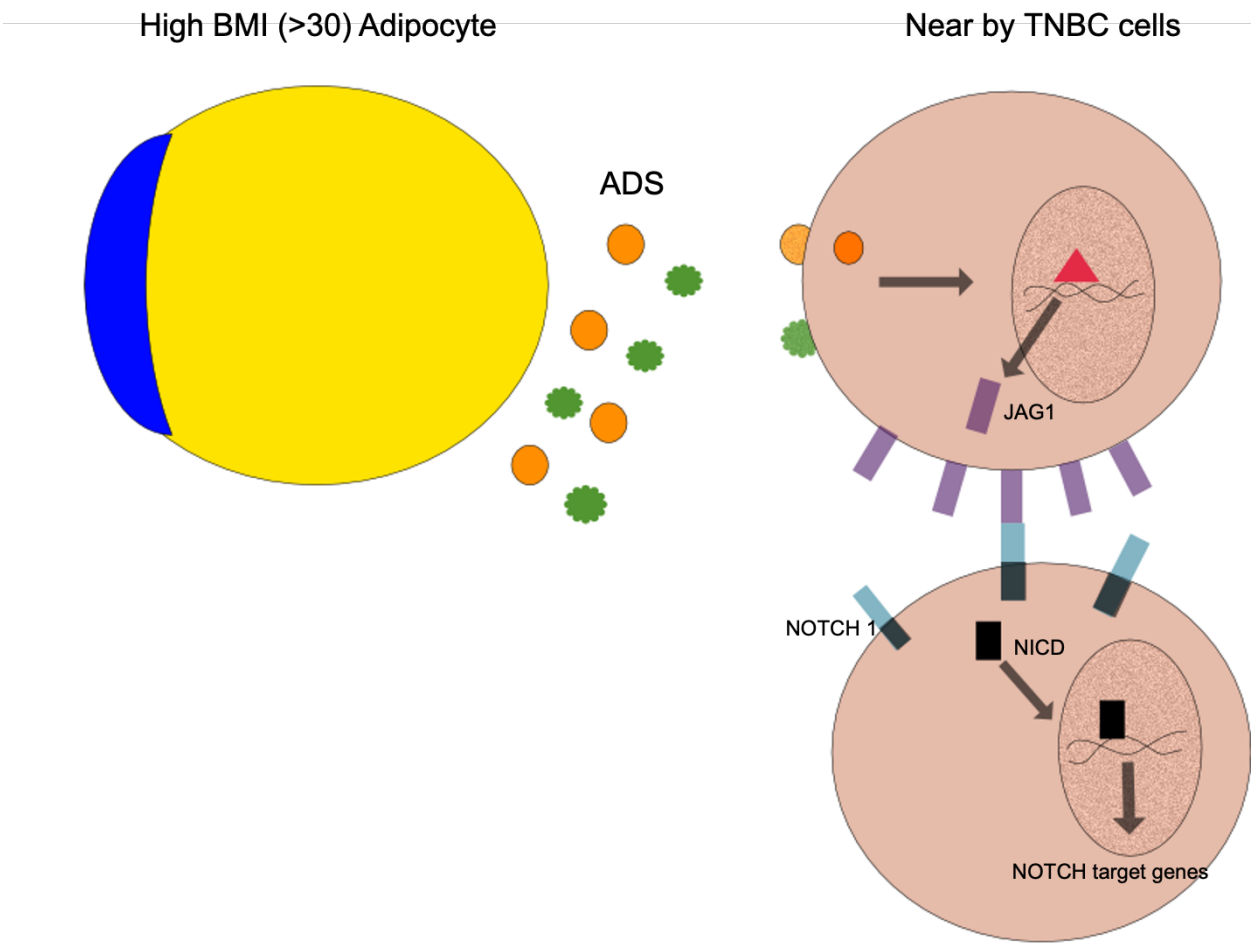
The NOTCH pathway, under normal conditions is crucial for development, however, hyperactivation has proven to be oncogenic (Lobry et al., 2014). More recently, NOTCH has been implicated in mediating crosstalk between tumor and the tumor microenvironment (Meurette & Mehlen, 2018). The NOTCH receptor is located on the cellular membrane. Upon ligand binding, the NOTCH receptor is activated and undergoes S2 cleavage by ADAMs10. After S2 cleavage, the activated NOTCH receptor then undergoes S3 cleavage by gamma secretase to creates the NOTCH intercellular domain (NICD) (Kopan, 2012). NICD then translocate to the nucleus where it regulates its target genes such as HES1, HEY1, MYC, and p21 (Borggrefe & Oswald, 2009). Activated NOTCH acts to repress p27, thus promoting CRC cell proliferation (Hristova et al., 2013). To prove this, Hristova et al knocked down NOTCH1 in CRC cancer cell line CaCO2.NOTCH knock down lead to an increase in p27 expression, and decreased cell proliferation by Brdu analysis (Hristova et al., 2013). Sonoshita et al demonstrated that NOTCH activation increased migration of in human primary colon cancer cells (Sonoshita et al., 2011). In addition, NOTCH activation in human primary CRC cells promoted a cancer stem cell phenotype (Lu et al., 2013).

There are two families of NOTCH ligands, delta like (Dll1, Dll3, Dll4) and serrate (Jagged1, and Jagged2) (D'Souza et al., 2010). Similar, to NOTCH, NOTCH ligands are transmembrane proteins expressed on the surface of neighboring cells (Kopan, 2012).

The specific ligand that has been most strongly linked to cancer is Jagged 1 (JAG1) (Xiu et al., 2020). Increased JAG1 expression has been linked to poor clinical prognosis in hepatocellular carcinoma, gastric carcinoma, TNBC, and CRC (Kunanopparat et al., 2021; Reedijk et al., 2008; Sugiyama et al., 2016; Yeh et al., 2009). In addition, increased expression of JAG1 is linked to increased tumor vasculature, leading to an increase in tumor growth (Pedrosa et al., 2015). These cancers are also linked to obesity (Table 1). Despite this link, the research linking obesity and JAG1/NOTCH signaling is limited. Our study is the first to demonstrate a strong link between obesity and increased JAG1 expression in TNBC (figure 7).

Figure 7

High BMI ADS Stimulates JAG1/Notch Activity



Note. High BMI adipocyte secretes adipokines, cytokines, leptin and exosomes that act on nearby TNBC cells. ADS acts on TNBC cells to induce signaling that regulates gene expression. Increased levels of JAG1 protein are then expressed. Increased JAG1 binds to its receptor NOTCH on a nearby cancer cell. Upon stimulation notch undergoes S2 cleavage to release notch intracellular domain (NICD). NICD then moves to the nucleus to act on its target genes, which in turn promotes the progression of cancer.

Conclusion

The link between obesity and increased cancer risk, poor clinical outcomes, increased metastasis, and poor responsiveness to cancer therapy, coupled with the high rates of obesity, translates to a clinical need for better cancer therapy in obesity (Park et al., 2014). The discovery that obesity increases circulating leucine and stimulates leucine uptake via LAT1 by cancer cells will foster the design of new therapies that selectively suppress leucine-mTOR signaling in obesity. While current LAT1 inhibitors seem to be well tolerated, phase 2 clinical trials are still underway (Okano et al., 2020). The selective inhibition of LAT1 in cancer will require a deeper mechanistic understanding of how LAT1 is uniquely regulated in cancer in obesity. There are no current studies into the effect of JPH203 on levels of other amino acid transporters such as LAT2 or LAT3. However, it could be possible that LAT1 blockers may lead to increased expression of LAT2 or LAT3. In addition, LAT1 is crucial for normal brain function, such as the transport for thyroid hormone and essential amino acids into the brain, and to mediate transport of L-dopa across the blood-brain barrier. This suggests targeting of LAT1 needs to be tissue specific to avoid serious side effects (Singh & Ecker, 2018). Based on our prior report showing that obese ADS increases LAT1 affinity for leucine in cancer cells (Thompson et al., 2021), we postulate that this increase in affinity may be due to phosphorylation of LAT1 via the obese adipose secretome. Thus, targeting LAT1 phosphorylation might be a way to selectively inhibit LAT1 activity in cancer cells in obesity. Further, obesity-mediated regulation of nutrient transport might not only apply to LAT1, and thus the activity of other transporters (such

as glutamine) transporters might also be uniquely regulated in cancer in obesity. Due to nutrient overlap it may never be possible to inhibit mTOR enough by solely targeting nutrient pathways. There is some evidence that changing the levels of substrates (nutrients) by exercise does have an anticancer effect (Jones et al., 2010).

Chapter 2

In Breast Cancer, a High (> 30) Body Mass Index is Associated with Changes in Peritumor Breast Adipose Tissue that Increases the Migration and Invasiveness of Triple-Negative Breast Cancer Cells

Cora E. Miracle[1], Chelsea Thompson[1], Krista Denning, Rebecca Russell, Logan Lawrence, Mary Legenza, Jacy Baxter[1], Paige Vanaman[1], Travis Salisbury[1]

Abstract

Breast cancer remains the most common cancer in women with multiple risk factors including smoking, genetics, environmental factors, and obesity. Smoking and obesity have been shown to be the top two risk factors for development of breast cancer, with smoking increasing the risk of development by 21% (Jones et al., 2017) and obesity by 20-40%, respectively (Munsell et al., 2014). This link has been established in the luminal estrogen receptor-positive breast cancer but remains understudied in triple-negative breast cancer (TNBC). TNBC is a breast cancer subtype characterized by the absence of estrogen and progesterone and HER2 receptors (Kumar & Aggarwal, 2016). Accounting for 10-20% of all breast cancers (Kumar & Aggarwal, 2016), TNBC is the deadliest breast cancer subtype. The 5 year survival rates for patients with TNBC are 8-16% lower than the 5 year survival rates for patients with estrogen receptor-positive breast tumors (Howard & Olopade, 2021). In addition, TNBC patients have early relapse rates (3-5 years after diagnosis) (Naik et al., 2019). Thus, new therapies are needed for TNBC that are efficacious in obesity (Howard & Olopade, 2021). Exploration into the link

between obesity and TNBC could potentially lead to new therapeutic intervention. In this paper, we show that peritumor breast adipose derived secretome (ADS) from patients with a high (>30) BMI is a stronger inducer of TNBC cell migration, invasiveness and JAG1 expression than peritumor breast ADS from patients with low (< 30) BMI.

1. Introduction

Breast cancer is the most common cancer in women worldwide with incidence rates expected to increase by 40% by the year 2040 (Arnold et al., 2022). Breast cancer is divided into four molecular subtypes: Luminal A, Luminal B, HER2+, and Triple Negative Breast Cancer (TNBC). TNBC is the deadliest breast cancer subtype as it does not possess the estrogen receptor (ER+), progesterone receptor (PR+), or HER2. The absence of these markers hinders the ability for clinical intervention. In addition, TNBC is known to have a high heterogeneity making it even more difficult to treat (Yin et al., 2020). While TNBC largely remains understudied compared to ER/PR+ breast tumors, it still accounts for 10-20% of breast cancer diagnoses (Kumar & Aggarwal, 2016). TNBC tends to occur in premenopausal women under 40 years old (Morris et al., 2007), and disproportionately occurs in women of color (Prakash et al., 2020). The survival after metastasis of TNBC is only 13.3 months (Chue & La Course, 2019). The factors that pre-dispose these women to such a deadly illness are currently under studied. Recently, clinical studies have shown a link between TNBC and obesity (Pierobon & Frankenfeld, 2013; Sun et al., 2017). One study showed that women who had been diagnosed TNBC were more likely to be overweight/obese (odds ratio =1.89) (Trivers et al., 2009). Further, a clinical study showed that of 183 TNBC patients, 63.7% were obese (Mowad et al., 2013). These clinical studies show a link between obesity and TNBC however the

mechanisms by which obesity promotes the progression and incidence of TNBC are unclear.

Obesity affects one in every three adults in the United States, with the prevalence rising each year. While obesity is commonly linked with diabetes and metabolic disease, obesity has also been linked to fourteen different cancers, including breast cancer. Those patients who are obese at the time diagnosis have been shown to have increased tumor grade, larger tumor size, poor prognosis, and increased risk of metastasis (Neuhouser et al., 2015; Thompson et al., 2021; Yang et al., 2011). The reason for this could be because obesity causes pathological changes within adipose tissue (AT) that in turn alters the levels of factors that are released from AT (Funcke & Scherer, 2019; Lengyel et al., 2018; Park et al., 2014). AT is an endocrine organ due to the numerous proteins and hormones it secretes. The secretions that are released from AT are referred to as being the adipose derived secretome (ADS). The complete set of factors in the ADS is unknown, however obesity changes the levels of adiponectin, leptin, adipokines, and cytokines in ADS. Prior studies have shown that ADS induces signaling in luminal ER breast cancer cells that promotes breast cancer cell migration and invasiveness. Based on these findings, we hypothesized that in obesity peritumor breast ADS acts on TNBC cells to induce signaling that promotes the migration and invasiveness of TNBC cells.

Recently, we published that peritumor breast AT obtained from women undergoing therapy for breast cancer secreted factors that induced signaling in luminal ER positive breast cancer cells that increased cancer cell migration and invasiveness. In this study, we extend those findings by showing peritumor breast ADS from breast cancer patients

with BMIs > than 30 is a stronger inducer of TNBC cell migration and invasiveness and signaling compared with peritumor breast ADS from women with BMIs < than 30. This new finding shows that high (> 30) BMI induces changes in AT in the breast tumor microenvironment that leads to a cancer promoting ADS that acts on TNBC cells to stimulate cancer cell migration and invasiveness. This finding supports the hypothesis that targeting AT in obesity might be a new way to suppress the progression of TNBC.

2. Results

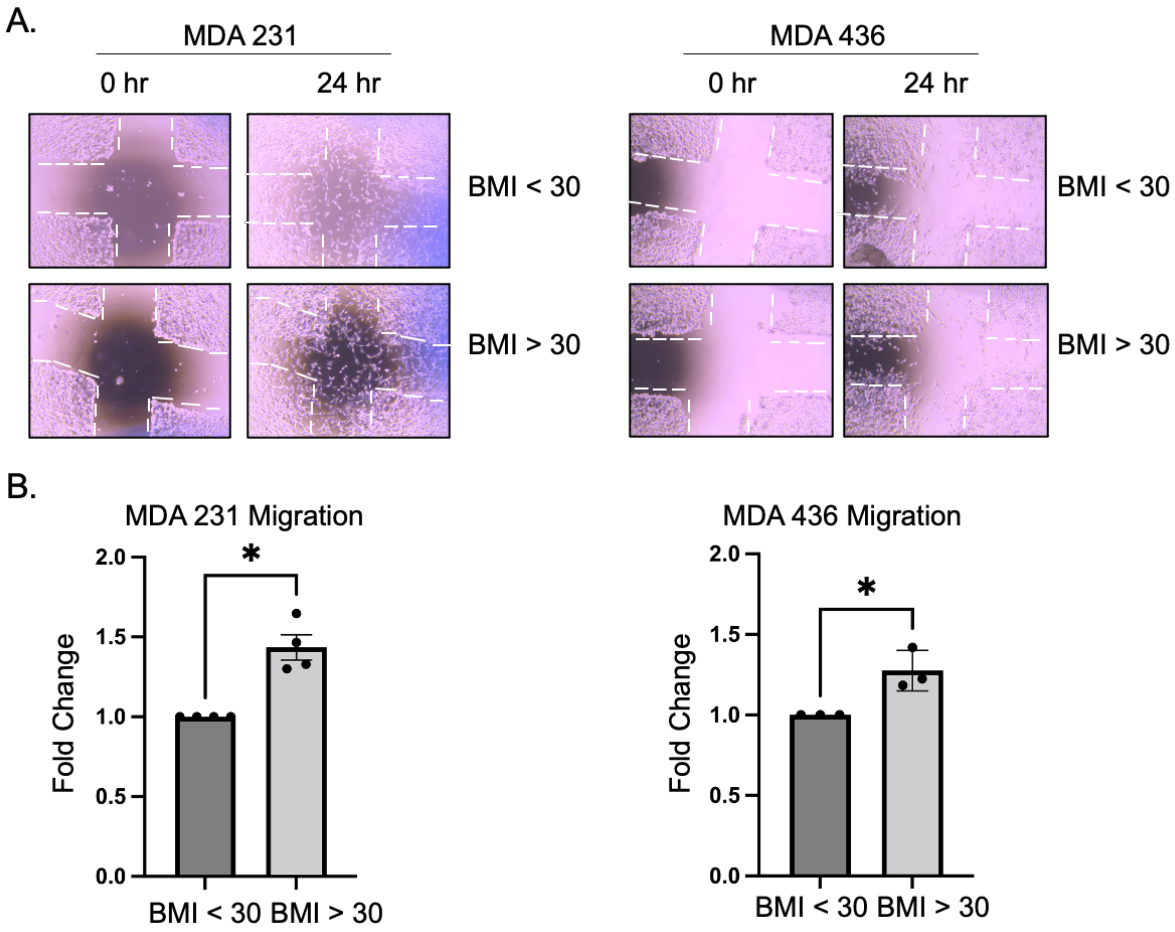
a. High (>30) BMI peritumor ADS Increases the Invasion and Migration of TNBC Cells

To investigate the role obesity plays in TNBC, we examined the effect of breast peritumor ADS on the migration and invasion of MDA-MB-231 and MDA-MB-436 TNBC cells. We hypothesized that the association of obesity with worsening of TNBC (Neuhouser et al., 2015; Thompson et al., 2021; Yang et al., 2011) is mediated by increased migration and invasiveness of TNBC cells in obesity. Freshly isolated breast peritumor AT samples (~100 mg) obtained from women with breast cancer were carefully cut into 5 equally sized pieces and cultured in serum free cell culture medium for 24 hours. The AT conditioned medium (termed ADS) was then collected and applied to TNBC cells to investigate the effects on TNBC cell migration and invasion. We analyzed the effect of BMI on TNBC cell by comparing responses induced by ADS from BMI > 30 with BMI < 30, which provided insight into the effect of obesity on TNBC cell migration and invasiveness. To assay migration, we applied peritumor breast ADS to MDA-MB-231 and MDA-MB-436 TNBC cells and quantified the migration of cancer cells across a “scratch” in a cell monolayer (wound healing assay). Results showed that at 24

hours post treatment, TNBC cells treated with high (> 30) BMI ADS migrated 1.2-1.4-fold faster than TNBC cells treated with low (< 30) BMI ADS (Figure 8 A and B).

Figure 8

The effect of BMI on the migration of TNBC cells



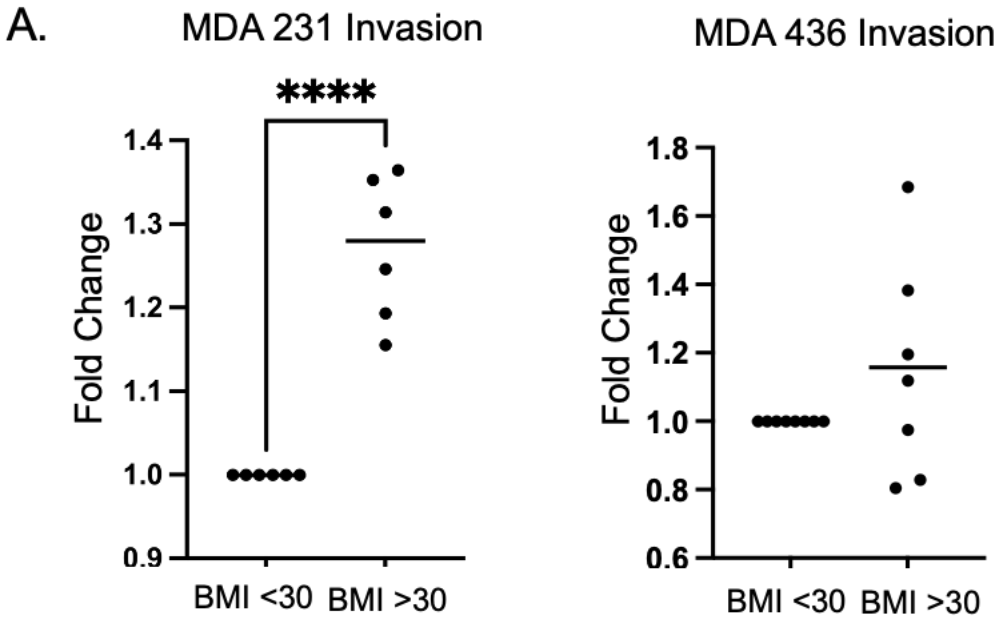
Note. Peritumor breast adipose tissue (AT) was obtained from women with breast cancer. Pieces of AT were cultured from 24 h. After the culture, the media were collected and centrifuged to remove tissue debris. The cleared media, which is the peritumor AT-derived secretome (ADS) was diluted 1:10 in media with 0.1% FBS and placed on TNBC cells for 24 h for migration (wound healing) (a, b). (a) representative images wound healing assay, (c) invasive activity was determined by Boyden Chamber Assay (see methods for details). Significant induction by BMI > 30 compared with BMI <

30, based on Student's t-test analysis, is indicated by *$p < 0.05$, and **** $p < 0.001$ (n= 4). The data are shown as the mean ± SEM (error bars).

Next, we sought to investigate the effect of BMI on peritumor breast ADS on TNBC cell invasiveness. To this end, the invasion of TNBC cells through a basement membrane towards cell culture medium containing peritumor ADS was analyzed using the Boyden Chamber assay (please see methods for details). MDA-MB-231 and MDA-MB-436 were plated on top of the membrane insert with peritumor breast ADS used as the chemoattractant in the lower chamber. The results showed that high (> 30) BMI induces greater (1.4-fold) TNBC cell invasiveness compared with low (< 30) BMI (Figure 9).

Figure 9

Effect of High BMI on the Invasion of TNBC.



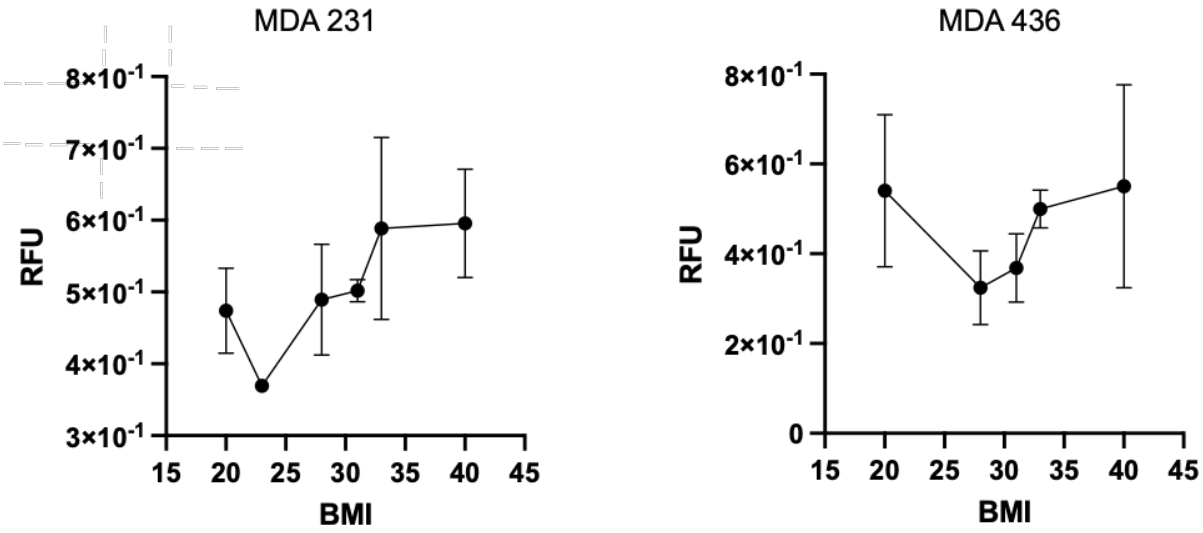
Note. Peritumor adipose tissue was obtained as described in figure 8. ADS was diluted 1:10 in media with 0.1% FBS and placed on TNBC cells for 24 h for invasion assay. (A) invasive activity was determined by Boyden Chamber Assay (see methods for details). Significant induction by BMI > 30 compared with BMI < 30, based on Student's t-test analysis, is indicated by **** $p < 0.001$ (n= 4). The data are shown as the mean ± SEM (error bars).

b. Correlation of BMI to invasive potential

To better ascertain the effect of BMI on TNBC cell invasiveness, we plotted BMI relative to fluorescence units from the invasion assay. We found that in MDA-MB-231 TNBC cells, the invasion rate trended to an increased as the BMI increased (Figure 10) (r =0.8116) (R^2 = 0.6587) (P-value =0.0499). In MDA-MB-436 cells, this correlation was not statistically significant but showed a trend (r = 0.1344) (0.01807) (P-value = 0.8293).

Figure 10

The Association of BMI With TNBC Cell Invasive Activity



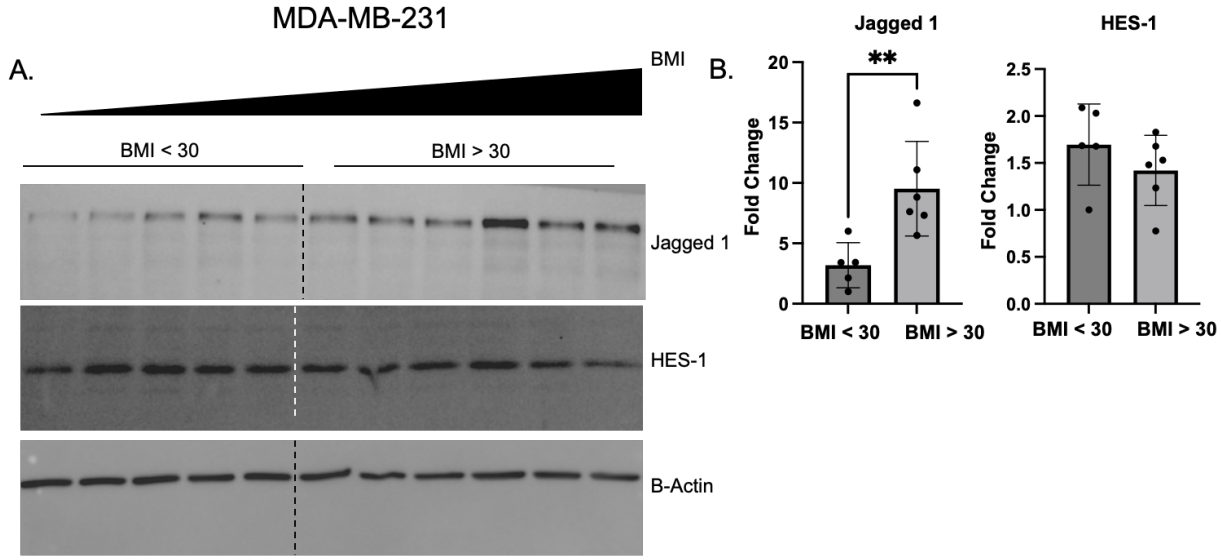
Note. A) A correlation between patient BMI and TNBC invasive activity (RFU values taken from the invasive assay shown in Figure 1) Pearson r (0.8116) R^2 (0.6587) P-value = (0.0499) Pearson r = (0.1344). R^2 (0.01807). P-value = (0.8293). RFU = Relative Fluorescence Units

c. High (>30) BMI Peritumor ADS Stimulates Increases in JAG1 Expression in TNBC Cells

Our functional data established that BMI induces changes in peritumor breast AT to stimulate TNBC cell migration and invasion with high (> than 30) BMI having a stronger effect than low (< 30) BMI. We hypothesize this BMI effect is mediated by peritumor breast ADS inducing signaling in TNBC cells that is more robust in response to high (> than 30) compared to low (< than 30) BMI. To investigate this hypothesis, we treated MDA-MB-231 and MDA-MB-436 with peritumor breast ADS over a range of BMI's (Figure 14 - Figure 20). We used antibodies that detect JAG1, HES1, phospho-ERK (Thr202/Tyr204), total ERK, phospho-NF-KB p65 (Ser536), total NF-KB, phospho-S6 (Ser235/236) and total S6 to probe cell extracts from TNBC cells treated with ADS for changes in JAG1/NOTCH, NF-KB, ERK, and mTOR complex 1 signaling, respectively. In MDA-MB 231 the pathways that showed significant increases in cells treated with ADS with a BMI > 30 were JAG1 (P-value =0.0094) and ERK (P-value =0.0149) (figure 11-14). However, in MDA-MB-436, there was no significant increase in the proteins and phospho-proteins associated with pathways mentioned above. Despite no significant increases, there was a trend to an increase in the signaling of the mTOR pathway (P-value = 0.0885).

Figure 11

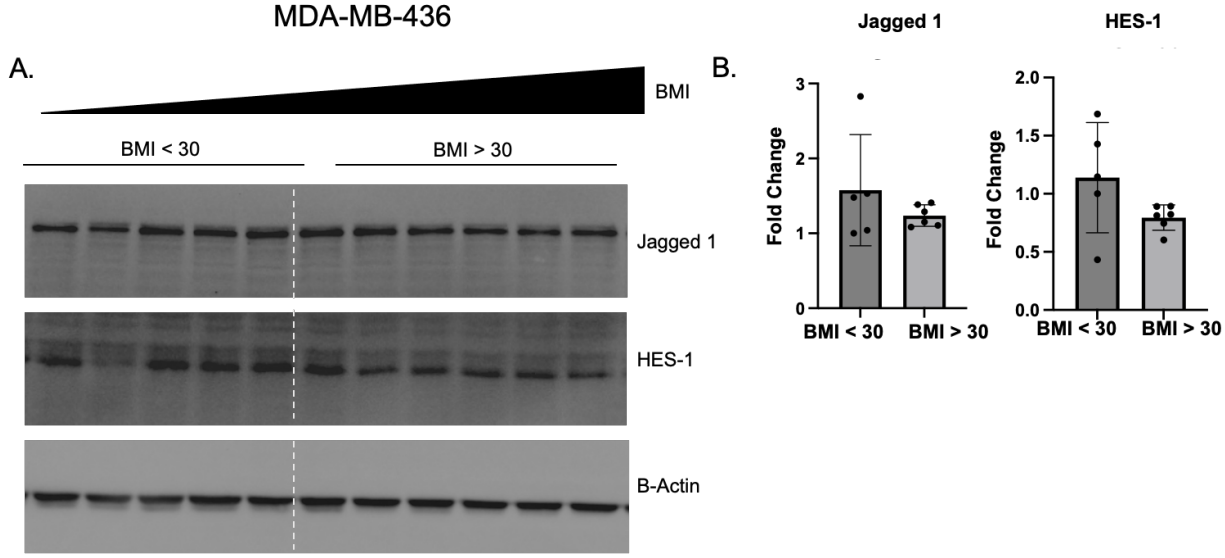
The Effect of BMI on JAG1/NOTCH Pathway in MDA-MB-231 TNBC Cells



Note. The method by which peritumor breast ADS from women with breast cancer was isolated and placed on TNBC cells for western blot studies is indicated in the legend of Figure 1. For western blot analysis, relative levels of proteins were calculated as the densitometric signal for a protein divided by the densitometric signal for β-actin loading control. *Indicates a significant ($P < 0.05$) increase in the levels of JAG1 in response to BMI > 30 (n = 6) compared with BMI < 30 (n = 5), as analyzed by Student's t-test. All data are mean ± SEM.

Figure 12

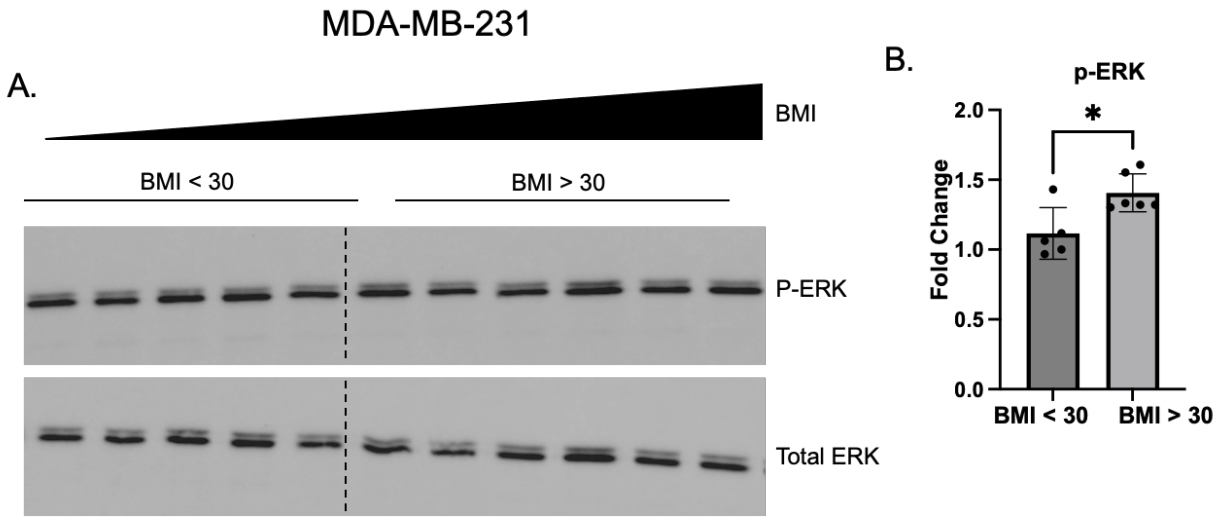
The Effect of BMI on JAG1/NOTCH Signaling in MDA-MB-436 TNBC Cells.



Note. The method by which peritumor breast ADS from women with breast cancer was isolated and placed on TNBC cells for western blot studies is indicated in the legend of Figure 1. For western blot analysis, relative levels of proteins were calculated as the densitometric signal for a protein divided by the densitometric signal for β-actin loading control. Statistical analysis, by Student's t-test, showed BMI had no effect on the expression of the proteins in the western blot experiment. All data are mean ± SE

Figure 13

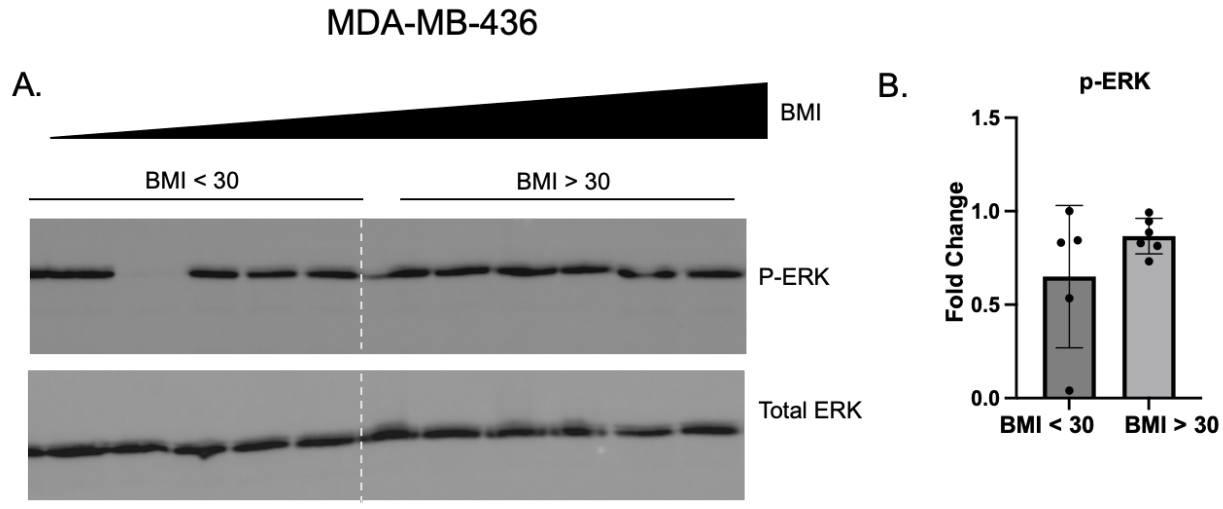
The Effect of BMI on ERK Signaling in MDA-MB-231 TNBC Cells.



Note. The method by which peritumor breast ADS from women with breast cancer was isolated and placed on TNBC cells for western blot studies is indicated in the legend of Figure 1. For western blot analysis, relative levels of proteins were calculated as the densitometric signal for a protein divided by the densitometric signal for β-actin loading control. *Indicates a significant ($P < 0.05$) increase in the phosphorylated ERK in response to BMI > 30 (n = 6) compared with BMI < 30 (n = 5), as analyzed by Student's t-test. All data are mean ± SEM.

Figure 14

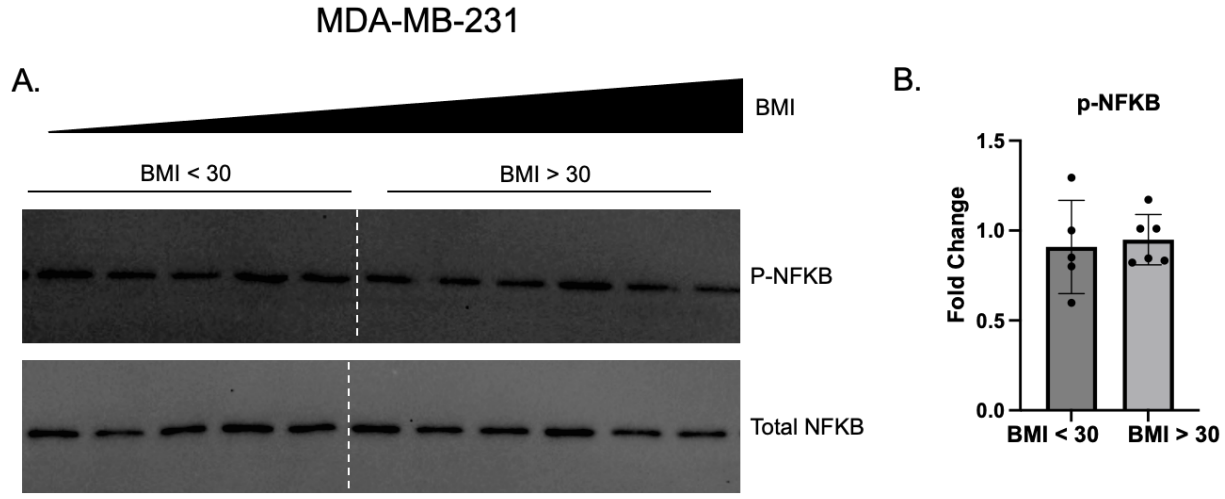
The Effect of BMI on ERK Signaling in MDA-MB-436 TNBC Cells.



Note. The method by which peritumor breast ADS from women with breast cancer was isolated and placed on TNBC cells for western blot studies is indicated in the legend of Figure 1. For western blot analysis, relative levels of proteins were calculated as the densitometric signal for a protein divided by the densitometric signal for β-actin loading control. Statistical analysis, by Student's t-test, showed BMI had no effect on the expression of the proteins in the western blot experiment. All data are mean ± SEM

Figure 15

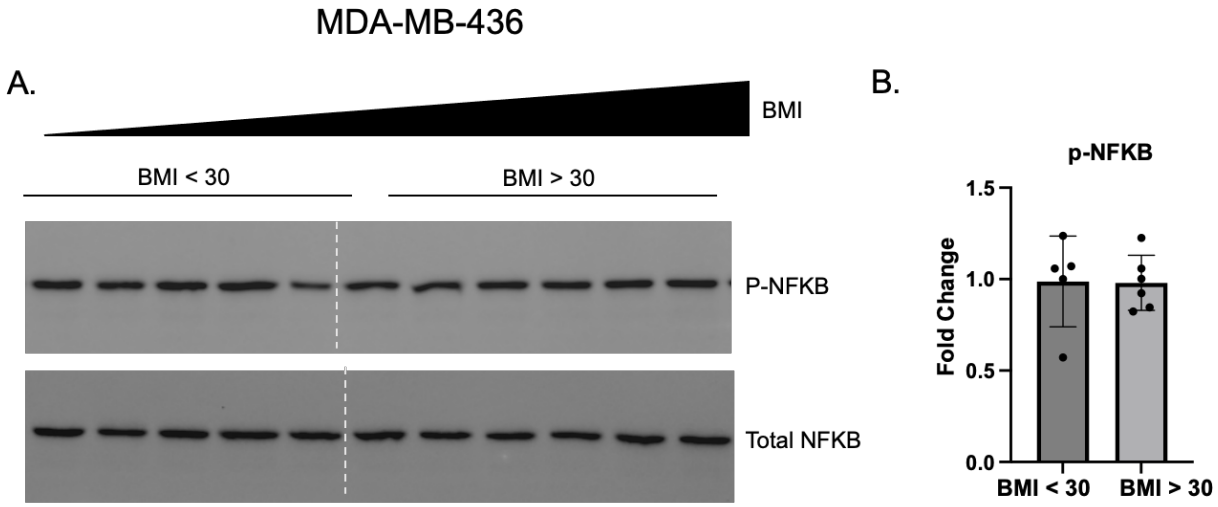
The Effect of BMI on NFKB Signaling in MDA-MB-231 TNBC Cells



Note. The method by which peritumor breast ADS from women with breast cancer was isolated and placed on TNBC cells for western blot studies is indicated in the legend of Figure 1. For western blot analysis, relative levels of proteins were calculated as the densitometric signal for a protein divided by the densitometric signal for β-actin loading control. Statistical analysis, by Student's t-test, showed BMI had no effect on the expression of the proteins in the western blot experiment. All data are mean ± SEM

Figure 16

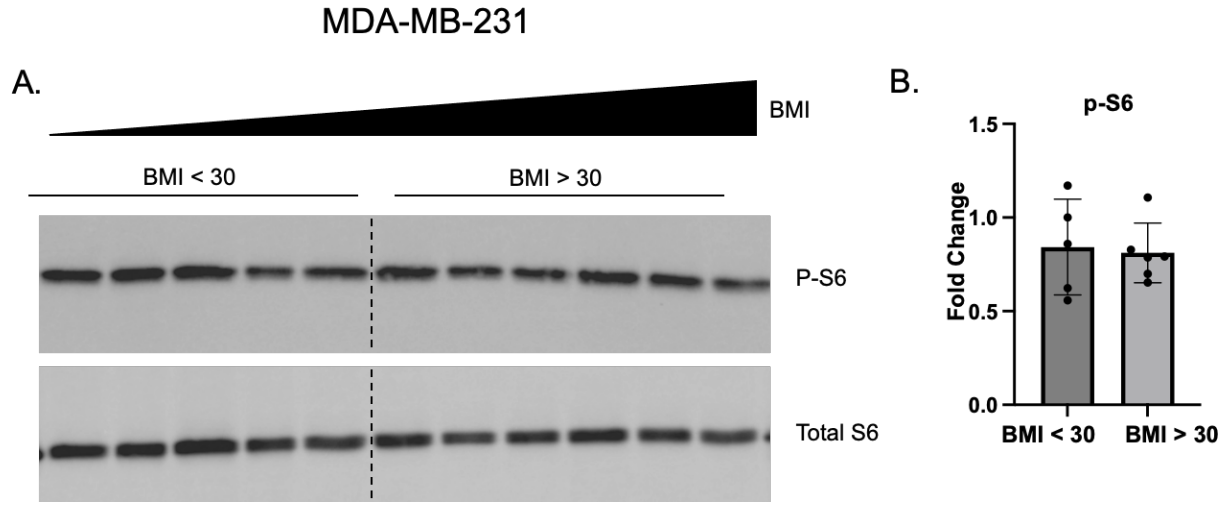
The Effect of BMI on NFKB Signaling in MDA-MB-436 TNBC Cells



Note. The method by which peritumor breast ADS from women with breast cancer was isolated and placed on TNBC cells for western blot studies is indicated in the legend of Figure 1. For western blot analysis, relative levels of proteins were calculated as the densitometric signal for a protein divided by the densitometric signal for β-actin loading control. Statistical analysis, by Student's t-test, showed BMI had no effect on the expression of the proteins in the western blot experiment. All data are mean ± SEM

Figure 17

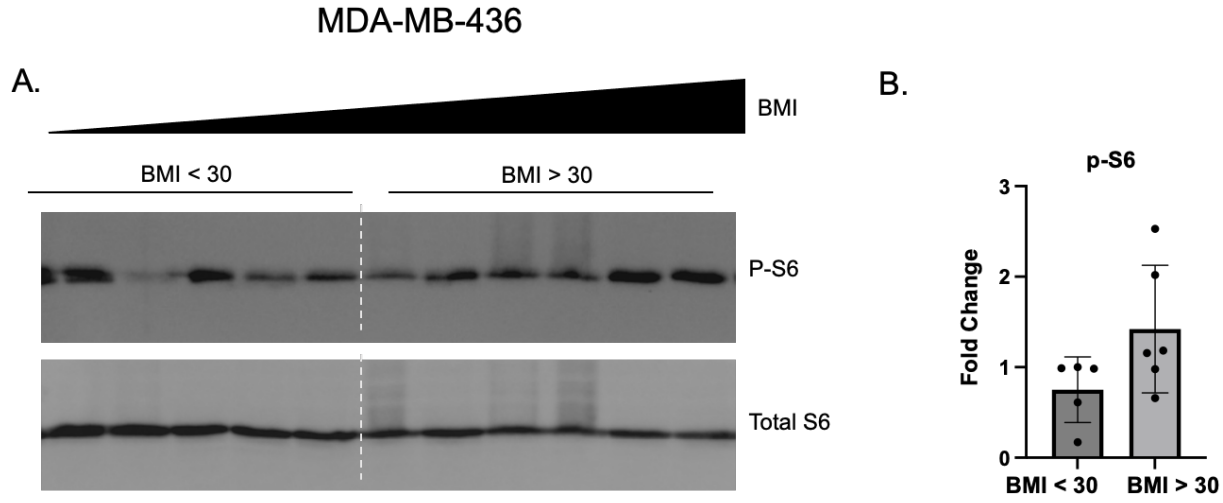
The Effect of BMI on mTOR Signaling in MDA-MB-231 TNBC Cells



Note. The method by which peritumor breast ADS from women with breast cancer was isolated and placed on TNBC cells for western blot studies is indicated in the legend of Figure 1. For western blot analysis, relative levels of proteins were calculated as the densitometric signal for a protein divided by the densitometric signal for β-actin loading control. Statistical analysis, by Student's t-test, showed BMI had no effect on the expression of the proteins in the western blot experiment. All data are mean ± SEM

Figure 18

The Effect of BMI on mTOR Signaling in MDA-MB-436 TNBC Cells



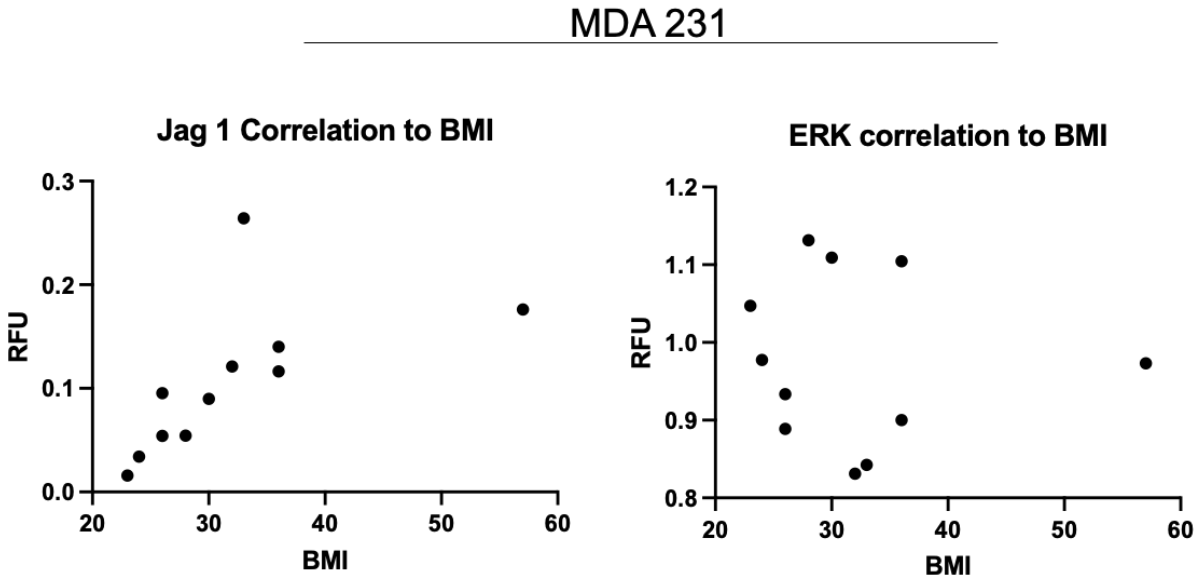
Note. The method by which peritumor breast ADS from women with breast cancer was isolated and placed on TNBC cells for western blot studies is indicated in the legend of Figure 1. For western blot analysis, relative levels of proteins were calculated as the densitometric signal for a protein divided by the densitometric signal for β-actin loading control. Statistical analysis, by Student's t-test, showed BMI had no effect on the expression of the proteins in the western blot experiment. All data are mean ± SEM

d. Correlation of BMI to Increases in JAG1 and ERK Signaling

Given the findings that showed MDA-MB-231 cells treated with peritumor breast ADS from high (> 30) BMI have higher JAG1 expression and ERK activity than cells treated with peritumor ADS from low (< 30) BMI, we assayed the potential for a correlation of BMI with JAG1 and ERK signaling. We found that as BMI increases the protein levels of JAG1 increased (figure 19) (P-value = 0.0488) (Pearson r = 0.6046) (R^2 = 0.3655). However, there was no correlation between BMI and protein levels of phosphorylated ERK (P-value = 0.6602) (Pearson r = -0.1498) (R^2 = 0.4758). In MDA-MB-436 there was a trend towards an increase in stimulation of the mTOR pathway. We found that in MDA-MB-436 cells, BMI was significantly correlated with protein levels of the mTOR complex 1 readout phosphorylated ribosomal protein S6 (P-value = 0.0188) (Pearson r = 0.6898) (R^2 = 0.4578).

Figure 19

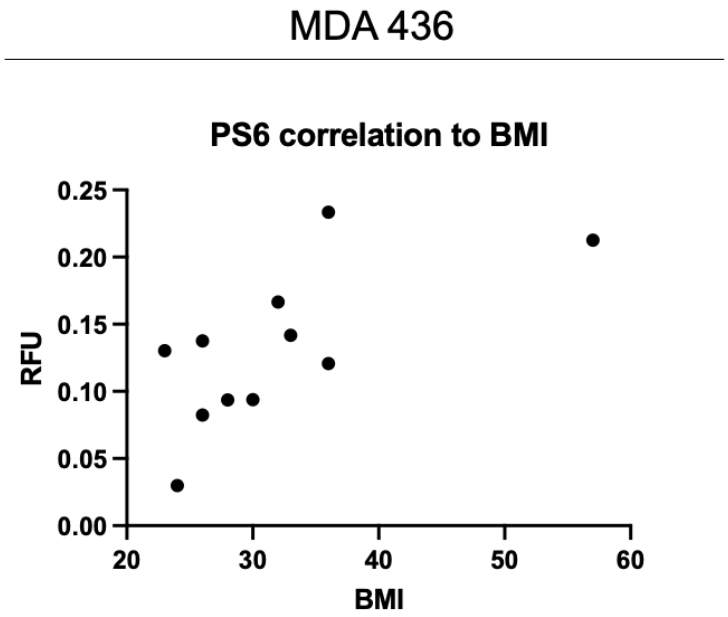
The Association of BMI with JAG1



Note. Correlation between BMI and western blot densitometric signals for JAG1 and ERK from the western blot experiments shown in Figure 11 and 13. JAG1 Pearson r (0.6046), R^2 (0.3655). P-value = (0.0488). (ERK Pearson r (-0.1498) R^2 (0.02243)). P-value = (0.6602).

Figure 20

The Association of BMI with pS6



Note. Correlation between BMI and western blot densitometric signals for pS6 from the western blot experiments shown in Figure 18. PS6 Pearson r (0.6898), R^2 (0.4758). P-value = 0.0188.

3. Discussion

This study is the first to demonstrate that high (> 30) BMI is associated with peritumor breast ADS having a stronger effect on the migration and invasiveness of TNBC cells. This finding provides new insight into the mechanism by which obesity is significantly associated with worsening of TNBC that was reported in clinical studies. This is significant, given that TNBC is the most aggressive breast cancer subtype and our findings viewed with clinical studies showing that obesity is associated with worsening TNBC collectively supports the hypothesis that obesity promotes the aggressiveness of TNBC by promoting TNBC cancer cell migration and invasiveness (Figure 11 and Figure 9). While MDA-MB-231 has increased migration and invasion, MDA-MB-436 only have increased migration in response to high (> 30) BMI. This finding indicates that the effect of obesity on TNBC cells is dependent on the genetics/mutations in TNBC cells. It also shows that different genes and pathways in TNBC cells mediate increases in TNBC cell migration and invasiveness, given that increases in MDA-MB-436 migration occurred without a corresponding increase in MDA-MB-436 invasiveness in response to high (>30) BMI. To further provide a strong association between BMI and MDA-MB-231 invasiveness, we plotted BMI compared with relative florescence units and found that increasing BMI is associated with increases in florescence. Given that increased fluorescence units in the Boyden Chamber Assay is a readout of higher invasiveness our finding showing that it is statistically significantly associated with increasing BMI provides further evidence that high BMI is positively linked with increasing TNBC cell migration and invasiveness. This

finding is the first to statistically link increasing BMI with increased invasion rate of TNBC cells.

After we established a link between increased invasiveness and BMI, we sought to investigate the activity of signaling pathways in TNBC cells that are fundamental to the progression of TNBC breast cancer and if such pathways are responsive to differences in BMI. The JAG1/Notch pathway promotes the progression of TNBC cells in part by stimulating the migration and invasiveness of TNBC cells. We and others have directly linked the activity of JAG1 signaling with the migration and invasiveness of TNBC cells, including MDA-MB-231 cells. Further, higher levels of JAG1 and associated NOTCH1 signaling in TNBC tumors significantly reduces the survival rates of women with this aggressive breast cancer subtype (Reedijk et al., 2005). Therefore, finding that among the pathways we assayed, JAG1 was significantly responsive to BMI in response to high (>30) BMI compared with low (< 30) in MDA-MB-231 breast cancer cells provides a new insight into how obesity might induce the invasiveness of TNBC cells. Of interest, is that we previously showed that BMI induced higher mTOR signaling in luminal ER positive breast cancer cells. The mTOR pathway however is not responsive to BMI in TNBC cells. This finding is not unexpected, given that luminal and TNBC cancer cells have different gene mutations and that JAG1-NOTCH signaling has stronger link with TNBC compared with luminal breast cancer.

As noted above, MDA-MB-231 cells are more responsive to changes in BMI than MDA-MB-436 cells. Specifically, increases in JAG1, phospho-ERK and invasiveness in response to high (> 30) BMI occurred in MDA-MB-231 cells but not MDA-MB-436 cells. We hypothesize that the different responses to BMI in TNBC cells are mediated by the

different genetic background of the two TNBC cell lines (Table 3). In this regard, MDA-MB-231 cells possess a KRAS mutation while MDA-MB-436 cells do not. KRAS mutations are more commonly found in premenopausal women with TNBC (Paranjape et al., 2011). In addition, pre-menopausal women who are obese tend to develop TNBC (Pierobon & Frankenfeld, 2013). This mutation could play a role in cancer aggressiveness seen in young obese TNBC patients and it might also explain why MDA-MB-231 cells are more responsive to BMI compared with MDA-MB-436 cells. MDA-MB-231 cells are a more heterogenous population that tends to express more cytokine receptors than MDA-MB-436 cells and that could also explain why MDA-MB-231 cells are more responsive to BMI (Norton et al., 2015) (Shen et al., 2020). While the specific receptors in TNBC cells that mediate the effects of BMI remain to be fully characterized prior studies show that cytokines in ADS are important paracrine factors in other cell models and thus differentially expressed cytokine receptors on MDA-MB-231 compared with MDA-MB-436 are good targets to investigate in future studies.

Table 3

Comparison Between TNBC Cell Lines

MDA-MB-231	MDA-MB-436
• Human cell line • 51-year-old white female with lung metastasis • Breast mammary gland • Adenocarcinoma • Tumorigenic • Express WNT7B oncogene • EGF and TNF alpha expression • KRAS mutation • Heterogenous population	• Human cell line • 43-year-old white female with lung metastasis • Breast mammary gland • Adenocarcinoma • Not tumorigenic • Express WNT7B oncogene • Tubulin and actin expression

Note. Difference between two different subtypes of TNBC cell lines.

Collectively, the findings of this study extend prior studies showing that high (> 30) BMI that occurs in obesity is a risk factor for worsening TNBC. We show a new mechanistic link between BMI, peritumor breast ADS and increases TNBC cell migration and invasiveness. Our finding that the increase in MDA-MB-231 migration and invasiveness is associated with increases in JAG1 is a new finding and provides mechanistic evidence to study the role of JAG1 as a pathway that mediates the cancer promoting effects of obesity on TNBC. Finding that MDA-MB-231 are more responsive to BMI than MDA-MB-436 provides a model to study mechanisms in TNBC cells that confer them responsive to peritumor ADS. Collectively, this study has provided new insight into the mechanistic links between obesity and TNBC cancer.

4. Methods

4.1 Cell Culture and Reagents:

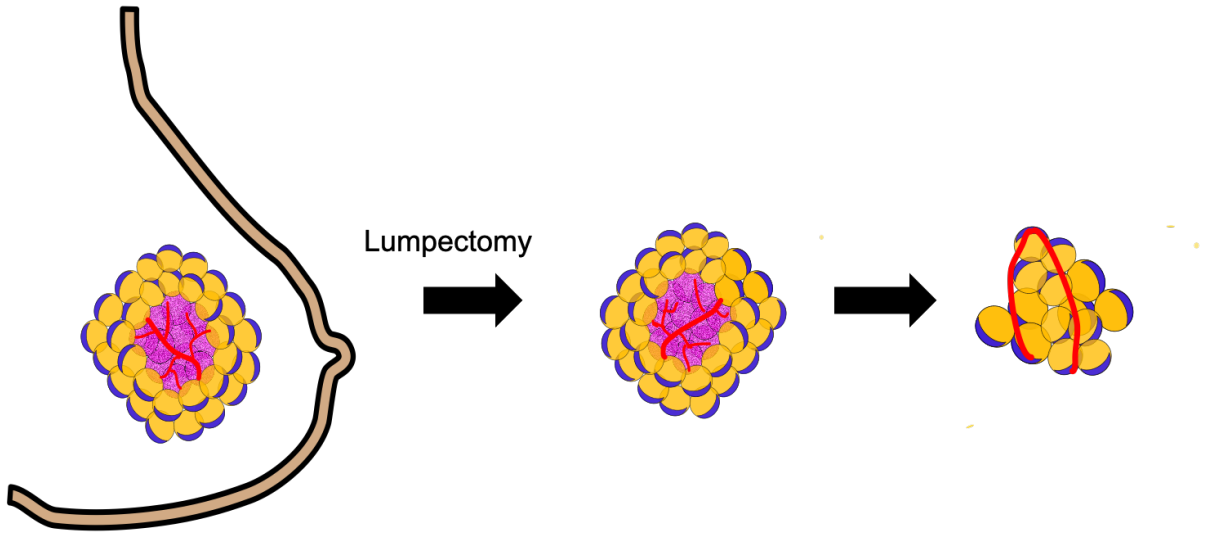
Triple negative breast cancer cells (MDA-MB-231, MDA-MB-436) were purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM/F12 media supplemented with 10% Fetal Bovine Serum (FBS) and Penicillin Streptomycin (P/S) antibiotics (P/S). Media, P/S, and FBS were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The incubator was set at 37 degrees Celsius with 5% CO2.

4.2 Breast Peritumor AT Derived Secretome:

De-identified peritumor breast AT patient samples were obtained from Edwards Comprehensive Cancer Center at Cabell Huntington hospital in Huntington, West Virginia. Peritumor breast AT (~ 100 mg) (figure 21) was gently cut into five equal pieces using sharp surgical scissors, and clean AT pieces (5 pieces/5 mL) were cultured for 24 hours at 37°C/5% C02. Media conditioned by peritumor AT was collected and briefly (~ 5 minutes) centrifuged to pellet tissue debris. Cutting, rinsing and tissue culture were done in serum free cell culture media (RPMI/F12). AT conditioned medium (termed the adipose derived secretome (ADS)) was diluted 1:10 in DMEM/F12/0.1% fetal bovine serum and applied to TNBC cells (figure 22). The 1:10 dilution dilutes potential harmful metabolites that might leach out from AT in cell culture. Patients with a BMI > 30 were categorized as obese, and those with a BMI < 30 were categorized as lean.

Figure 21

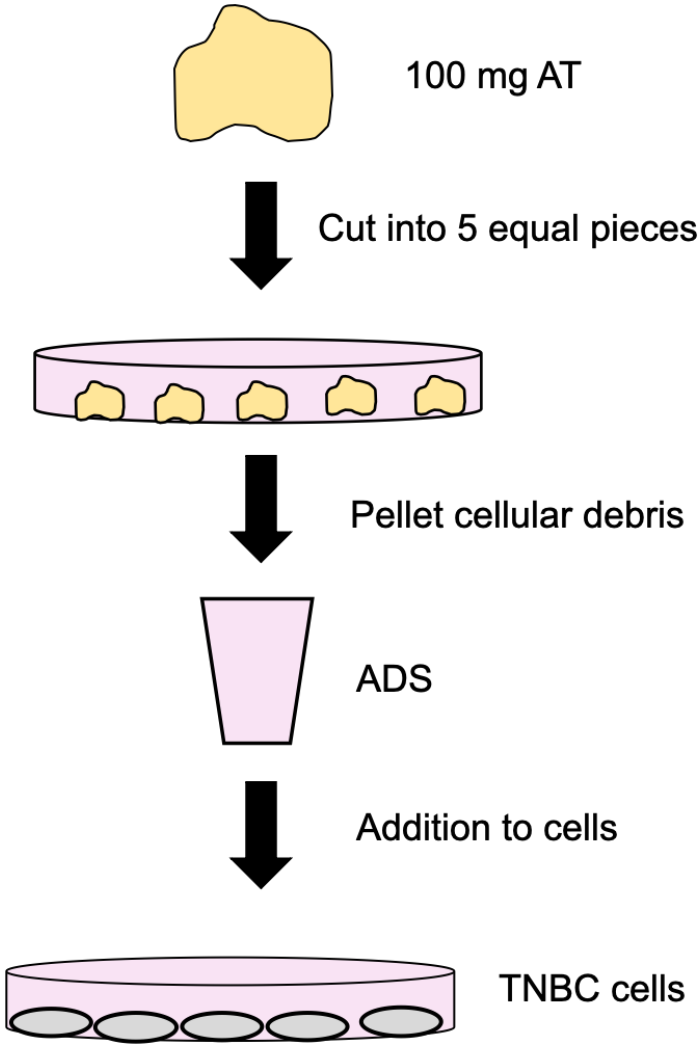
Patient Derived Peri-Tumor Adipose Tissue



Note. Patient with breast cancer undergoes lumpectomy. Tumor is removed along with surrounding adipose tissue about 5-10cm away. Tumor is removed. Result is ~100 mg of peri-tumor adipose tissue.

Figure 22

Formation of Adipose Derived Secretome



Note. Patient peritumor adipose tissue is rinsed and cut into 5 equally sized pieces. Adipose pieces cultured in phenol red free DMEM for 24 hours. After 24 hours media is collected in an Eppendorf tube and deemed adipose derived secretome (ADS). ADS at 10% is then added to cells.

4.3 Western Blot Analysis

TNBC cells (MDA-MB-231, MDA-MB-436) were plated in 60 mm dishes and allowed to reach confluency. Cells were treated with 10% ADS for 24 hours. Cells were lysed in 400 uL 1X Radio-Immunoprecipitation Assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (Cell Signaling Technology, Danvers, MA, USA). Cells were incubated with RIPA buffer for 5 minutes then transferred to microtubes. Cell lysates were sonicated for 15 seconds on ice. Cell lysates were boiled for 10 minutes in Lamelli buffer plus B-mercaptoethanol prior to SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated in primary antibody overnight (β-actin dilution 1:5000, NFKB 1:1000 dilution, P-NFKB 1:1000 dilution, PS6 dilution 1:1000, Total S6 dilution 1:1000, P-ERK 1:1000 dilution, Total ERK, 1:1000 dilution, Jagged-1 1:500 dilution). Membranes were washed three times at five minutes per wash in 1X Tris Buffered Saline 0.1% Tween 20 (TBST). Blots were incubated with secondary antibody (1:2000) for 2 hours at room temperature while rocking, and then rinsed three times with TBST (ten minutes per rinse). Proteins were detected using chemiluminescence (Bio-Rad Laboratories, Hercules, CA, USA). All proteins were normalized to loading control B-Actin. Bands were quantified using image lab software. Antibodies were purchased from cell signaling technologies (Danvers, MA, USA).

4.6 Migration Assay (Wound Assay)

MDA-MB-231 and MDA-MB-436 cells were plated in 60 mm dishes and allowed to reach 100% confluency. Once cells reached 100% confluency, cells were washed

with 1 mL of PBS. A 200 uL pipet tip was then used to create a cross section wound and cells were treated with ADS. Images of cells within the center of the cross section were taken at time 0 and 24 hours with a Leica Microscope. Area of the gap was calculated using image J program. Fold change was calculated from the area of the gap measured at time 0 and time 24 hours.

4.7 Invasion

Boyden Chamber from Millipore sigma were used (St. Louis, MO, USA) (CA #: ECM508). Fifty thousand cells were plated in the top chamber membrane in serum free media. DMEM/10% FBS supplemented with 10% ADS was placed in the lower chamber as the chemoattractant. After 24 hours, cells that migrated from upper chamber to the lower chamber through the Matrigel-based membrane insert were rinsed and stained following the Boyden Chamber Protocol provided in invasion kit.

4.9 Statistical Analysis

Two-tailed paired and unpaired t-tests were used to determine statistically significant differences between to two groups. One-way ANOVA was used when comparing more than two groups. Statistics were done using Graph-pad Prism.

5. Conclusions

This study shows that in women with breast cancer high (>30) BMI is associated with increased TNBC cell migration and invasiveness. This BMI effect is transduced through peritumor AT secreted factors. The TNBC cell response to BMI, however, is dependent on the genetic background of the TNBC cells. In our study, the TNBC cell line with a KRAS mutation and high expression levels of cytokine receptors was more responsive to BMI compared with the TNBC cell line with normal KRAS and lower

expression of cytokine receptors. Our results support the hypothesis that worsening TNBC progression in obesity is in part mediated by changes in peritumor breast AT secreted factors that act on TNBC cells to induce increased cancer cell migration and invasiveness. Further, this BMI effect could in part be mediated by increases in JAG1-NOTCH signaling in a subset of TNBC cells.

Acknowledgements:

We wish to acknowledge Dr. Piyali Dasgupta for allowing use of lab equipment.

Chapter 3

The Effect of High (>30) BMI Peritumor Breast ADS on mTOR Signaling in TNBC Cells

1. Introduction

The importance of high (>30) BMI peritumor breast adipose tissue on the tumor microenvironment was shown in Chapter 2. Our results followed the clinical pattern seen in obese patients, indicating the critical importance for continuation of this study. In this chapter, we aim to explore what factors are in the peritumor breast (ADS) and measure their effects on the stem cell marker CD44 and the mTOR pathway in TNBC cells.

CD44

Breast cancer stem cells (BCSC) are a small population within the breast tumor that have the ability to self-renew and proliferate. These cells are mainly responsible for tumor initiation, migration, drug resistance and recurrence (X. Zhang et al., 2020). Breast cancer stem cell markers are expressed on the cell surface of BCSC. One of the key BCSC markers for TNBC cancer is the stem cell marker CD44. CD44 is a transmembrane protein commonly expressed on the surface of cancer stem cells. Hyaluronan, CD44 ligand, binds to CD44 and stimulates cell proliferation, cell survival, and cell motility (Chen et al., 2018). Chekhun et al examined 132 patients with breast cancer of different molecular subtypes (Chekhun et al., 2015). They found CD44 has been correlated with metastatic lymph node involvement as well as basal like status

(TNBC). CD44 is responsible for cell-to-cell communication and has been shown to activate the mTOR pathway. A study recently showed that anti-CD44 antibodies inhibited mTOR signaling, indicating a role of CD44 in the stimulation of mTOR (Gadhoum et al., 2016).

mTOR

Mammalian target of rapamycin (mTOR) is a cytoplasmic protein kinase that plays a large role in cell growth, cell survival, and metabolism (Hua et al., 2019). This role is played across multiple organs and cells within the body. MTOR genetic mutations are found in 60% of tumors that have increased activity of this pathway (Engelman, 2009). In TNBC, mTOR has also been shown to contribute to resistance to drug therapy (Khan et al., 2019). The activity of mTOR is stimulated by growth factors, amino acids and glucose (Guerrero-Zotano et al., 2016). Obesity is considered a nutrient overload state, however, the direct connection between obesity and increases in mTOR signaling in TNBC has not been published. With this knowledge, mTOR inhibitors such as everolimus and sirolimus, have been used clinically to treat estrogen receptor positive (ER+) breast cancer in conjunction with endocrine therapy (Schmid et al., 2019). Unfortunately, mTOR inhibitors have significant side effects which limit their use for cancer therapy. The adverse side effects of mTOR inhibitors include cytopenia, stomatitis, interstitial lung disease, lymphocele, dyslipidemia, and poor wound healing (Chang & Barbas, 2021). Dyslipidemia can serve as a deadly side-effect as those who are obese are at a higher risk of cardiovascular events, adding additional dyslipidemia from mTOR inhibitors, this risk is increased even more, potentially leading to heart attack and death. The cause of these side effects is not fully known but could be due to

the prevention of clonal T cell expansion by mTOR inhibitors (Millan et al., 2006). If we discover factors within ADS that are responsible for the increased mTOR signaling in TNBC, we can target these factors individually, and thus prevent the adverse side effects seen with mTOR inhibitors. Based on previous literature, we hypothesized that the key factors known to be increased in obesity; IL-6, Leptin, and extracellular vesicles, may play a role in stimulating the activity of the mTOR pathway in TNBC cells.

Interleukin 6

IL-6 is primarily linked to auto-immune disease, multiple sclerosis, and rheumatoid arthritis. This pro-inflammatory cytokine is also known to induce the formation of Th17 cells (Kimura & Kishimoto, 2010). Naïve CD4+ T cells differentiate to Th17 cells due to IL-6 signaling through STAT3 (Harbour et al., 2020). Th17 or T helper 17 cells assists in host protection against microbes and aids B cells in the production of antibodies (Mitsdoerffer et al., 2010; Tesmer et al., 2008). While these functions are the most common, Th17 is becoming a larger player in the tumor environment. Th17 cells have been found to have the highest density within the tumor (Bailey et al., 2014), however their function within the tumor is unknown. With this knowledge, it can be hypothesized that increased IL-6 plays a large role in the recruitment of these cells to the tumor, demonstrating its role in cancer. In addition, IL-6 is becoming more known for its role in obesity. In circulation, about 15-35% of IL-6 is produced by AT (Mohamed-Ali et al., 1997). Obese patients are in a constant state of low-grade inflammation (Wang & He, 2018), as a result, serum levels of IL-6 are increased in obese patients as compared to lean (7.69 +/- 5.06 pg/mL in obesity vs 1.28 +/- 0.85 pg/mL in lean) (Roytblat et al., 2000). Obese AT hypertrophies in response to hypoxic conditions.

These hypoxic conditions are due to the growth of the adipocytes being faster than the angiogenesis (Eder et al., 2009). As a result genes encoding IL-6 are upregulated (Eder et al., 2009). A previous study showed that Chinese women with increased levels of IL-6 correlated with breast cancer stage as well as lymph node involvement (Ma et al., 2017). In this study, 110 women with various types of ductal breast cancer (HER2+, ER+, PR+) and 30 healthy women's serum were examined for levels of IL-6, IL-8, and TNF alpha. They found that these inflammatory cytokines were correlated with increased staging as well as lymph node metastasis. The limitation of this study is that they only included estrogen receptor positive, progesterone receptor positive, or HER2 + breast cancer, TNBC was not included. This study prompted us to investigate the effects of IL-6 on mTOR signaling in TNBC cancer cells.

Leptin

Leptin is a hormone encoded by the LEP gene. Leptin is elevated in obesity and is secreted by adipose tissue and breast cancer cells. Recent reports have shown that leptin leads to chemoresistance and poor survival of patients with estrogen-receptor-negative breast cancer (Lipsey et al., 2020). In this study, authors treated TNBC cells with chemotherapy drugs such as paclitaxel or cisplatin. When co-treated with leptin, TNBC cells had higher survival as compared to TNBC cells treated with chemotherapy drugs alone (Lipsey et al., 2020). This study also found that increased expression of leptin target genes such as CDK8 and NANOG are correlated with lower survival in ER- breast cancer patients. This did not hold true for ER+ patients (Lipsey et al., 2020). This suggests that leptin plays a different role in ER- breast cancer as compared to ER+ breast cancer. In addition, leptin has been proposed to be an important biomarker

associated with lymph node involvement, and recurrence rate (Khabaz et al., 2017; Sanchez-Jimenez et al., 2019). Cytoplasmic immunohistochemical staining of patient samples with breast fibroadenoma or normal breast tissue indicated that leptin staining was correlated to tumor grade, stage, and lymph node involvement (Khabaz et al., 2017). Leptin has been shown to be an important growth factor for breast cancer cells that induces signaling that increases the proliferation of luminal ER+ breast cancer cells. This was seen in MCF7 xenografts, leptin activated p53/FOXO3A (Nepal et al., 2015). Cancer cells are known to have activated autophagy. Inactivation of tumor suppressor genes increase autophagy, thus promoting tumor survival (Mathew et al., 2007). P53/FOXO3A is an autophagic pathway that is key for tumor growth both in vivo and in vitro. In addition to promoting tumor survival via autophagy, leptin has also been shown to inhibit apoptosis of breast cancer cells (Nepal et al., 2015; Sanchez-Jimenez et al., 2019). Another important determinant of the prognosis of breast cancer is the number of cancer stem cells (CSC) as well as the ability to undergo epithelial mesenchymal transition (EMT). Bowers et al explored the effects of diet induced obesity and leptin on breast tumor formation. In this study MMTV-Wnt-1 transgenic mice (known to form spontaneous tumors that mimic basal like breast cancer) were fed a high fat diet to induce obesity. These mice demonstrated poor survival, increased KI67 proliferation marker, and higher leptin levels as compared to lean mice. This study demonstrated that diet induced obese mice had increased leptin signaling, leading to increased tumor CSC and EMT gene signature (Bowers et al., 2018), indicating leptin plays an important role in the pathogenesis of breast cancer.

Microparticles

Microparticles or “nanoparticles” are secreted from almost every cell type in the body. These lipid bilayer enclosed particles are thought to be involved in cell-to-cell communication (Crewe et al., 2018). A previous study demonstrated that adipose tissue plays a large role in communicating metabolic health to distant tissues (Kusminski et al., 2016). In an update to this paper, exosomes were found to not only participate in cell-to-cell communication but also play a role in endocrine signaling, by regulating gene expression in distant tissues (Thomou et al., 2017). In obesity, the function of adipose tissue is dysregulated, leading to changes in the levels and types of adipokines that are released by adipose tissue (Unamuno et al., 2018). While changes in the levels and types of adipokines and cytokines that are released by adipose tissue in obesity has been published, how obesity alters the levels and types of microparticles released by adipose tissue is a new field of study.

In this chapter, we hypothesized that in obesity, the levels of leptin, IL-6, and extracellular vesicles that are released from peritumor breast adipose tissue act on TNBC cells to stimulate increases in mTOR signaling. We propose that identifying which specific peritumor breast adipose factor(s) induce mTOR signaling in TNBC cells will foster new targets or repurpose existing therapies to treat TNBC in obesity.

2. Methods

2.1 TNBC Cell Lines

MDA-MB-231 and MDA-MB-436 TNBC cell lines were purchased from ATCC and cultured in DMEM/F12 media supplemented with 10 % FBS and 5% penicillin streptomycin.

2.1 Peritumor Breast Adipose Tissue Secretome (ADS) Model

Methods to isolate peritumor breast adipose tissue was stated in Chapter 2 (4.2 Breast peri-tumor AT derived secretome, pg. 47).

2.2 Treatment of TNBC Cells With Peritumor ADS

ADS was added to MDA-MB-231 or MDA-MB-436 TNBC cells were 100% confluent. ADS was diluted 1:10 in serum free DMEM/F12 with 5% penicillin streptomycin and applied to TNBC cells for 24 hours. These methods were performed as stated in Chapter 2 (section 4.2 Breast peritumor AT derived secretome, pg. 47).

2.2 Adipokine Array

Peritumor breast ADS (isolation described above—section 2.1 peritumor breast adipose tissue secretions, pg. 57) was examined for changes in the levels of growth factors and cytokines that are associated with obesity using a human adipokine array purchased from R&D systems (Minneapolis, MN, USA).

2.3 Migration assay

Migration assay was conducted as stated in Chapter 2 (4.6 Migration Assay, pg 48.) Changes in cell migration was quantified using image J software.

2.4 IL6 Treatment

IL-6 was purchased from Cell Signaling Technologies (Danvers, MA). IL-6 was added at a concentration of 1ng/mL or 10ng/mL to MDA-MB-231 or MDA-MB-436 cancer cells. We used the concentration of 1ng/mL or 10ng/mL as it has been shown in literature that this dose is enough to induce a significant increase in MDA-MB-231 cell rolling, a key process in metastasis of cancer cells (Geng et al., 2013).

2.5 Leptin ELISA

Human Leptin ELISA was purchased from Invitrogen (Waltham, MA, USA) (catalog number: KAC2281). We followed ELISA kit protocols that were provided with the kit. Optical density was measured at 450 nm and concentration was calculated using graph pad prism interpolation software.

2.6 Leptin Treatment

Human recombinant leptin was purchased from R&D systems (CA: 398-LP-01M) (Minneapolis, MN, USA). Cells were plated to 100% confluency then treated for 24 hours in serum free media at the following concentrations: 30pg/mL, 70 pg/mL.

2.7 IL6 and Leptin Synergy

IL-6 and leptin were applied to MDA-MB-231 or MDA-MB-426 cancer cells at 1 pg/mL (IL-6) and 30 ng/mL (Leptin) alone, and then 1pg/mL and 30ng/mL together.

2.8 Western Blot Analysis

TNBC cells (MDA-MB-231, MDA-MB-436) were plated in 60 mm dishes and allowed to reach confluency. Cells were treated with IL-6, Leptin, or IL-6 and Leptin for 24 hours. After 24-hours cells were washed with 1mL of PBS. Cells were lysed in 400 uL of 1X RIPA buffer with protease and phosphatase inhibitors. Cells were incubated with RIPA for 5 minutes. Cell lysates were then sonicated on ice for 15 seconds. Prior to running the western blot, cell lysates were boiled for 10 minutes in Lamelli buffer plus B-mercaptoethanol. Western blots were performed using SDS/Page and polyvinylidene difluoride membranes. Membranes were incubated in primary antibody overnight (Beta-Actin dilution 1:5000, PS6 dilution 1:5000, Total S6 dilution 1:5000, PNDRG1 1:2000, NDRG1 1:2000, CD44 1:2000). Proteins were detected using chemiluminescence (Bio-Rad Laboratories, Hercules, CA, USA). Phosphorylated proteins were normalized to

total proteins. Quantification was conducted with image lab software. Antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA).

2.9 Microparticle Isolation

One milliliter of peritumor breast ADS was spun for 2,000 X G for 15 minutes. Supernatant from this spin was taken and centrifuged at 20,000 X G for 60 minutes. This supernatant was taken for ADS without microparticles. The invisible pellet containing microparticles was re-suspended in filtered PBS. The presence of microparticles was ensured by NTA analysis.

2.10 Treatment of TNBC Cells With Microparticles

MDA-MB-231 cells were plated in 60 mm dishes and allowed to reach confluency. ADS containing and lacking microparticles was diluted 1:10 in serum free cell culture media and applied to MDA-MB-231 cells for 24 hours. Cells were then lysed in RIPA lysis buffer supplemented with protease and phosphatase inhibitors for western blot analysis.

2.11 Statistical Tests

Statistics were run in Graph-Pad prism. Student's paired T-test were used to compare differences between two groups, and ANOVA followed by post hoc analysis was used to compared differences between more than two groups.

3. Results

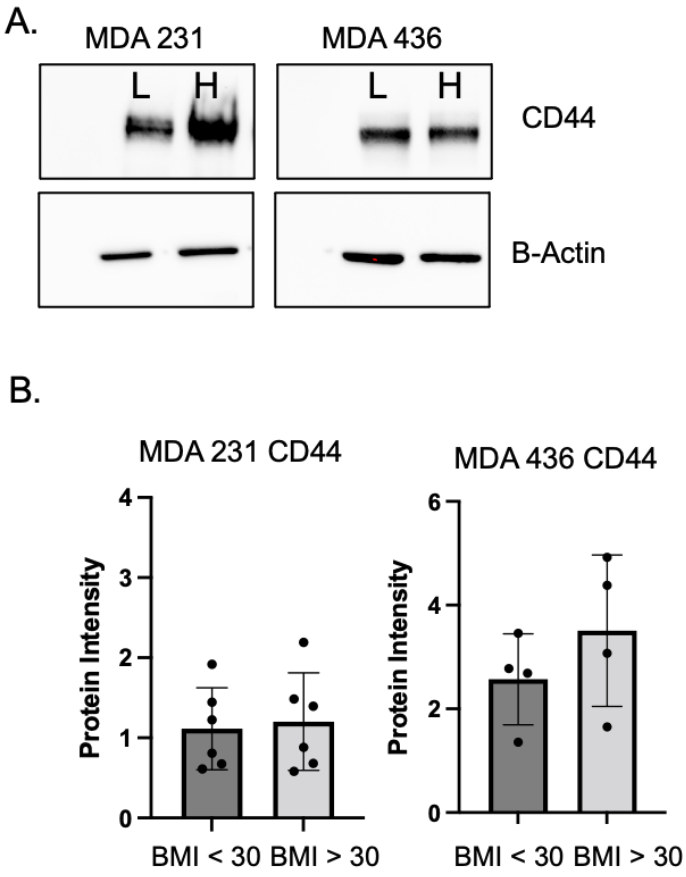
3.1 Role of Peritumor Breast ADS on Breast Cancer Stem Cell Marker CD44

The breast cancer stem cell marker CD44 has been linked to poor prognosis of patients with breast cancer (Wang et al., 2017). We sought to test the effect of peritumor breast ADS on CD44 levels in TNBC cell lines (figure 23). Our results show

there is no difference in CD44 levels in TNBC cells treated with lean (BMI < 30) compared with obese (BMI > 30) ADS (P-value =0.79) (N=6) (P-value =0.31) (N=4)

Figure 23

Effect of Obese ADS on Levels of Breast Cancer Stem Cell Marker CD44



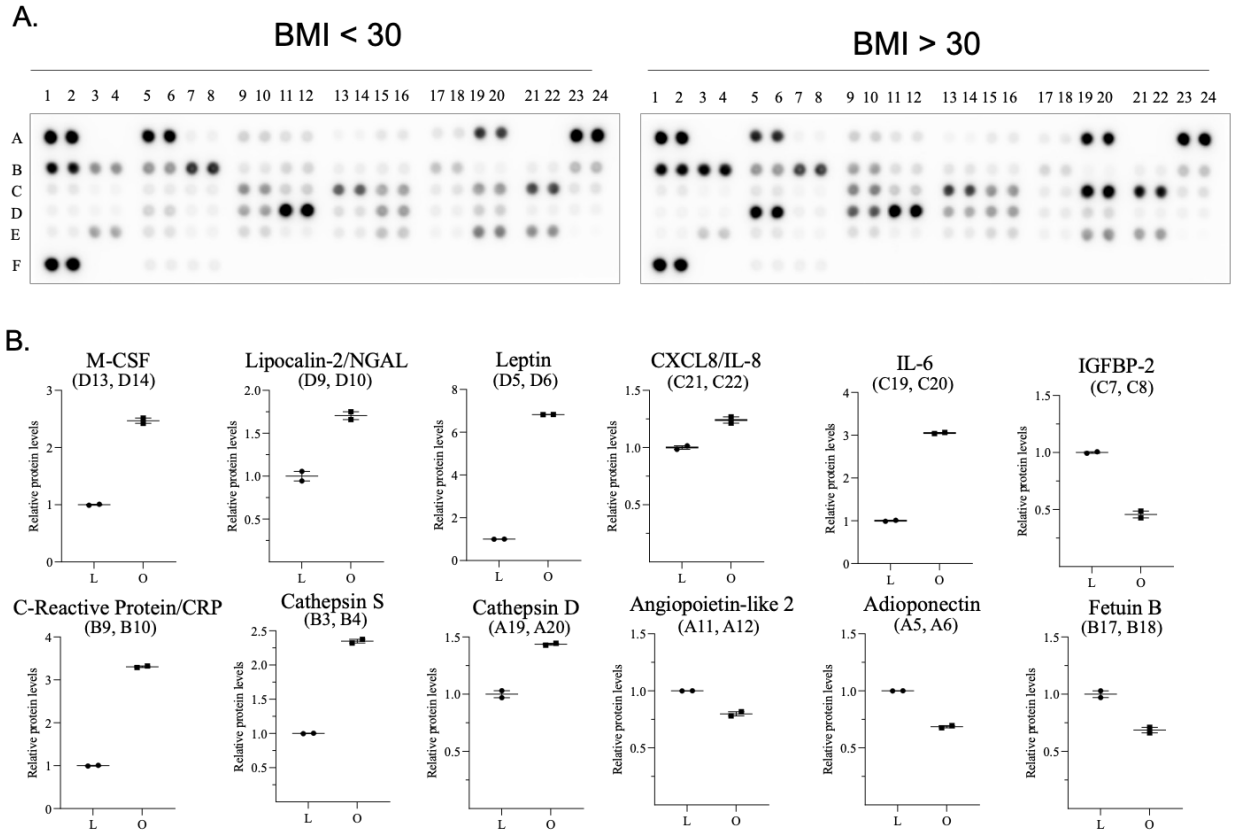
Note. A) Representative western blot of MDA-MB-231 or MDA-MB-436 cells being treated with Lean or Obese ADS. B) Quantification of western blot analysis (N=6) (P-value =0.79) (N=4) (P-value =0.31).

3.2 Contents of Peritumor Breast ADS

We used an adipokine array to determine the levels of obesity-associated cytokines and growth factors in peritumor breast ADS (figure 2). The results showed that there were multiple differences between low (BMI < 30) and high (BMI > 30) peritumor breast ADS. Of interest was that higher levels of Capthesin D, a growth factor, was associated with high (> 30) BMI (figure 25). This growth factor degrades the extracellular matrix and therefore higher levels of Capthesin D could in part be the mechanism by which peritumor breast ADS from women with BMI's is a strong inducer of TNBC cell invasiveness (chapter 2, figure 9). Regarding cytokines, the results showed that the levels of IL-6 and leptin were higher in peritumor breast ADS from high (> 30) compare with low (< 30) BMI (figure 24). This finding indicates that BMI induces obesity associated changes in peritumor breast ADS.

Figure 24

Differences Between Lean Adipose Derived Secretome and Obese Adipose Derived Secretome



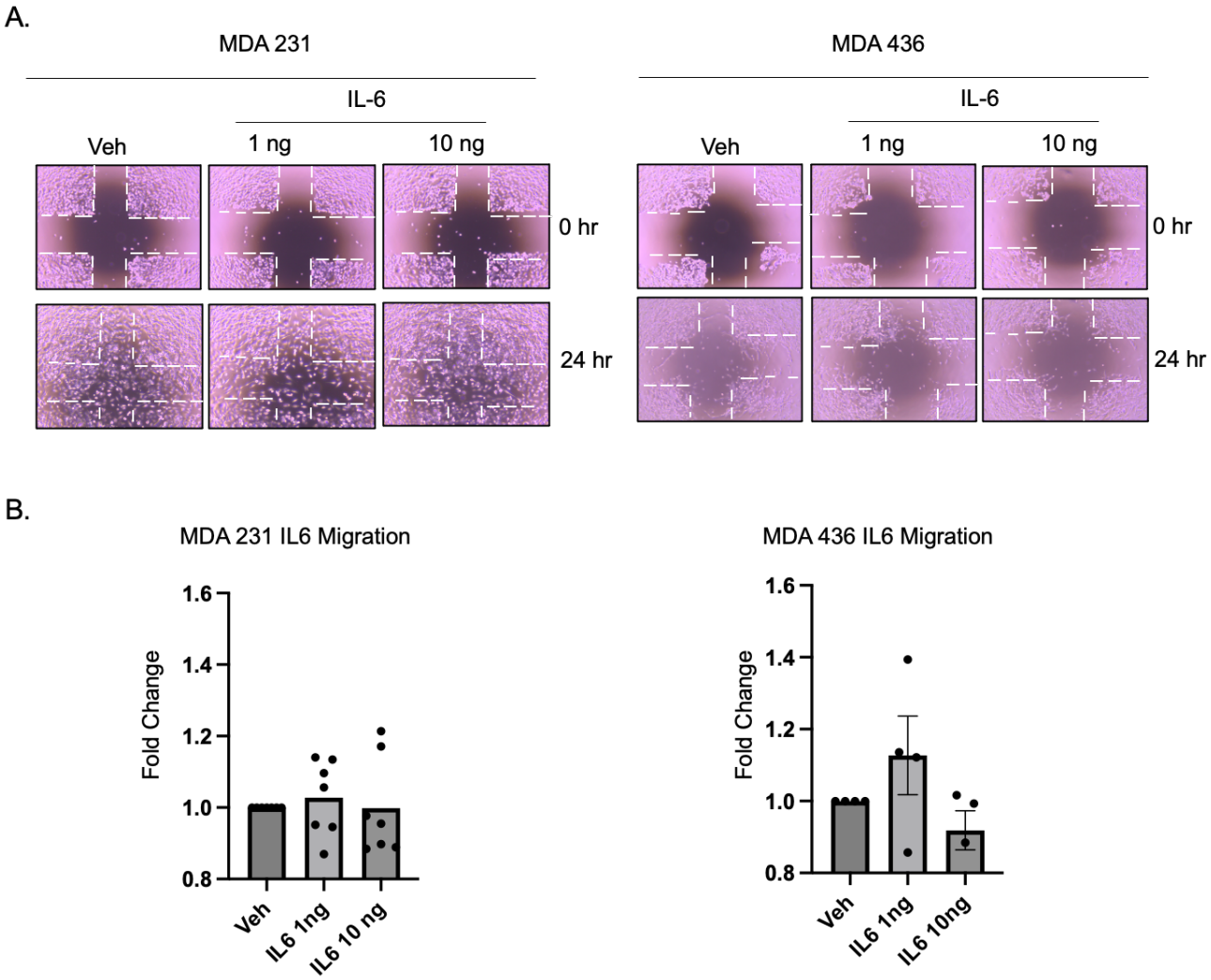
Note. A) Representative images of the adipokine blots. The levels of 58 proteins were assessed. B) Relative protein levels of the proteins that demonstrated different levels between lean ADS and Obese ADS (N=2).

3.3 The Effect of IL-6 on the Migration of TNBC Cells

Given that increased BMI increased the levels of IL-6 in peritumor breast ADS, we assayed the effect of recombinant human IL-6 on the migration of TNBC cells. The results showed that IL-6 at a concentration of 1 ng/mL or 10 ng/mL, had no effect on the migration of MDA-MB-231 or MDA-MB-436 cells at 24 hours post treatment (figure 25).

Figure 25

Inflammatory Cytokine IL6 role in Migration of TNBC Cells



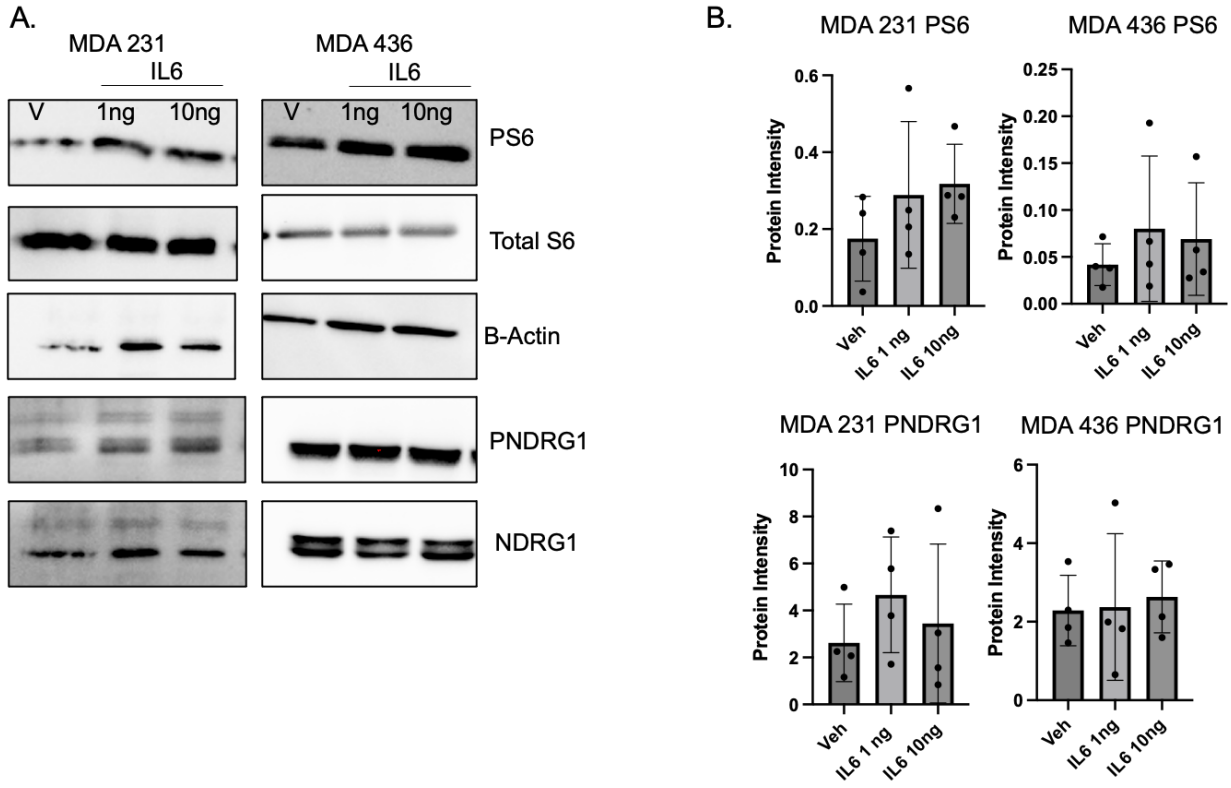
Note. A) Representative images of scratch assay in MDA-MB-231 or MDA-MB-436 cells at time 0 hr and 24 hr after treatment. B) Quantification of gap closure represented as fold change. (P-value = 0.82) (P-value = 0.16)

3.4 IL-6 Effect on mTOR in TNBC

Next, we sought to determine if IL-6 acts on TNBC cells to induce mTOR signaling. The results showed that IL-6 at 1 ng/mL and 10 ng/mL induced a trend towards an increase in mTOR complex 1 (P-value = 0.35) (P-value = 0.63) and mTOR complex 2 (P-value = 0.55) (P-value = 0.92) signaling, as measured by the phosphorylation of S6 (Ser 235/236) and the phosphorylation NDRG1 (Thr 346), respectively (figure 26). The results however were not significant (figure 27). The stem cell marker CD44 was significantly increased in MDA-MB-231, but not MDA-MB-436, in response to IL-6 1 ng/mL treatment for 24-hour treatment) (figure 27). Collectively, these findings indicate that IL-6 at 1 ng/mL and 10 ng/mL do not stimulate mTOR activity in MDA-MB-231 and MDA-MB-436 cells (figure 26). Of interest is that IL-6 at 1ng/mL did stimulate a significant increase in the expression of the breast cancer stem cell marker, CD44, in MDA-MB-231 cells.

Figure 26

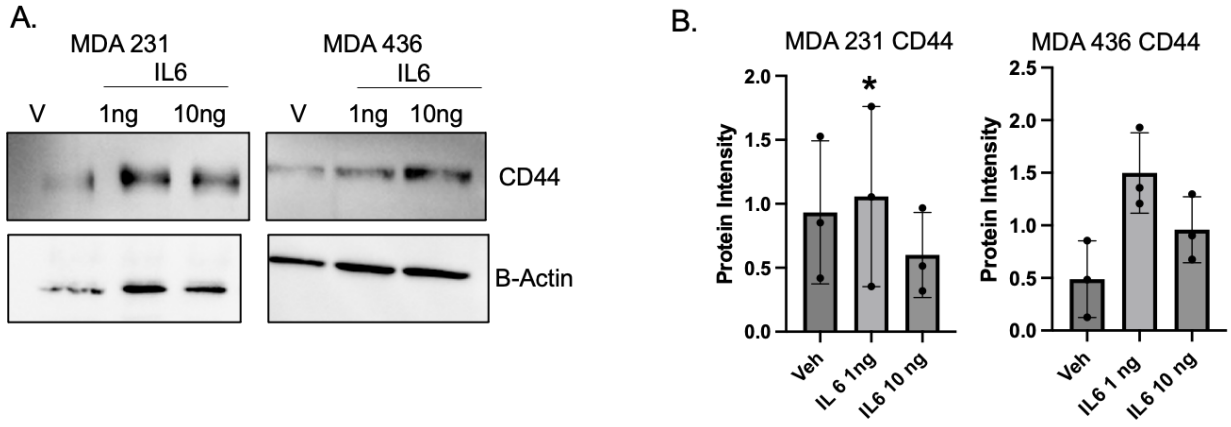
Role of IL6 on mTOR Signaling in TNBC Cells



Note. A) Representative western blot images of MDA-MB-231 or MDA-MB-436 cells treated with IL6. B) Quantification of western blot analysis using protein intensity. (N=4) (N=3) (N=4)

Figure 27

Role of IL6 on CD44 Levels in TNBC Cells



Note. A) Representative western blot images of MDA-MB-231 or MDA-MB-436 cells treated with IL6. B) Quantification of western blot analysis using protein intensity. (N=3)

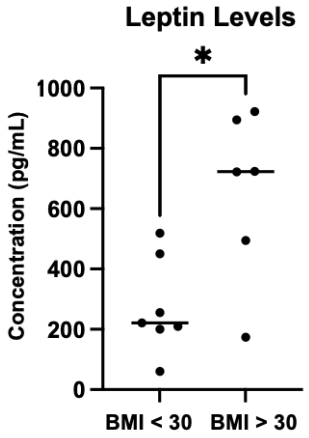
3.5 The Levels of Leptin in Peritumor Breast ADS

Next, we sought to determine the specific concentration of leptin in peritumor breast ADS to investigate a potential correlation between the cytokine and increased BMI. The results show higher concentrations of leptin in the high BMI (> 30) compared with low BMI (< 30) peritumor ADS (Figure 29). The concentration in high BMI samples was double the amount in lean BMI samples (655 pg/mL compared to 274 pg/mL, respectively) (figure 28).

Figure 28

Leptin Levels are Higher in Obese Peritumor Fat Compared to Lean

A.



B.

Treatment	Mean	Stnd Dev
Lean ADS	273.749 pg/mL	+/- 157.815
Obese ADS	655.029 pg/mL	+/- 281.009

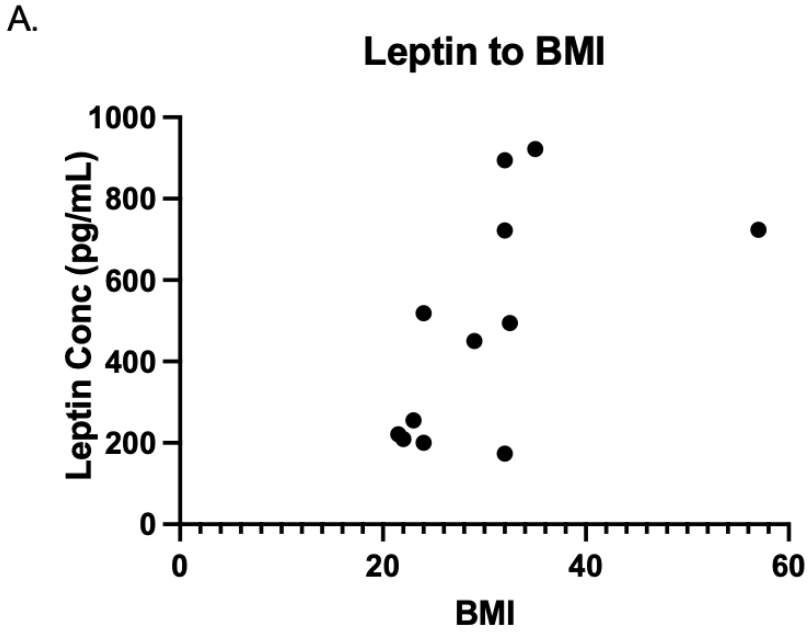
Note. A). Graphical representation of Leptin values measured in patient peritumor samples with a BMI > 30 (OADS) and a BMI < 30 (LADS). Concentration measured in picograms per milliliter.

3.6 Leptin Correlation to BMI

With the knowledge that leptin is increased in obesity (figure 29), we wanted to demonstrate a correlation between BMI and leptin levels. To do this, we plotted leptin concentration against the corresponding patient BMI. Despite leptin being significantly increased in BMI >30 (Figure 28) there is no correlation between leptin levels and individual BMI (Pearson R = 0.5726) (R^2 = 0.3279) (P-value = 0.3279) (figure 29).

Figure 29

Leptin Correlation to BMI



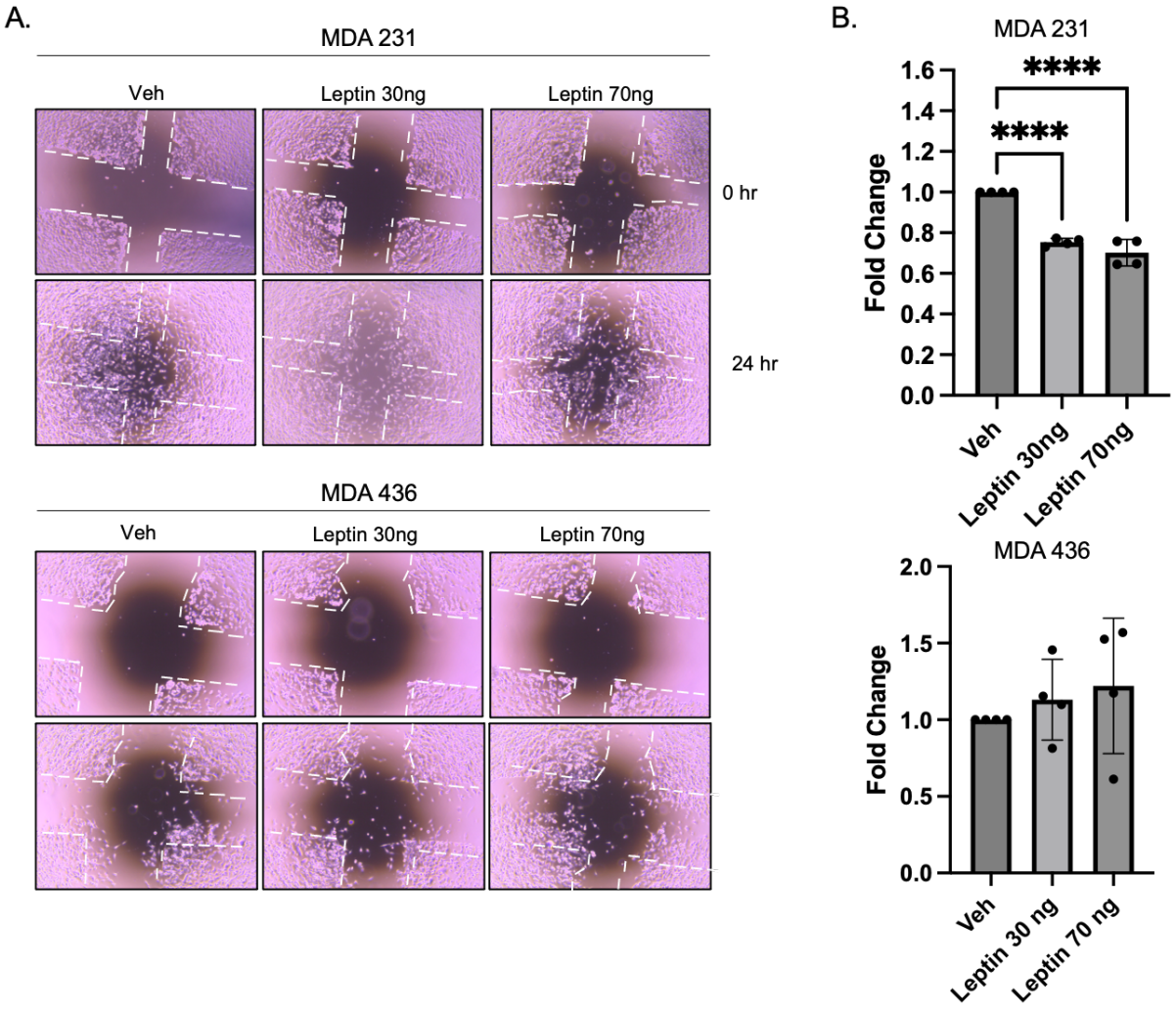
Note. A). Leptin levels as measure by ELISA correlated to their corresponding BMIs. (Pearson R = 0.5726) (R^2 = 0.3279) (P-value = 0.3279)

3.7 Effect of Leptin on Migration and Invasion of TNBC Cells

Next, we tested whether leptin was sufficient to induce the migration of TNBC cells. The results showed that leptin applied to cells at 30 and 70 ng/mL was not sufficient to induce the migration of MDA-MB-231 or MDA-MB-436 cells (figure 30). These doses were chosen based on the range of serum leptin levels in obese patients (Kazmi et al., 2013).

Figure 30

Effect of Leptin on Migration in TNBC Cells



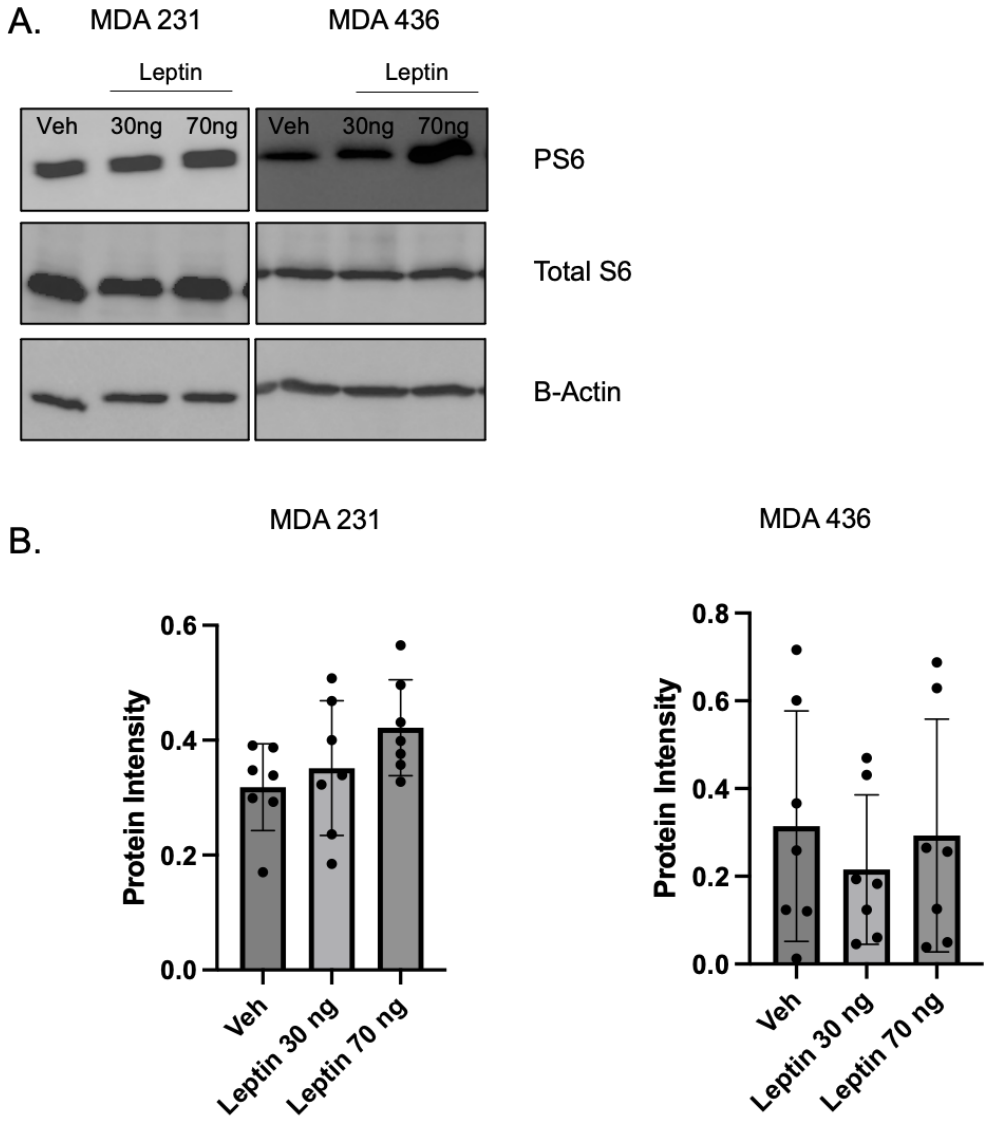
Note. A) Representative images (N=4) of MDA 231/436 wound healing assay treated Leptin 30ng or Leptin 70ng. Images taken at 0 hours and 24 hours. B) Fold change based on the closure of the wound (P-value = 0.0001 and 0.59 respectively).

3.8 Leptin Signaling in TNBC Cells

Considering that leptin has been shown to induce mTOR signaling in CRC and endometrial cancer (Carino et al., 2008; Wang et al., 2012), we sought to determine if the cytokine could induce mTOR signaling in TNBC cells. The results showed that leptin at 30 ng/mL or 70 ng/mL did not significantly increase mTOR signaling in TNBC cells as determined by no change in the levels of the mTOR complex 1 readout, phospho-S6 (Ser235/236) when analyzed by Western blot experiments (figure 31).

Figure 31

Effect of Leptin on the Phosphorylation of Ribosomal Protein S6



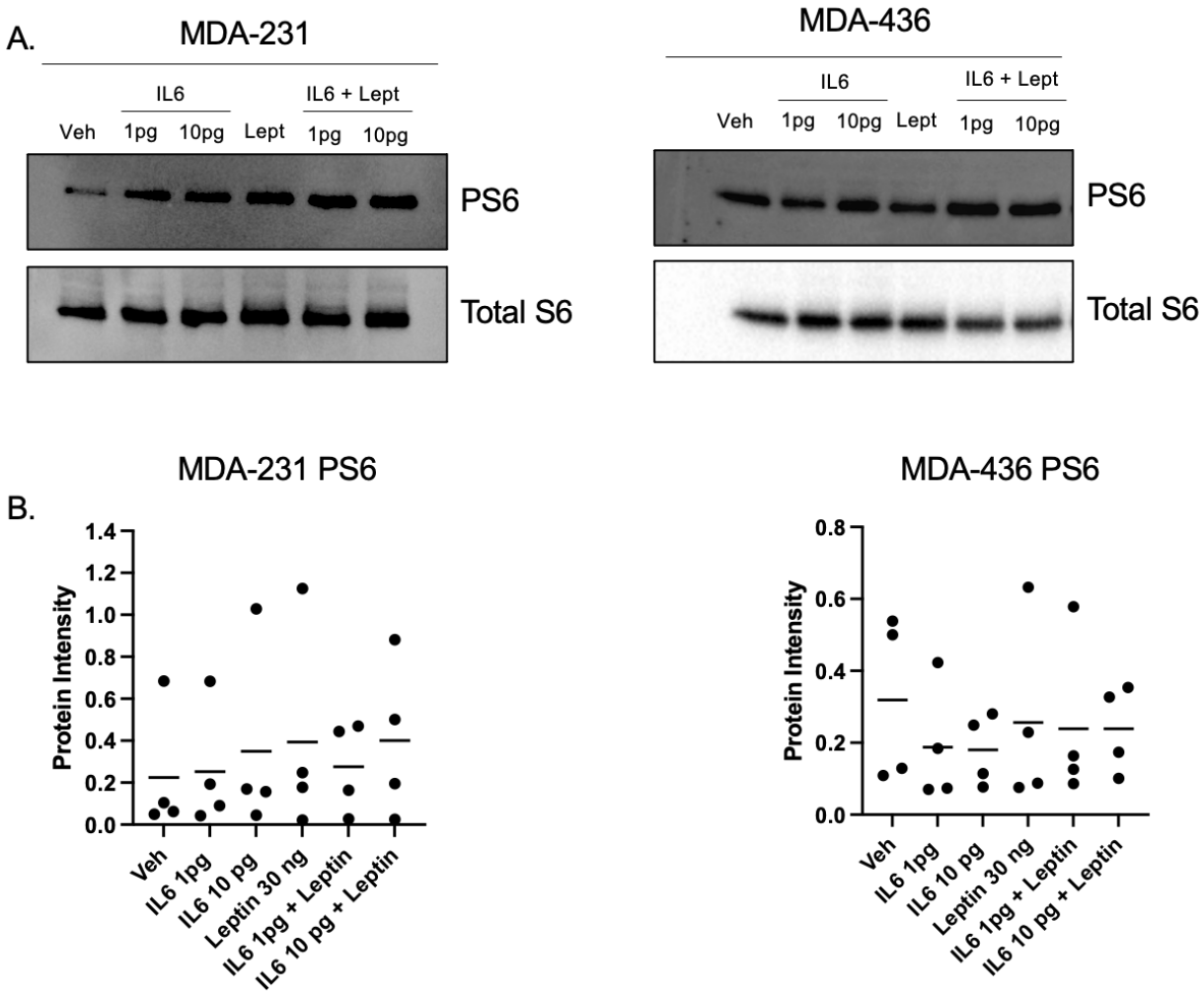
Note. A) Representative western blot images of MDA-MB-231 or MDA-MB 436 cells treated with leptin at a concentration of 30 ng or 70 ng. B) Quantification of western blots (N = 7) (P-value = 0.46) (P-value = 0.54)

3.9 IL6 and Leptin Synergy

Next, we cotreated TNBC cells with leptin dose at obese plasma conditions (30 ng/mL) (Kazmi et al., 2013) and IL-6 dose at obese plasma conditions (1 pg/mL or 10 pg/mL) (Roytblat et al., 2000) and assayed mTOR signaling in TNBC cells. The results showed that co-treatment with IL-6 and leptin did not significantly increase mTORC1 signaling in TNBC cells (figure 32).

Figure 32

IL6 + Leptin Synergy



Note. A) Representative western blot images of MDA-MB-231 or MDA-MB-436 cells treated with IL6. B) Quantification of IL6 and Leptin treatment in TNBC cell lines. (P-value = 0.97) (P-value = 0.92)

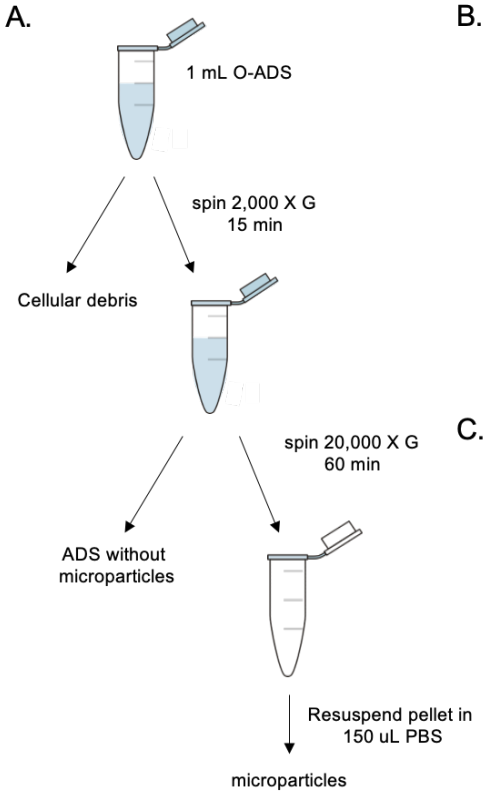
3.10 Concentration of Microparticles within ADS

Considering the finding that two major obesity-associated cytokines (leptin and IL-6) did not induce mTORC1 signaling in TNBC cells, we questioned if AT-secreted microparticles induce mTOR. Due to the hypertrophy of obese adipose tissue, we hypothesized that there would be a larger release of microparticles in obese AT as compared to lean AT. We found that obese ADS had roughly two times as many microparticles per milliliter (4.8475×10^7 particles/mL vs 2.01×10^7 particles per mL) as compared to their lean counterpart (figure 33). Based on this finding, we then sought to explore the effects of microparticles on signaling in our system.

Figure 33

Microparticle Quantity in O-ADS vs L-ADS

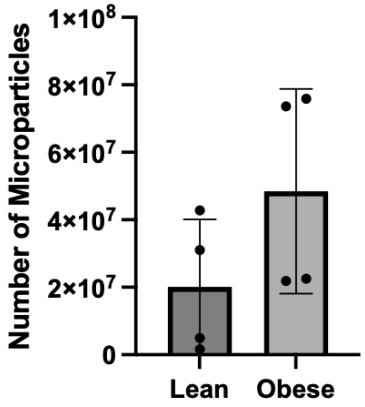
A.



B.

BMI	Average Microparticle Conc. [particles/mL]
BMI > 30	48,475,000
BMI < 30	20,102,500

C.



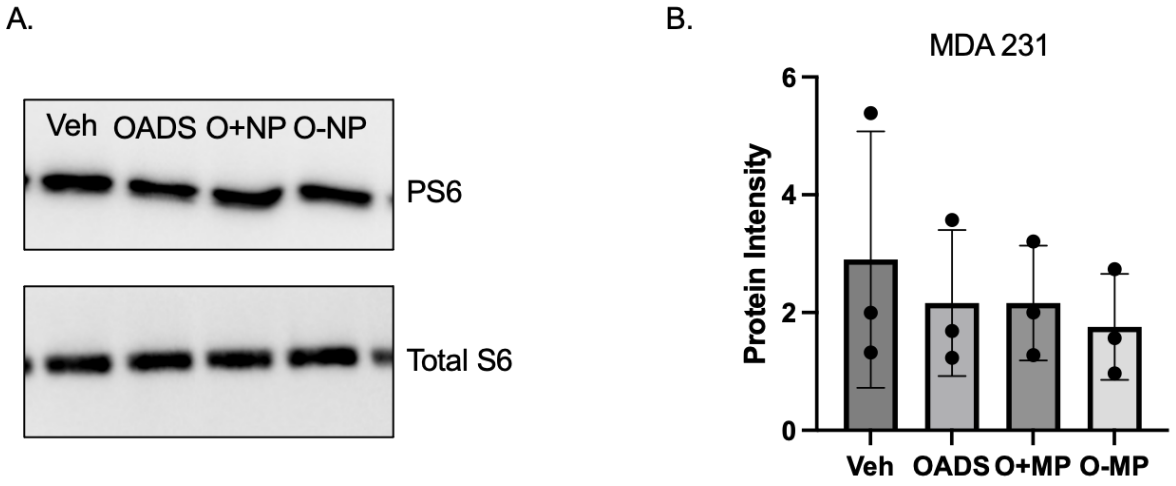
Note. A) Schematic of isolation of microparticles B) Average concentration (particles/mL) of microparticles from four separate patients with BMI > 30 and BMI < 30. C) Graphical representation of the number of microparticles in each individual sample.

3.11 Role of Microparticles on mTOR Signaling in TNBC Cells

Adipose tissue-derived microparticles derived from subcutaneous fat promote the progression of melanoma (Lazar et al., 2016). We therefore assessed the role of microparticles derived from peritumor breast ADS on mTOR signaling in TNBC cells. The results showed no difference in mTORC1 activation in MDA-MB-231 cells in response to microparticles obtained from peritumor breast ADS compared to MDA-MB-231 cells treated with vehicle (baseline mTOR complex 1 activity) (figure 34).

Figure 34

Effect of Microparticles on mTOR Signaling in Obese Patients.



Note. A) Representative western blot in MDA-231 treated with vehicle, OADS, OADS microparticles, OADS without microparticles. B) Quantitative analysis of western blot. (P-value = 0.79)

4. Discussion

This study extends our prior study in this dissertation (chapter 2) showing that BMI regulates TNBC cell migration, invasiveness and signaling by modifying peritumor breast ADS. Specifically, the findings in chapter 2 showed that high BMI (>30) is stronger inducer of TNBC cell migration, invasiveness and JAG1 signaling than low BMI (< 30). In this study, we hypothesized that the two major obesity-associated cytokines, leptin, and IL-6, would act on TNBC cells to stimulate mTORC1 signaling and that this would be associated with an increase in cancer cell migration and invasiveness. Our results, however, do not support this hypothesis. The findings showed that although leptin and IL-6 are higher in high (> 30) BMI samples (figure 24), these cytokines alone and together are not sufficient to induce mTOR complex signaling in TNBC cells (MDA-MB-231 and MDA-MB-436) (figure 27, 31, 32). Further, neither IL-6 nor leptin were sufficient to stimulate the migration of TNBC cells (figure 25, 30). We also tested the role of microparticles. The results showed that the concentration of microparticles was 2-fold higher in the high (> 30) compared to the low (< 30) BMI samples. The direct application of purified microparticles obtained from high (> 30) BMI samples however did not induce mTORC1 signaling in TNBC cells (figure 35).

This study did provide a new insight into IL-6 effects in TNBC cells that could have implications regarding the mechanism by which obesity promotes the aggressiveness of TNBC. The finding that IL-6 is sufficient to increase the levels of CD44 in TNBC cells would be anticipated to promote epithelial mesenchymal transition (EMT) and cancer stemness (Xu et al., 2015). Published data showing that CD44 promotes the metastasis of breast cancer and is a poor prognostic marker for breast cancer supports our

hypothesis that IL-6 plays a role in TNBC progression (X. Liu et al., 2019). The finding that high BMI (>30) peritumor breast ADS did not increase the level of CD44 in TNBC cells however does not support our hypothesis that induction of CD44 is the mechanism by which obesity promotes TNBC cell progression (figure 23). It is possible the concentrations of IL-6 in peritumor ADS are too low to induce CD44 in MDA-MB-231 cells that express relatively high basal levels of CD44 (figure 26).

Our next step was to determine if IL-6 and Leptin, which are obesity-associated cytokine, are sufficient to induce the migration of TNBC cells. The adipokine array results showing that these two cytokines are higher in high (>30) BMI samples than low (<30) BMI samples supports our hypothesis that leptin and IL-6 promote TNBC in obesity (figure 24). The result also provided mechanistic evidence supporting our hypothesis that high BMI acts on peritumor breast AT to induce the release of an obesity-associated secretome that in turn acts through a paracrine mechanism to induce signaling in proximal breast tumors. The adipokine array results also showed that in addition to leptin and IL-6 the levels 7 other cytokines and growth factors were higher in the high (>30) BMI samples compared with the low (<30) ADS samples (M-CSF, lipocalin2/NGAL, leptin, CXCL8/IL-8, IL-6, cathepsin S, cathepsin D, and C-reactive protein/CRP) (figure 25). Performing experiments to test if IL-6 is sufficient to stimulate the migration and invasiveness of TNBC cells was supported by prior clinical data showing that the serum levels of IL-6 are significantly higher in obese patients compared to lean patients (7.69 +/- 5.06 pg/mL, 1.28 +/- 0.85 pg/mL respectively) (Roytblat et al., 2000). In addition, IL-6 has also been shown to play a significant role in breast cancer stemness, angiogenesis, and therapy resistance (C. Zhao et al., 2020).

With this knowledge, and the results of our adipokine array (figure 24), we sought to investigate the effects of IL-6 on the migration of TNBC cells. In our system, we found no effect of IL-6 treatment (1ng/mL and 10ng/mL) on the migration of MDA-MB-231 or MDA-MB-436 TNBC cells (figure 25).

We then sought to determine the effect of IL-6 on mTOR signaling. We measured the effect of IL-6 on the phosphorylation of ribosomal protein S6 (PS6) and the phosphorylation of N-myc downstream regulated 1 (NDRG1), as read outs of mTOR complex 1 and 2 signaling, respectively. Our results indicated that IL-6 had no significant effect on the stimulation of mTOR (figure 26). While IL-6 was increased in high (>30) BMI samples, our data indicates that it alone is not sufficient to stimulate mTOR signaling. We therefore co-treated TNBC cells with IL-6 and leptin and discovered that combining these two cytokines did not induce mTOR complex 1 signaling in TNBC cells (figure 31).

The premise to investigate the effect of leptin on TNBC cell migration and signaling is based on prior reports showing that leptin stimulates the progression of luminal ER positive breast cancer. Many studies demonstrate that leptin is increased in the serum of obese patients (range of 28.2-77.4 ng/mL) (Kazmi et al., 2013). Leptin has been shown to be proinflammatory and is thus believed to have a role in migration and invasion of tumor cells (Paz-Filho et al., 2011; Sundaram et al., 2013).To determine if leptin is responsible for the increased migration, invasion, and mTOR signaling we first sought to measure the levels of leptin in peritumor breast ADS. We found that the concentration of leptin in high (>30) BMI samples was significantly higher than the concentration of leptin in low (<30) BMI samples (figure 33). We were the first to show

that BMI acts on peritumor breast AT to promote the secretion of leptin, which in turn plays a role in the breast tumor microenvironment. We therefore investigated if leptin is sufficient to induce migration and mTORC1 signaling in TNBC cells. The results showed that leptin at physiologically relevant serum concentrations (30 to 70 ng/mL) is not sufficient to increase the migration of TNBC cells or stimulate increases in mTORC1 signaling (figure 31, 32). Conversely, in MDA-MB-231 leptin decreased the migration potential (P-value < 0.0001). This is contrary to previously published data, however, previous studies used concentrations of Leptin that are not physiologically relevant (100ng/mL or 200ng/mL). Our data suggests that the role that obese serum leptin levels play may not be as important as previously reported. The results also showed that leptin is not sufficient to increase the activity of mTORC1 signaling in TNBC cells.

The signaling factors released by adipose tissue is obesity is still an active area of new study and new ADS findings will provide a better insight into the mechanisms by which obesity promotes breast cancer. The complexity of the ADS is that factors in ADS are likely to interact to drive signaling and cellular effects in other tissues, including cancer cells. Thus, the limitation of studying the possibility that one ADS factor is sufficient to induce signaling in cancer cells is that it fails to address the potential interaction between several factors that are co-secreted by AT. That end, our findings indicted that IL-6 and Leptin alone are not sufficient to induce mTORC1 signaling in TNBC cells, and that co-treatment with both factors still falls short of inducing mTOR signaling (figure 32). Future studies will require testing the effect of an obesity-associated adipokine cocktail that potentially mimics the effect of peritumor ADS.

AT secretes over 600 hundred proteins (Kita et al., 2019). In addition to proteins, AT also secretes microparticles that are composed of exosomes and micro-vesicles. These microparticles act through paracrine and endocrine pathways to induce signaling in other tissues, including tumors (Crewe et al., 2018). We therefore hypothesized that microparticles in peritumor ADS act on TNBC cells to stimulate mTORC1 signaling. Microparticles were isolated rather than exosomes as we did not have enough volume from patient samples to isolate enough exosomes. The results showed that in high BMI samples, ADS purified microparticles and total ADS induce similar levels of mTOR signaling that was higher than the level induced by ADS lacking microparticles (figure 34). This suggests that microparticles secreted from peritumor breast AT from high (>30) BMI patients induces mTORC1 signaling in TNBC cells.

5. Conclusions

This study shows that leptin and IL-6, which are obesity-associated cytokines, that are elevated in high (>30) BMI samples are not sufficient to induce TNBC cell migration or mTORC1 signaling in TNBC cells. Finding that high (>30) BMI induces peritumor breast adipose tissue to secrete more exosomes than low (< 30) BMI indicates these secreted factors might play a mechanistic role in obesity in TNBC cancer. Support for this hypothesis stems from the finding that peritumor ADS lacking exosomes is a weak inducer of mTORC1 in TNBC cells compared with peritumor ADS containing exosomes. Based on these findings, we propose future studies need to further investigate synergy between AT-derived adipokines/exosomes on TNBC cells in the context of peritumor AT.

Chapter 4

Summary of Findings and Future Directions

1. Introduction

Obesity has quickly become a global epidemic. Obesity is measured as a body mass index (BMI) of greater than or equal to 30. According to the world health organization, approximately 41.9% of people are obese, with half of the population expected to be obese by the year 2030. It has been shown that obesity is more common in low-income individuals, mainly driven by food insecurity (Nettle et al., 2017). This association holds true for the Appalachian population. The average income of the Appalachian people is 10,000 dollars less than the national average. Many studies have linked poverty and low income to obesity (Rogers et al., 2015; Zukiewicz-Sobczak et al., 2014). The combination of low income and high poverty are likely socioeconomic factors that contribute to higher rates of obesity in Appalachia compared with the national average. Women are twice as likely to be obese as compared to men (Kapoor et al., 2021), making Appalachian women specifically at risk. Obesity is linked to a multitude of conditions such as, cardiovascular disease, stroke, osteoarthritis, dyslipidemia, and fourteen different cancers, including breast cancer. Breast cancer is the most common cancer in women and is responsible for 42,000 deaths each year. TNBC is the breast cancer subtype that has the worst prognosis and is more likely to occur in obese young women than postmenopausal lean women. (Sizemore & Rudisill, 2021). Further, TNBC is more aggressive in obese than in lean women at the time of diagnosis. Given that

nearly half of women are obese and that breast cancer is the most common cancer in women, defining the mechanisms that link obesity with the increased progression of breast cancer has translational significance. Given this importance, we have investigated mechanisms by which BMI acts through peritumor breast adipose tissue to increase the migration and invasiveness of TNBC cells. My long-term objectives are to provide a better insight into the mechanistic links between obesity and TNBC with the goal of using this knowledge to improve cancer therapy to better serve the community.

2. Summary of Findings

In this thesis, our group was the first to demonstrate the effect of high BMI peritumor ADS on TNBC. In order to do this, we used a new novel approach to mimic high BMI conditions, that of patient derived peritumor adipose tissue. The characteristics of our de-identified patient samples can be found in Table 4. Our study was the first to use this isolation method and the first to investigate the effect of BMI of peritumor breast ADS on the migration, invasiveness and signaling in TNBC. Our peritumor breast ADS model provided many novel, clinically relevant results. We showed that peritumor breast ADS derived from patients with a high BMI stimulated the invasion of TNBC MDA-MB-231 cells more than peritumor breast ADS from patients with lean BMI (figure 9). Invasion is the first step in metastasis of cancer (Novikov et al., 2021). This finding provides mechanistic insight into clinical data showing that obesity is linked to increase risk of metastasis in TNBC (Naik et al., 2019). The next step in the metastasis process is migration. The peritumor breast ADS from patients with high BMI induced greater TNBC cell migration compared with peritumor breast ADS from patients with lower BMI (figure 8). We hypothesize that ADS induces these effects through two mechanisms. The first

being that high BMI peritumor breast ADS acts as a chemoattractant and thus stimulates the migration of TNBC cells within the tumor microenvironment. We hypothesize the chemoattractant protein monocyte chemoattractant protein 1 (MCP1) is released from peritumor breast AT, and it acts on TNBC cells to induce cancer cell migration (Jiao et al., 2009). Consistent with this hypothesis is that MCP1 promotes cancer progression (Liu et al., 2020). Our second hypothesis is that factors in peritumor breast ADS binds receptors on the cell surface of cancer cells, stimulating signaling pathways that stimulate TNBC cell migration and invasiveness. To provide mechanistic data supporting our hypothesis, we sought to investigate the effect of high BMI (> 30) compared with low (< 30) BMI on signaling pathways in TNBC cells. We selected pathways that are involved in the initiation and progression of cancer. The first signaling pathway we investigated was the mTOR pathway. The mTOR complex 1 pathway is stimulated by growth factors and nutrients (amino acids and glucose). Further, mTORC1 regulates cell size, protein translation, and cell metabolism (promoting the synthesis of proteins, lipids and nucleotides) and therefore is a pathway that has been linked with increased cancer progression (Harachi et al., 2018). Since obesity is a nutrient overload state, we hypothesized that peritumor breast ADS from high (> 30) BMI patients would be a stronger inducer of mTORC1 activity in TNBC cells than peritumor breast ADS from lower (< 30) BMI patients. The results showed that high and low BMI peritumor ADS induces the same amount of mTORC1 activity in TNBC cells, which suggests that mTOR activity in TNBC cells is not responsive to BMI. However, further analysis of the data, showed that for MDA-MB-436 cells, mTOR stimulation is correlated to an increasing in BMI. Prior studies have shown that obesity promotes the

activity of mTORC1 activity in other types of cancer cells, including luminal estrogen receptor positive breast cancer cells (Dann et al., 2007). Thus, the BMI effect on mTORC1 activity in cancer is context dependent. Regarding this, we discovered in a prior publication that mTORC1 responsiveness to BMI in luminal estrogen receptor positive breast cancer cells was dependent on the concentration of leucine in cell culture medium (Liang et al., 2021). Leucine is a potent agonist for mTORC1 activity, and the levels of leucine in cell culture media are supraphysiological (approximately 3-fold higher than serum levels). Thus, high leucine-mediated induction of mTORC1 might have masked a BMI effect on mTORC1 activity in TNBC cells.

Table 4

Patient Information

Patient ID	BMI	Age	Receptor Status
1252	20	68	ER+, PR+, HER2+
1059	22	22	ER+, PR+
772	23	48	ER+, PR+
1292	23	34	ER+, PR+
902	24	44	ER+, PR+
1009	26	59	ER+, PR+
1697	26	42	ER+, PR+
1221	28	73	ER+
1729	28	53	ER+
810	30	51	-
1613	31	70	ER+
928	32	59	ER+, PR+
1698	33	43	-
1632	40	64	ER+
1599	40	59	ER+, PR+
1676	57	39	ER+, PR+

Note. De-identified patient ID numbers with correlation BMI, age, and receptor status of their breast cancer.

The next pathway we investigated was the ERK pathway. High levels of ERK have been shown to indicate poor survival rates in patients with TNBC (Bartholomeusz et al., 2012). Based on this information we sought to explore the effects of high BMI ADS on ERK levels. We found that high BMI increased ERK expression in MDA-MB-231 TNBC cells but not MDA-MB-436 cells (figure 13 and figure 14). Increased activity of the NFKB pathway in TNBC promotes the progression of this breast cancer subtype. The NFKB pathway is commonly linked to inflammation, and obese individuals are in a constant

state of low-grade inflammation (Monteiro & Azevedo, 2010; Taniguchi & Karin, 2018). NFKB signaling is commonly stimulated by cytokines such as IL-1, IL-6, and IL-23 (Liu et al., 2017). In our study (figure 24) and previous studies, IL-6 has been shown to be released into the ADS (Wueest & Konrad, 2018). Based on these facts we hypothesized that high BMI peritumor breast ADS treatment would increase NFKB signaling in TNBC cells. Contrary to our hypothesis, high BMI ADS treatment did not influence total or phosphorylated NFKB protein levels in MDA-MB-231 or MDA-MB-436 cells, thus indicating that NFKB signaling was not altered (figure 15 and figure 16). This result was surprising. However, NFKB is also known to lead to a more aggressive phenotype of breast cancer by promoting the epithelial mesenchymal transition (EMT). The cells we use in our model have already undergone EMT, suggesting that the NFKB levels in these cells are already high and potentially that's why it is not responsive to a paracrine mechanism (Pires et al., 2017). The final pathway that we investigated was JAG1/NOTCH pathway. The JAG1/NOTCH pathway plays a large role in the development of cancer. JAG1/NOTCH, once activated, carries out a signaling cascade that in turn promotes proliferation, metastasis, drug-resistance, and angiogenesis (Xiu et al., 2020). Based on its prominent role in TNBC, we investigated the effects of high BMI peritumor breast ADS treatment on JAG1 protein expression in TNBC cells (Giuli et al., 2019). Our results showed that high (>30) BMI peritumor breast ADS significantly increased the expression of JAG1 (by 3-fold) in MDA-MB-231 but not in MDA-MB-436 TNBC cells (figure 11 and figure 12). We also performed a linear regression analysis and the results showed that JAG1 expression is correlated with increasing BMI (figure 19). This result shows that increasing BMI has an effect on peritumor breast ADS that in

turn induces greater expression of JAG1 in TNBC cells. We are the first to link increasing BMI to increased expression of JAG1 in TNBC cells. JAG1, being the known ligand of the NOTCH1 receptor, we sought to measure the protein levels of a NOTCH1 target gene HES1, as a read out of activated NOTCH1 signaling in response to high BMI peritumor breast ADS. The results showed that despite increases in JAG1 expression in response to high BMI, there was not a corresponding increase in HES1 protein in TNBC cells treated with peritumor breast ADS (figure 11, figure 12). HES1 however, is only one of the many target genes of NOTCH. We therefore propose a more thorough assessment of NOTCH targets, such as HEY5, CCND1, CDKN1 is needed to investigate the link more fully between increased JAG1 with activation of NOTCH1 signaling in TNBC cells stimulated with peritumor breast ADS (Kandasamy et al., 2010).

The strength of our study is its translational potential, given that peritumor breast ADS was obtained from women with breast cancer and the ADS was applied to human TNBC cells. Finding that the high BMI peritumor breast ADS induces the migration and invasiveness of TNBC, which is the breast cancer subtype with the worst prognosis, provides a new mechanistic link between obesity and TNBC progression. This is the first study to demonstrate link BMI, peritumor breast ADS with increased TNBC cell migration and invasiveness. Based on the importance of these results we sought to continue our study and determine if high BMI can be linked to an increase in the expression of BCSC marker CD44. CD44 is a well-known stem cell marker that is present in over 50% of triple negative breast cancers (Giatromanolaki et al., 2011). Increased expression of CD44 is also linked to a poor prognosis in TNBC

(Giatromanolaki et al., 2011). The results showed that high BMI peritumor did not stimulate an increase in CD44 in TNBC cells (figure 23). The upregulation of CD44 is mainly stimulated by extracellular factors or activation of STAT3 and NFKB (Xu et al., 2015). Our previous results showed that high BMI had no effect on NFKB signaling, possibly explaining why we did not see an effect on CD44 expression.

The goal of this study was to determine new potential therapeutic interventions. By investigating contents of peritumor breast ADS (figure 24) and finding that high BMI peritumor breast ADS stimulated JAG1 expression, suggests that agents targeting JAG1 signaling might be useful for treating TNBC in obese patients. Identifying the specific factor(s) in peritumor breast ADS that induces JAG1 signaling would provide a target to inhibit to suppress JAG1-mediated signaling in TNBC cells. Based on these results, we are the first study to make a mechanistic connection between human ADS and the aggressiveness of TNBC. To this end, we performed an adipokine array on peritumor breast ADS obtained from patients with high (BMI>30) compared with low (BMI<30). The adipokine array showed differences between low and high BMI peritumor breast ADS (figure 24). For instance, M-CSF, IL-6, C-reactive protein, and cathepsin S. Based on the results of our adipokine array we selected two proteins that play a large role in obesity: the inflammatory cytokine IL-6 and the hormone leptin. Prior reports show that the levels of IL-6 and leptin in plasma are higher in obesity compared with lean patients. Our data now shows that BMI is also associated with increased release of leptin and IL-6 from peritumor breast ADS, which in turn could have a paracrine effect on breast cancer cells. We therefore investigated the effects of IL-6 on migration in TNBC cells. Regardless of concentration (1ng/mL or 10 ng/mL) IL-6 had no effect on

the migration on TNBC cells compared with vehicle. IL-6 has been previously shown to act as a chemoattractant to monocytes, however our data shows that the cytokine did not increase the migration of TNBC cells, which is consistent with a prior report (Clahsen & Schaper, 2008). While IL-6 had no effect on migration, in MDA-MB-231 IL-6 at 1 ng/mL increased the expression of CD44 by ~ 3-fold. IL-6 is a potent stimulator of STAT3, which plays a role in the regulation of CD44 (Xu et al., 2015). A recent study demonstrated anti-CD44 antibodies inhibit mTORC1 and mTORC2 (Gadhoum et al., 2016). This study suggests a role CD44 in stimulation of the mTOR pathway. Based on our previous results showing that IL-6 1 ng/mL increased CD44 expression, we hypothesized that this increase may lead to increases in the mTOR pathway. We then sought to investigate if IL-6 induces the mTORC1 pathway in TNBC cells. The results showed that IL-6 does not stimulate mTORC1 or mTORC2 signaling in MDA-MB-231 TNBC cells (figure 26). From these findings, we propose that IL-6 alone is not sufficient to mimic the effects of peritumor breast ADS and thus it alone is not mediating the effects of BMI, however, it might work in synergy with other proteins in peritumor ADS to promote TNBC progression.

The next protein we investigate is the hormone leptin. Leptin resistance and increases in serum leptin levels are hallmarks of obesity (Obradovic et al., 2021). The primary function of leptin is to regulate nutrient intake as well as maintenance of body mass (Obradovic et al., 2021). The role of leptin in obesity is established, however, there is less known regarding the role of leptin in TNBC. Leptin has been shown to promote metastasis in mouse models with patient derived TNBC xenografts, which indicates breast tumors are responsive to leptin, in vivo (Sabol et al., 2019). To

investigate the role of leptin, we first sought to determine the concentration of leptin in peritumor breast ADS. The results showed that the concentration of leptin in high BMI peritumor breast ADS was significantly higher than the concentration of leptin in low BMI peritumor breast ADS (figure 28). To test the effects of leptin on TNBC cell migration, we used physiologically relevant doses. Obese patients have serum levels in a range of 50-70 ng/mL (Kazmi et al., 2013). At these physiological conditions, leptin reduced the migration of MDA-MB-231 and had no effect in MDA-MB-436 cells (figure 30) and did not stimulate mTORC1 activity in either TNBC cell line (figure 31). Given that IL-6 and leptin are currently secreted from peritumor AT, we hypothesized that concurrent treatment of TNBC cells with leptin and IL-6 would stimulate mTORC1 activity. To test this hypothesis, we added IL-6 (1ng/mL or 10ng/mL) and leptin (30 ng/mL) together and measured the stimulation of the mTOR pathway. The results showed that concurrent treatment with IL-6 and leptin did not stimulate the mTORC1 pathway in TNBC cells. This finding shows that IL-6 + leptin is not sufficient to mimic the effect of peritumor breast ADS on mTORC1 signaling in TNBC cells. We also performed TNBC cell migration studies and the finding showed that concurrent treatment of leptin with IL-6 does not stimulate the migration of TNBC cells (figure 32).

Based on published studies showing that AT secreted micro-vesicles, I hypothesized that increases in BMI would stimulate peritumor breast AT to secrete increased levels of micro-vesicles, and that this in turn would increase signaling in TNBC cells (Lazar et al., 2016). Nanoparticles, contain micro-vesicles and exosomes, are 30 nm -1.0 um in size and function as cell-cell communicators (Stahl et al., 2019; Tai et al., 2018). In addition, nanoparticles transfer bioactive molecules from the peritumor microenvironment to

cancer cells (Tai et al., 2018). In a recent study, nanoparticles were shown to play a large role in the promotion of melanoma (Lazar et al., 2016). In this paper exosomes derived from obese or lean mice were added to SKMEL28 or 1205Lu melanoma cells. Obese exosomes increased migration and invasion at a higher level compared to lean exosomes (Lazar et al., 2016). Based on these findings, we isolated nanoparticles from patient derived peritumor breast AT (figure 33). After isolation we compared the number of nanoparticles in peritumor breast ADS from high and low BMI patients. While there was not a statistically significant difference, there was a trend towards an increase in nanoparticles in high BMI peritumor breast ADS (figure 33). We diluted the nanoparticles 1:10 in cell culture medium and applied them to TNBC cells in cell culture. The results showed the nanoparticles alone maintain mTORC1 signaling to the same level as complete peritumor breast ADS (figure 34). In accordance with our prior results (figure 20 and figure 21), peritumor breast ADS did not stimulate mTORC1 activity above baseline (figure 34). The inability of peritumor breast ADS to induce mTORC1 could be due to high constitutive mTORC1 activity in TNBC cells.

3. Limitations

The limitation of this study is that our that the majority of our de-identified patient samples are middle-age white females. Our cell lines were obtained from middle-age white females as well. TNBC is most common in the African American population (Newman & Kaljee, 2017). Our results would be better rounded if different races were included in our study.

Another limitation of this study was in the isolation of nanoparticles. Due to the size of our adipose tissue and the amount of tissue we had we were not able to isolate

exosomes alone, we had to isolate a mixture of nanoparticles that contained microparticles (0.1um-100uM) and exosomes (30 nm -100 nm) (Stahl et al., 2019). We were also unable to repeat this experiment due to the low level of samples and the fact that the isolation protocol uses an entire sample.

4. Conclusions

Collectively, our findings provide a new mechanistic link between obesity and the progression of TNBC, by showing that BMI induces changes in peritumor AT that in turn influences the effect of peritumor breast ADS on TNBC cells, such that the cancer cells are more invasive and have stronger migratory activity. We are also the first to demonstrate that BMI correlates with increased levels of JAG1 in TNBC cells in response to and the mTOR readout phosphorylated ribosomal protein S6 (in the correlation study).

5. Future experiments

5.1 The Effect of High (>30) BMI and Peritumor Breast ADS on Matrix Metalloprotease Protein Expression in TNBC Cells.

5.1.1 Rationale

Matrix metalloproteases (MMP) are enzymes that are involved in the digestion and breakdown of the extracellular matrix (Jablonska-Trypuc et al., 2016). This family of enzymes specifically digests type IV collagen, a large component of the basement membrane (Song et al., 2000). Given their mechanism of action, these proteins promote the invasiveness of cancer cells. In our previous experiments, we have demonstrated that high BMI (> 30) peritumor breast ADS treated TNBC cells exhibit increased migration compared with TNBC cells treated with low (<30) peritumor breast ADS

(figure 1). We hypothesize that MMPs are released by peritumor breast ADS and/or peritumor breast ADS induces signaling in TNBC cells that increases the expression of MMPs.

5.1.2 Hypothesis

High (>30) BMI peritumor breast ADS will induce a greater increase in the expression of MMPs in TNBC cells compared with low (<30) BMI peritumor breast ADS.

5.1.3 Experimental Procedure

MDA-MB 231 and MDA-MB-436 cells will be treated with high (> 30) and low (< 30) BMI peritumor breast ADS. After 24 hours, the expression of MMPs (MMP2 and MM9) in TNBC cell culture media and TNBC cell extract will be analyzed by Western blot analysis. MMP2 and MMP9 antibodies will be purchased from Cell Signaling Technologies (Danvers, MA).

5.1.4 Pitfalls/Alternative Experiments

It is possible that high (>30) BMI peritumor ADS will not stimulate an increase in the expression of MMP2 and MMP9 in TNBC cells. We would then design experiments to investigate if high (> 30) BMI peritumor breast ADS acts on TNBC cells to increase MMP2 and MMP9 activity. The gel zymography technique is used to measure the activity of MMPs and the technique would be used to investigate the alternative hypothesis that high (>30) BMI peritumor breast ADS acts on TNBC cells to increase the activity of MMPs.

5.2 Obese Adipose Derived Secretome Effect on Breast Cancer Stemness

5.2.1 Rationale

BCSC play a large role in the resistance to hormone therapy, radiotherapy, and chemotherapy (Palomeras et al., 2018). The expression of multiple different stem cell markers is indicative of breast cancer poor prognosis (X. Liu et al., 2019; Panigoro et al., 2020). Based on our previous experiments and previous literature, high BMI is also linked to poor prognosis of TNBC (Sun et al., 2017). Based on these studies, we hypothesize that high BMI peritumor breast ADS induces signaling in TNBC cells that increase the expression of BCSC markers.

5.2.2 Hypothesis

MDA-MB-231 and MDA-MB-436 cells treated with high BMI peritumor breast ADS will express higher levels of breast cancer stem cells markers on their cell surface as compared to cells treated with low BMI.

5.2.3 Experimental Procedure

TNBC breast cancer cells will be treated with low or high BMI peritumor breast ADS for 24 hours. After 24 hours a single cell suspension of cells will be created and then flow cytometry will be performed. Stem cell markers CD44 and ALDH1 will be probed for as well as the absence of CD24. The presence/absence of these markers will show changes in stem cell activity in TNBC (O'Conor et al., 2018). Cell populations will be sorted by CD44, ALDH1 and CD24 and then analyzed for changes in the percentage of TNBC cells that express these markers in response to peritumor breast ADS.

5.2.4 Pitfalls/Alternative Experiments

Flow cytometry is a sensitive and effective technique for measuring cell surface CSC markers. If we are not able to detect changes, we can attempt to use the

ALDEFLOUR assay, which is specifically for detecting CSC marker ALDH1. ALDEFLOUR assay works by adding a fluorescent aldehyde to cellular samples. Cells that express the ALDH enzyme will be able to convert this aldehyde into a carboxylic acid. This carboxylic acid is retained within the cell and then read by a flow cytometer (L. Zhou et al., 2019). There is a possibility that BMI does not regulate the expression of BSCS markers. Or it regulates the expression of one marker, but not the other markers. Another explanation for no difference could be that the levels of BCSC within MDA-MB-231 or MDA-MB-436 cells may already be overexpressed and consequently unresponsive to exogenous stimuli. If we do not see a difference in the ALDEFLOUR assay, we will examine different concentrations and time point of ADS treatment as only one concentration and time point (10% at 24 hours) has been tested. In addition, if a significance is not seen it may be due to the small sample size of our patient derived peritumor ADS.

5.3 Characterization of the BMI Regulated Transcriptome in TNBC Cells.

5.3.1 Rationale

RNA sequencing followed by pathway analysis will be used to determine BMI regulated transcriptomes and associated pathways in TNBC cells treated with low and high BMI peritumor breast ADS. The premise for this study is that the JAG1/Notch pathway is a key regulator of gene transcription (Sanchez-Iranzo et al., 2022). The RNA-seq approach will provide an unbiased approach to determine BMI sensitive genes in TNBC cells.

5.3.2 Hypothesis

We hypothesize that gene readouts of JAG1/NOTCH pathway such as HEY1, HES5, CCND1, will be increased in TNBC cells treated with peritumor breast ADS from patients with high BMIs. Given our results showing that high BMI peritumor breast ADS stimulates TNBC cell migration, we hypothesize that genes associated with cancer cell migration such as vascular endothelial growth factor (VEGF), glucose 6 phosphate isomerase/autocrine motility factor (GPI/AMF), and chemokine C-C motif. Ligand 2 (CCL22) will be increased in response to high BMI peritumor breast ADS.

5.3.3 Experimental Procedure

MDA-MB-231 and MDA-MB-436 cells treated with high BMI peritumor ADS for 24 hours will be lysed and total RNA will be purified by Qiagen RNA columns and purified RNA will be submitted to the MU Genomics Core for library prep and RNA-sequencing, and to the bioinformatics for data analysis.

5.3.4 Pitfalls/Alternative Experiments

The number of differentially expressed genes might be overwhelming. We will use pathway analysis to identify significantly associated pathways. We have only selected one time point and therefore will miss several gene expression changes. The 24-hour time point is based on our western blot data showing changes in JAG1. The gene expression changes could be primary, secondary, and tertiary effects, and thus mechanism of regulation will be unknown. This is a hypothesis generating experiment, and not designed to provide mechanistic details regarding how genes are being regulated. If there are no changes in RNA expression of TNBC cells treated with high BMI peritumor ADS, we will perform a 3 hour and 48-hour time point experiment.

5.4 Determine the Expression of Cell Curface NOTCH Receptors on TNBC Cells

5.4.1 Rationale

High BMI peritumor breast ADS induces increases in JAG1 expression in TNBC cells, which is associated with increases in cancer cell migration and invasion (figure 8, figure 9, figure 11, and figure 12). Given that JAG1 binds to it cell surface receptor, NOTCH1, NOTCH2, NOTCH3, and NOTCH4 (Lobry et al., 2014), one (or all these receptors) are likely expressed on the surface of TNBC cells. Knowing which JAG1 receptor is expressed on TNBC cells will provide mechanistic evidence to target it with a therapeutic NOTCH receptor targeting antibody.

5.4.2 Hypothesis

We hypothesize that NOTCH receptors are expressed on MDA-MB-231 and MDA-MB-436 TNBC cells.

5.4.3 Experimental Procedure

Immunofluorescence staining of TNBC cells for NOTCH 1, NOTCH 2, NOTCH 3, and NOTCH 4 will be performed.

5.4.4 Pitfalls/Alternative Experiments

Characterizing receptors is difficult, and antibodies for receptors may not be specific for each NOTCH receptor. The NOTCH receptors may not be expressed on the surface of TNBC cells. If we are unable to do immunofluorescence staining, we use a western blot approach.

5.5 Mass Spectrometry

5.5.1 Rationale

Mass spectrometry can be used to determine the proteins in peritumor breast ADS. One limitation of peritumor breast ADS is that many of the proteins in ADS are

unknown and therefore it is difficult to determine the mechanism by which peritumor breast ADS stimulates cancer cell migration and invasiveness. The adipokine array showed preliminary evidence that certain growth factors and cytokines are in peritumor ADS (figure 25). Mass spectrometry will extend the adipokine array results and potentially provide insight into the mechanism(s) by which peritumor breast ADS regulates TNBC.

5.5.2 Hypothesis

We hypothesize that high BMI peritumor breast ADS will contain growth factors, cytokines, and hormones that are associated with obesity and mediate the effects of peritumor breast ADS on TNBC cells.

5.5.3 Experimental Procedure

High BMI peritumor ADS sample will be prepared as stated in chapter 2 and prepared for mass spectrometry using the EasyPrep Mini sample prep kit from Thermofisher Scientific (CA #A40006).

5.5.4 Pitfalls/Alternative Experiments

The number of proteins identified by Mass spectrometry might be overwhelming. We will use pathway analysis to find statistically significant associations between differentially expressed proteins and pathways. We will use a published kit (Haun et al., 2019), to purify proteins for Mass Spectrometry and therefore we do not anticipate problems with preparing protein extract.

5.6 Determine if Peritumor Breast ADS Stimulated Increases in JAG1 in TNBC Cells Stimulates the Migration of TNBC Cells.

5.6.1 Rationale

We and others have shown that JAG stimulates TNBC cell migration and invasiveness (Piwarski et al., 2020). Peritumor breast ADS stimulates JAG1 expression, cell migration and invasiveness when applied to TNBC cells (figure 8, figure 9, figure 11 and figure 12). Our objective is to make a connection between peritumor breast ADS, JAG1, and increased migration and invasiveness of TNBC cells.

5.6.2 Hypothesis

We hypothesize that peritumor breast ADS stimulated increases in JAG1 will stimulate the migration and invasiveness of TNBC cells.

5.6.3 Experimental Procedure

Short interfering RNA against JAG1 will be transfected into MDA-MB-231 and MDA-MB-436 prior to the application of peritumor breast ADS. Cell migration and cell invasion will be assayed by wound healing and boyden chamber techniques, respectively. Western blot and real time PCR analysis will be used to verify short interfering RNA mediated knockdown of JAG1 protein and mRNA, respectively.

5.6.4 Pitfalls/Alternative Experiments

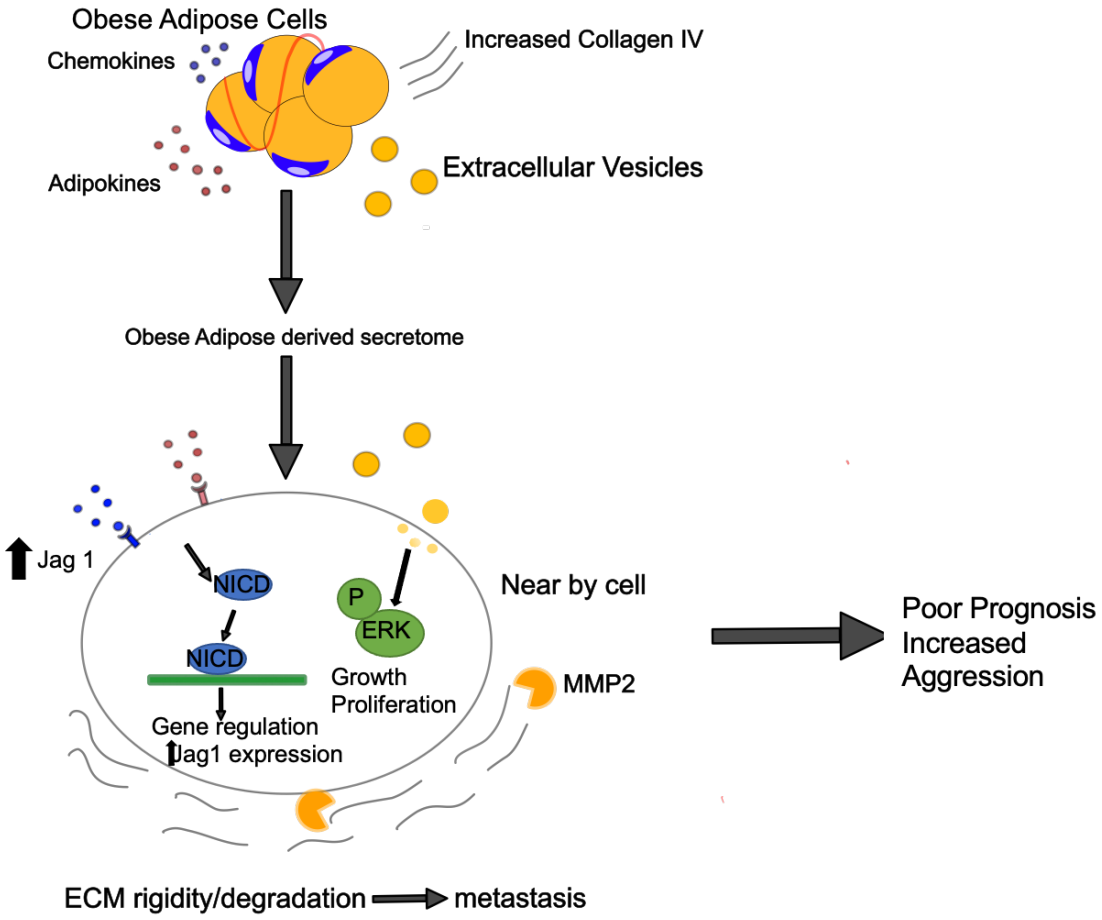
It is possible that peritumor breast ADS induces cell migration and invasiveness through a JAG1 independent pathway. This would be an important result considering that prior reports have shown that JAG1 is sufficient to stimulate the migration and invasiveness of TNBC cells. If JAG1 is not important, we analyze RNA-seq and proteomic data to develop an alternative hypothesis to determine what signaling pathway mediates increases in TNBC cell migration and invasion in response to high BMI peritumor breast ADS.

6. Proposed Model

Based on our findings, we have created a proposed model (figure 36) showing the mechanism by which high (>30) BMI associated peritumor breast ADS promotes TNBC. I hypothesize that low grade inflammation in obesity induces changes to AT that in turn changes the types and/or levels of chemokines, adipokines, extracellular vesicles, and collagen IV that are secreted by peritumor breast AT. This BMI-associated change in the peritumor ADS acts on TNBC cells through a paracrine mechanism. The ADS regulation includes cytokines interacting with cytokine receptors on TNBC cells, JAG1 interacting with NOTCH receptors, and the fusion of extracellular vesicles with the plasma membrane of TNBC cells. The induction of JAG1 in TNBC cells might be sufficient to induce a poor prognosis, however, it is likely JAG1 plus additional ADS stimulated pathways in TNBC interact to stimulate the progression of TNBC. The increase in JAG1 acts through its NOTCH receptors to stimulate the cell cycle inducer CCND1, which in turn promotes the proliferation of TNBC cells (Cohen et al., 2010). In addition, NOTCH signaling increases the number of cancer stem cells, promotes drug resistance, invasion, and migration. Furthermore, the increased secretion of collagen makes the extracellular matrix more rigid (Riching et al., 2014). The increased rigidity of the extracellular matrix promotes malignancy and metastasis (Pickup et al., 2014). The hypothesized increase in secretion of MMPs and the increased rigidity leads to an increased ability for the cancer cell to metastasize. The cumulation of all these events lead to an increase in aggression of triple negative breast cancer as well as a poorer prognosis in obesity.

Figure 35

Proposed Model of O-ADS Promotion of TNBC



Note. Fundamental alterations in peri-tumor obese adipose tissue leads to increase release of Collagen IV, extracellular vesicles, chemokines, and adipokines. These alterations and increased release of these proteins compromises the obese adipose derived secretome (O-ADS). O-ADS then acts on nearby tumor. O-ADS binds to receptors and/or fuses with the plasma membrane. These particles then lead to S2 cleavage of the NOTCH intracellular domain, which then translocates to the nucleus to

regulate gene expression and increase JAG1 protein levels. In addition, O-ADS acts to stimulate the phosphorylation of ERK which then leads to increased growth and proliferation. All of these events combined leads to increased aggression of TNBC as well as a poorer prognosis.

References

Agostini, D., Natalucci, V., Baldelli, G., De Santi, M., Donati Zeppa, S., Vallorani, L., Annibalini, G., Lucertini, F., Federici, A., Izzo, R., Stocchi, V., & Barbieri, E. (2018). New insights into the role of exercise in inhibiting mTOR signaling in triple-negative breast cancer. *Oxid Med Cell Longev*, *2018*, 5896786. https://doi.org/10.1155/2018/5896786

Altomare, I., Bendell, J. C., Bullock, K. E., Uronis, H. E., Morse, M. A., Hsu, S. D., Zafar, S. Y., Blobe, G. C., Pang, H., Honeycutt, W., Sutton, L., & Hurwitz, H. I. (2011). A phase II trial of bevacizumab plus everolimus for patients with refractory metastatic colorectal cancer. *Oncologist*, *16*(8), 1131-1137. https://doi.org/10.1634/theoncologist.2011-0078

Arnold, M., Morgan, E., Rumgay, H., Mafra, A., Singh, D., Laversanne, M., Vignat, J., Gralow, J. R., Cardoso, F., Siesling, S., & Soerjomataram, I. (2022). Current and future burden of breast cancer: Global statistics for 2020 and 2040. *Breast*, *66*, 15-23. https://doi.org/10.1016/j.breast.2022.08.010

Avgerinos, K. I., Spyrou, N., Mantzoros, C. S., & Dalamaga, M. (2019). Obesity and cancer risk: Emerging biological mechanisms and perspectives. *Metabolism*, *92*, 121-135. https://doi.org/10.1016/j.metabol.2018.11.001

Bae, J. Y., Shin, K. O., Woo, J., Woo, S. H., Jang, K. S., Lee, Y. H., & Kang, S. (2016). Exercise and dietary change ameliorate high fat diet induced obesity and insulin resistance via mTOR signaling pathway. *J Exerc Nutrition Biochem*, *20*(2), 28-33. https://doi.org/10.20463/jenb.2016.06.20.2.4

Bailey, S. R., Nelson, M. H., Himes, R. A., Li, Z., Mehrotra, S., & Paulos, C. M. (2014). Th17 cells in cancer: the ultimate identity crisis. *Front Immunol*, *5*, 276. https://doi.org/10.3389/fimmu.2014.00276

Bardou, M., Barkun, A. N., & Martel, M. (2013). Obesity and colorectal cancer. *Gut*, *62*(6), 933-947. https://doi.org/10.1136/gutjnl-2013-304701

Bartholomeusz, C., Gonzalez-Angulo, A. M., Liu, P., Hayashi, N., Lluch, A., Ferrer-Lozano, J., & Hortobagyi, G. N. (2012). High ERK protein expression levels correlate with shorter survival in triple-negative breast cancer patients. *Oncologist*, *17*(6), 766-774. https://doi.org/10.1634/theoncologist.2011-0377

Beckner, M. E., Stracke, M. L., Liotta, L. A., & Schiffmann, E. (1990). Glycolysis as primary energy source in tumor cell chemotaxis. *J Natl Cancer Inst*, *82*(23), 1836-1840. https://doi.org/10.1093/jnci/82.23.1836

Betsunoh, H., Fukuda, T., Anzai, N., Nishihara, D., Mizuno, T., Yuki, H., Masuda, A., Yamaguchi, Y., Abe, H., Yashi, M., Fukabori, Y., Yoshida, K., & Kamai, T. (2013). Increased expression of system large amino acid transporter (LAT)-1 mRNA is associated with invasive potential and unfavorable prognosis of human clear cell renal cell carcinoma. *BMC Cancer*, *13*, 509. https://doi.org/10.1186/1471-2407-13-509

Betz, C., & Hall, M. N. (2013). Where is mTOR and what is it doing there? *J Cell Biol*, *203*(4), 563-574. https://doi.org/10.1083/jcb.201306041

Borggrefe, T., & Oswald, F. (2009). The Notch signaling pathway: transcriptional regulation at Notch target genes. *Cell Mol Life Sci*, *66*(10), 1631-1646. https://doi.org/10.1007/s00018-009-8668-7

Bouloumie, A., Drexler, H. C., Lafontan, M., & Busse, R. (1998). Leptin, the product of Ob gene, promotes angiogenesis. *Circ Res*, *83*(10), 1059-1066. https://doi.org/10.1161/01.res.83.10.1059

Bowers, L. W., Rossi, E. L., McDonell, S. B., Doerstling, S. S., Khatib, S. A., Lineberger, C. G., Albright, J. E., Tang, X., deGraffenried, L. A., & Hursting, S. D. (2018). Leptin Signaling Mediates Obesity-Associated CSC Enrichment and EMT in Preclinical TNBC Models. *Mol Cancer Res*, *16*(5), 869-879. https://doi.org/10.1158/1541-7786.MCR-17-0508

Calle, E. E. (2007). Obesity and cancer. *BMJ*, *335*(7630), 1107-1108. https://doi.org/10.1136/bmj.39384.472072.80

Calle, E. E., & Kaaks, R. (2004). Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer*, *4*(8), 579-591. https://doi.org/10.1038/nrc1408

Calle, E. E., & Thun, M. J. (2004). Obesity and cancer. *Oncogene*, *23*(38), 6365-6378. https://doi.org/10.1038/sj.onc.1207751

Cameron, A. J., Linch, M. D., Saurin, A. T., Escribano, C., & Parker, P. J. (2011). mTORC2 targets AGC kinases through Sin1-dependent recruitment. *Biochem J*, *439*(2), 287-297. https://doi.org/10.1042/BJ20110678

Camino, T., Lago-Baameiro, N., Martis-Sueiro, A., Couto, I., Santos, F., Baltar, J., & Pardo, M. (2020). Deciphering Adipose Tissue Extracellular Vesicles Protein Cargo and Its Role in Obesity. *Int J Mol Sci*, *21*(24). https://doi.org/10.3390/ijms21249366

Cammisotto, P. G., & Bendayan, M. (2007). Leptin secretion by white adipose tissue and gastric mucosa. *Histol Histopathol*, *22*(2), 199-210. https://doi.org/10.14670/HH-22.199

Carino, C., Olawaiye, A. B., Cherfils, S., Serikawa, T., Lynch, M. P., Rueda, B. R., & Gonzalez, R. R. (2008). Leptin regulation of proangiogenic molecules in benign and cancerous endometrial cells. *Int J Cancer*, *123*(12), 2782-2790. https://doi.org/10.1002/ijc.23887

Carosi, J. M., Fourrier, C., Bensalem, J., & Sargeant, T. J. (2022). The mTOR-lysosome axis at the centre of ageing. *FEBS Open Bio*, *12*(4), 739-757. https://doi.org/10.1002/2211-5463.13347

Castano, C., Kalko, S., Novials, A., & Parrizas, M. (2018). Obesity-associated exosomal miRNAs modulate glucose and lipid metabolism in mice. *Proc Natl Acad Sci U S A*, *115*(48), 12158-12163. https://doi.org/10.1073/pnas.1808855115

Chang, Y.-C., & Barbas, A. (2021). 1 - The surgical and immunosuppressive basis for infections in the pediatric solid organ transplant recipient. In W. J. Steinbach, M. D. Green, M. G. Michaels, L. A. Danziger-Isakov, & B. T. Fisher (Eds.), *Pediatric Transplant and Oncology Infectious Diseases* (pp. 1-9.e3). Elsevier. https://doi.org/https://doi.org/10.1016/B978-0-323-64198-2.00010-5

Chekhun, S. V., Zadvorny, T. V., Tymovska, Y. O., Anikusko, M. F., Novak, O. E., & Polishchuk, L. Z. (2015). capital ES, CyrillicD44+/CD24- markers of cancer stem cells in patients with breast cancer of different molecular subtypes. *Exp Oncol*, *37*(1), 58-63. https://www.ncbi.nlm.nih.gov/pubmed/25804234

Chen, C., Zhao, S., Karnad, A., & Freeman, J. W. (2018). The biology and role of CD44 in cancer progression: therapeutic implications. *J Hematol Oncol*, *11*(1), 64. https://doi.org/10.1186/s13045-018-0605-5

Chen, J. S., Wang, Q., Fu, X. H., Huang, X. H., Chen, X. L., Cao, L. Q., Chen, L. Z., Tan, H. X., Li, W., Bi, J., & Zhang, L. J. (2009). Involvement of PI3K/PTEN/AKT/mTOR pathway in invasion and metastasis in hepatocellular carcinoma: Association with MMP-9. *Hepatol Res*, *39*(2), 177-186. https://doi.org/10.1111/j.1872-034X.2008.00449.x

Choi, Y. K., & Park, K. G. (2018). Targeting Glutamine Metabolism for Cancer Treatment. *Biomol Ther (Seoul)*, *26*(1), 19-28. https://doi.org/10.4062/biomolther.2017.178

Chue, B. M., & La Course, B. D. (2019). Case report of long-term survival with metastatic triple-negative breast carcinoma: Treatment possibilities for metastatic disease. *Medicine (Baltimore)*, *98*(16), e15302. https://doi.org/10.1097/MD.0000000000015302

Chun, K. A., Kocarnik, J. M., Hardikar, S. S., Robinson, J. R., Berndt, S. I., Chan, A. T., Figueiredo, J. C., Lindor, N. M., Song, M., Schoen, R. E., Hayes, R. B., Potter, J. D., Nassir, R., Bezieau, S., Le Marchand, L., Slattery, M. L., White, E., Peters, U., & Newcomb, P. A. (2018). Leptin gene variants and colorectal cancer risk: Sex-specific associations. *PLoS One*, *13*(10), e0206519. https://doi.org/10.1371/journal.pone.0206519

Clahsen, T., & Schaper, F. (2008). Interleukin-6 acts in the fashion of a classical chemokine on monocytic cells by inducing integrin activation, cell adhesion, actin polymerization, chemotaxis, and transmigration. *J Leukoc Biol*, *84*(6), 1521-1529. https://doi.org/10.1189/jlb.0308178

Clement, E., Lazar, I., Muller, C., & Nieto, L. (2017). Obesity and melanoma: could fat be fueling malignancy? *Pigment Cell Melanoma Res*, *30*(3), 294-306. https://doi.org/10.1111/pcmr.12584

Clinton, S. K., Giovannucci, E. L., & Hursting, S. D. (2020). The World Cancer Research Fund/American Institute for Cancer Research Third Expert Report on Diet, Nutrition, Physical Activity, and Cancer: Impact and Future Directions. *J Nutr*, *150*(4), 663-671. https://doi.org/10.1093/jn/nxz268

Cohen, B., Shimizu, M., Izrailit, J., Ng, N. F., Buchman, Y., Pan, J. G., Dering, J., & Reedijk, M. (2010). Cyclin D1 is a direct target of JAG1-mediated Notch signaling in breast cancer. *Breast Cancer Res Treat*, *123*(1), 113-124. https://doi.org/10.1007/s10549-009-0621-9

Collaborators, G. B. D. O., Afshin, A., Forouzanfar, M. H., Reitsma, M. B., Sur, P., Estep, K., Lee, A., Marczak, L., Mokdad, A. H., Moradi-Lakeh, M., Naghavi, M., Salama, J. S., Vos, T., Abate, K. H., Abbafati, C., Ahmed, M. B., Al-Aly, Z., Alkerwi, A., Al-Raddadi, R., . . . Murray, C. J. L. (2017). Health Effects of Overweight and Obesity in 195 Countries over 25 Years. *N Engl J Med*, *377*(1), 13-27. https://doi.org/10.1056/NEJMoa1614362

Coppin, C. (2010). Everolimus: the first approved product for patients with advanced renal cell cancer after sunitinib and/or sorafenib. *Biologics*, *4*, 91-101. https://doi.org/10.2147/btt.s6748

Crewe, C., Joffin, N., Rutkowski, J. M., Kim, M., Zhang, F., Towler, D. A., Gordillo, R., & Scherer, P. E. (2018). An Endothelial-to-Adipocyte Extracellular Vesicle Axis Governed by Metabolic State. *Cell*, *175*(3), 695-708 e613. https://doi.org/10.1016/j.cell.2018.09.005

D'Souza, B., Meloty-Kapella, L., & Weinmaster, G. (2010). Canonical and non-canonical Notch ligands. *Curr Top Dev Biol*, *92*, 73-129. https://doi.org/10.1016/S0070-2153(10)92003-6

Dan, H. C., Ebbs, A., Pasparakis, M., Van Dyke, T., Basseres, D. S., & Baldwin, A. S. (2014). Akt-dependent activation of mTORC1 complex involves phosphorylation of mTOR (mammalian target of rapamycin) by IkappaB kinase alpha (IKKalpha). *J Biol Chem*, *289*(36), 25227-25240. https://doi.org/10.1074/jbc.M114.554881

Dann, S. G., Selvaraj, A., & Thomas, G. (2007). mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. *Trends Mol Med*, *13*(6), 252-259. https://doi.org/10.1016/j.molmed.2007.04.002

De Pergola, G., & Silvestris, F. (2013). Obesity as a major risk factor for cancer. *J Obes*, *2013*, 291546. https://doi.org/10.1155/2013/291546

De Rosa, V., Procaccini, C., Cali, G., Pirozzi, G., Fontana, S., Zappacosta, S., La Cava, A., & Matarese, G. (2007). A key role of leptin in the control of regulatory T cell proliferation. *Immunity*, *26*(2), 241-255. https://doi.org/10.1016/j.immuni.2007.01.011

Demory Beckler, M., Higginbotham, J. N., Franklin, J. L., Ham, A. J., Halvey, P. J., Imasuen, I. E., Whitwell, C., Li, M., Liebler, D. C., & Coffey, R. J. (2013). Proteomic analysis of exosomes from mutant KRAS colon cancer cells identifies

intercellular transfer of mutant KRAS. *Mol Cell Proteomics*, *12*(2), 343-355. https://doi.org/10.1074/mcp.M112.022806

Deng, Z. B., Poliakov, A., Hardy, R. W., Clements, R., Liu, C., Liu, Y., Wang, J., Xiang, X., Zhang, S., Zhuang, X., Shah, S. V., Sun, D., Michalek, S., Grizzle, W. E., Garvey, T., Mobley, J., & Zhang, H. G. (2009). Adipose tissue exosome-like vesicles mediate activation of macrophage-induced insulin resistance. *Diabetes*, *58*(11), 2498-2505. https://doi.org/10.2337/db09-0216

Deutsch, A., Feng, D., Pessin, J. E., & Shinoda, K. (2020). The Impact of Single-Cell Genomics on Adipose Tissue Research. *Int J Mol Sci*, *21*(13). https://doi.org/10.3390/ijms21134773

Dodd, K. M., & Tee, A. R. (2012). Leucine and mTORC1: a complex relationship. *Am J Physiol Endocrinol Metab*, *302*(11), E1329-1342. https://doi.org/10.1152/ajpendo.00525.2011

Doyle, L. M., & Wang, M. Z. (2019). Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells*, *8*(7). https://doi.org/10.3390/cells8070727

Duan, W., Shen, X., Lei, J., Xu, Q., Yu, Y., Li, R., Wu, E., & Ma, Q. (2014). Hyperglycemia, a neglected factor during cancer progression. *Biomed Res Int*, *2014*, 461917. https://doi.org/10.1155/2014/461917

Duran, R. V., & Hall, M. N. (2012). Glutaminolysis feeds mTORC1. *Cell Cycle*, *11*(22), 4107-4108. https://doi.org/10.4161/cc.22632

Dutta, D., Ghosh, S., Pandit, K., Mukhopadhyay, P., & Chowdhury, S. (2012). Leptin and cancer: Pathogenesis and modulation. *Indian J Endocrinol Metab*, *16*(Suppl 3), S596-600. https://doi.org/10.4103/2230-8210.105577

Eder, K., Baffy, N., Falus, A., & Fulop, A. K. (2009). The major inflammatory mediator interleukin-6 and obesity. *Inflamm Res*, *58*(11), 727-736. https://doi.org/10.1007/s00011-009-0060-4

Ekstrom, E. J., Bergenfelz, C., von Bulow, V., Serifler, F., Carlemalm, E., Jonsson, G., Andersson, T., & Leandersson, K. (2014). WNT5A induces release of exosomes containing pro-angiogenic and immunosuppressive factors from malignant melanoma cells. *Mol Cancer*, *13*, 88. https://doi.org/10.1186/1476-4598-13-88

Ellulu, M. S., Patimah, I., Khaza'ai, H., Rahmat, A., & Abed, Y. (2017). Obesity and inflammation: the linking mechanism and the complications. *Arch Med Sci*, *13*(4), 851-863. https://doi.org/10.5114/aoms.2016.58928

Emont, M. P., Jacobs, C., Essene, A. L., Pant, D., Tenen, D., Colleluori, G., Di Vincenzo, A., Jorgensen, A. M., Dashti, H., Stefek, A., McGonagle, E., Strobel, S., Laber, S., Agrawal, S., Westcott, G. P., Kar, A., Veregge, M. L., Gulko, A., Srinivasan, H., . . . Rosen, E. D. (2022). A single-cell atlas of human and mouse white adipose tissue. *Nature*, *603*(7903), 926-933. https://doi.org/10.1038/s41586-022-04518-2

Endo, H., Hosono, K., Uchiyama, T., Sakai, E., Sugiyama, M., Takahashi, H., Nakajima, N., Wada, K., Takeda, K., Nakagama, H., & Nakajima, A. (2011). Leptin acts as a growth factor for colorectal tumours at stages subsequent to tumour initiation in murine colon carcinogenesis. *Gut*, *60*(10), 1363-1371. https://doi.org/10.1136/gut.2010.235754

Engelman, J. A. (2009). Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*, *9*(8), 550-562. https://doi.org/10.1038/nrc2664

Feng, J., Qiu, S., Zhou, S., Tan, Y., Bai, Y., Cao, H., Guo, J., & Su, Z. (2022). mTOR: A Potential New Target in Nonalcoholic Fatty Liver Disease. *Int J Mol Sci*, *23*(16). https://doi.org/10.3390/ijms23169196

Flegal, K. M., Kruszon-Moran, D., Carroll, M. D., Fryar, C. D., & Ogden, C. L. (2016). Trends in Obesity Among Adults in the United States, 2005 to 2014. *Jama*, *315*(21), 2284-2291. https://doi.org/10.1001/jama.2016.6458

Francisco, V., Pino, J., Campos-Cabaleiro, V., Ruiz-Fernandez, C., Mera, A., Gonzalez-Gay, M. A., Gomez, R., & Gualillo, O. (2018). Obesity, Fat Mass and Immune System: Role for Leptin. *Front Physiol*, *9*, 640. https://doi.org/10.3389/fphys.2018.00640

Frezza, E. E., Wachtel, M. S., & Chiriva-Internati, M. (2006). Influence of obesity on the risk of developing colon cancer. *Gut*, *55*(2), 285-291. https://doi.org/10.1136/gut.2005.073163

Funcke, J. B., & Scherer, P. E. (2019). Beyond adiponectin and leptin: adipose tissue-derived mediators of inter-organ communication. *J Lipid Res*, *60*(10), 1648-1684. https://doi.org/10.1194/jlr.R094060

Furuya, M., Horiguchi, J., Nakajima, H., Kanai, Y., & Oyama, T. (2012). Correlation of L-type amino acid transporter 1 and CD98 expression with triple negative breast cancer prognosis. *Cancer Sci*, *103*(2), 382-389. https://doi.org/10.1111/j.1349-7006.2011.02151.x

Fuster, J. J., Ouchi, N., Gokce, N., & Walsh, K. (2016). Obesity-Induced Changes in Adipose Tissue Microenvironment and Their Impact on Cardiovascular Disease. *Circ Res*, *118*(11), 1786-1807. https://doi.org/10.1161/CIRCRESAHA.115.306885

Gadhoum, S. Z., Madhoun, N. Y., Abuelela, A. F., & Merzaban, J. S. (2016). Anti-CD44 antibodies inhibit both mTORC1 and mTORC2: a new rationale supporting CD44-induced AML differentiation therapy. *Leukemia*, *30*(12), 2397-2401. https://doi.org/10.1038/leu.2016.221

Garcia-Cruz, E., Piqueras, M., Huguet, J., Peri, L., Izquierdo, L., Musquera, M., Franco, A., Alvarez-Vijande, R., Ribal, M. J., & Alcaraz, A. (2012). Low testosterone levels are related to poor prognosis factors in men with prostate cancer prior to treatment. *BJU Int*, *110*(11 Pt B), E541-546. https://doi.org/10.1111/j.1464-410X.2012.11232.x

Geng, Y., Chandrasekaran, S., Hsu, J. W., Gidwani, M., Hughes, A. D., & King, M. R. (2013). Phenotypic switch in blood: effects of pro-inflammatory cytokines on breast cancer cell aggregation and adhesion. *PLoS One*, *8*(1), e54959. https://doi.org/10.1371/journal.pone.0054959

Gesierich, S., Berezovskiy, I., Ryschich, E., & Zoller, M. (2006). Systemic induction of the angiogenesis switch by the tetraspanin D6.1A/CO-029. *Cancer Res*, *66*(14), 7083-7094. https://doi.org/10.1158/0008-5472.CAN-06-0391

Giatromanolaki, A., Sivridis, E., Fiska, A., & Koukourakis, M. I. (2011). The CD44+/CD24- phenotype relates to 'triple-negative' state and unfavorable prognosis in breast cancer patients. *Med Oncol*, *28*(3), 745-752. https://doi.org/10.1007/s12032-010-9530-3

Giuli, M. V., Giuliani, E., Screpanti, I., Bellavia, D., & Checquolo, S. (2019). Notch Signaling Activation as a Hallmark for Triple-Negative Breast Cancer Subtype. *J Oncol*, *2019*, 8707053. https://doi.org/10.1155/2019/8707053

Gleason, C. E., Lu, D., Witters, L. A., Newgard, C. B., & Birnbaum, M. J. (2007). The role of AMPK and mTOR in nutrient sensing in pancreatic beta-cells. *J Biol Chem*, *282*(14), 10341-10351. https://doi.org/10.1074/jbc.M610631200

Guerrero-Zotano, A., Mayer, I. A., & Arteaga, C. L. (2016). PI3K/AKT/mTOR: role in breast cancer progression, drug resistance, and treatment. *Cancer Metastasis Rev*, *35*(4), 515-524. https://doi.org/10.1007/s10555-016-9637-x

Guertin, D. A., Stevens, D. M., Saitoh, M., Kinkel, S., Crosby, K., Sheen, J. H., Mullholland, D. J., Magnuson, M. A., Wu, H., & Sabatini, D. M. (2009). mTOR complex 2 is required for the development of prostate cancer induced by Pten loss in mice. *Cancer Cell*, *15*(2), 148-159. https://doi.org/10.1016/j.ccr.2008.12.017

Hafliger, P., & Charles, R. P. (2019). The L-Type Amino Acid Transporter LAT1-An Emerging Target in Cancer. *Int J Mol Sci*, *20*(10). https://doi.org/10.3390/ijms20102428

Hafliger, P., Graff, J., Rubin, M., Stooss, A., Dettmer, M. S., Altmann, K. H., Gertsch, J., & Charles, R. P. (2018). The LAT1 inhibitor JPH203 reduces growth of thyroid carcinoma in a fully immunocompetent mouse model. *J Exp Clin Cancer Res*, *37*(1), 234. https://doi.org/10.1186/s13046-018-0907-z

Haggar, F. A., & Boushey, R. P. (2009). Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clin Colon Rectal Surg*, *22*(4), 191-197. https://doi.org/10.1055/s-0029-1242458

Haining, Z., Kawai, N., Miyake, K., Okada, M., Okubo, S., Zhang, X., Fei, Z., & Tamiya, T. (2012). Relation of LAT1/4F2hc expression with pathological grade, proliferation and angiogenesis in human gliomas. *BMC Clin Pathol*, *12*, 4. https://doi.org/10.1186/1472-6890-12-4

Halberg, N., Khan, T., Trujillo, M. E., Wernstedt-Asterholm, I., Attie, A. D., Sherwani, S., Wang, Z. V., Landskroner-Eiger, S., Dineen, S., Magalang, U. J., Brekken, R. A., & Scherer, P. E. (2009). Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. *Mol Cell Biol*, *29*(16), 4467-4483. https://doi.org/10.1128/MCB.00192-09

Hales, C. M., Carroll, M. D., Fryar, C. D., & Ogden, C. L. (2017). Prevalence of Obesity Among Adults and Youth: United States, 2015-2016. *NCHS Data Brief*(288), 1-8.

Han, J., Zhang, L., Guo, H., Wysham, W. Z., Roque, D. R., Willson, A. K., Sheng, X., Zhou, C., & Bae-Jump, V. L. (2015). Glucose promotes cell proliferation, glucose uptake and invasion in endometrial cancer cells via AMPK/mTOR/S6 and MAPK signaling. *Gynecol Oncol*, *138*(3), 668-675. https://doi.org/10.1016/j.ygyno.2015.06.036

Harachi, M., Masui, K., Okamura, Y., Tsukui, R., Mischel, P. S., & Shibata, N. (2018). mTOR Complexes as a Nutrient Sensor for Driving Cancer Progression. *Int J Mol Sci*, *19*(10). https://doi.org/10.3390/ijms19103267

Harbour, S. N., DiToro, D. F., Witte, S. J., Zindl, C. L., Gao, M., Schoeb, T. R., Jones, G. W., Jones, S. A., Hatton, R. D., & Weaver, C. T. (2020). T(H)17 cells require ongoing classic IL-6 receptor signaling to retain transcriptional and functional identity. *Sci Immunol*, *5*(49). https://doi.org/10.1126/sciimmunol.aaw2262

Haun, C. T., Vann, C. G., Osburn, S. C., Mumford, P. W., Roberson, P. A., Romero, M. A., Fox, C. D., Johnson, C. A., Parry, H. A., Kavazis, A. N., Moon, J. R., Badisa, V. L. D., Mwashote, B. M., Ibeanusi, V., Young, K. C., & Roberts, M. D. (2019). Muscle fiber hypertrophy in response to 6 weeks of high-volume resistance training in trained young men is largely attributed to sarcoplasmic hypertrophy. *PLoS One*, *14*(6), e0215267. https://doi.org/10.1371/journal.pone.0215267

Hayase, S., Kumamoto, K., Saito, K., Kofunato, Y., Sato, Y., Okayama, H., Miyamoto, K., Ohki, S., & Takenoshita, S. (2017). L-type amino acid transporter 1 expression is upregulated and associated with cellular proliferation in colorectal cancer. *Oncol Lett*, *14*(6), 7410-7416. https://doi.org/10.3892/ol.2017.7148

Higuchi, K., Sakamoto, S., Ando, K., Maimaiti, M., Takeshita, N., Okunushi, K., Reien, Y., Imamura, Y., Sazuka, T., Nakamura, K., Matsushima, J., Furihata, T., Ikehara, Y., Ichikawa, T., & Anzai, N. (2019). Characterization of the expression of LAT1 as a prognostic indicator and a therapeutic target in renal cell carcinoma. *Sci Rep*, *9*(1), 16776. https://doi.org/10.1038/s41598-019-53397-7

Holmes, M. D., Chen, W. Y., Feskanich, D., Kroenke, C. H., & Colditz, G. A. (2005). Physical activity and survival after breast cancer diagnosis. *JAMA*, *293*(20), 2479-2486. https://doi.org/10.1001/jama.293.20.2479

Honkala, S. M., Motiani, P., Kivela, R., Hemanthakumar, K. A., Tolvanen, E., Motiani, K. K., Eskelinen, J. J., Virtanen, K. A., Kemppainen, J., Heiskanen, M. A., Loyttyniemi, E., Nuutila, P., Kalliokoski, K. K., & Hannukainen, J. C. (2020). Exercise training improves adipose tissue metabolism and vasculature

regardless of baseline glucose tolerance and sex. *BMJ Open Diabetes Res Care*, *8*(1). https://doi.org/10.1136/bmjdrc-2019-000830

Houghton, P. J. (2010). Everolimus. *Clin Cancer Res*, *16*(5), 1368-1372. https://doi.org/10.1158/1078-0432.CCR-09-1314

Howard, F. M., & Olopade, O. I. (2021). Epidemiology of Triple-Negative Breast Cancer: A Review. *Cancer J*, *27*(1), 8-16. https://doi.org/10.1097/PPO.0000000000000500

Hristova, N. R., Tagscherer, K. E., Fassl, A., Kopitz, J., & Roth, W. (2013). Notch1-dependent regulation of p27 determines cell fate in colorectal cancer. *Int J Oncol*, *43*(6), 1967-1975. https://doi.org/10.3892/ijo.2013.2140

Hua, H., Kong, Q., Zhang, H., Wang, J., Luo, T., & Jiang, Y. (2019). Targeting mTOR for cancer therapy. *J Hematol Oncol*, *12*(1), 71. https://doi.org/10.1186/s13045-019-0754-1

Ichinoe, M., Yanagisawa, N., Mikami, T., Hana, K., Nakada, N., Endou, H., Okayasu, I., & Murakumo, Y. (2015). L-Type amino acid transporter 1 (LAT1) expression in lymph node metastasis of gastric carcinoma: Its correlation with size of metastatic lesion and Ki-67 labeling. *Pathol Res Pract*, *211*(7), 533-538. https://doi.org/10.1016/j.prp.2015.03.007

Iwaya, T., Yokobori, T., Nishida, N., Kogo, R., Sudo, T., Tanaka, F., Shibata, K., Sawada, G., Takahashi, Y., Ishibashi, M., Wakabayashi, G., Mori, M., & Mimori, K. (2012). Downregulation of miR-144 is associated with colorectal cancer progression via activation of mTOR signaling pathway. *Carcinogenesis*, *33*(12), 2391-2397. https://doi.org/10.1093/carcin/bgs288

Jablonska-Trypuc, A., Matejczyk, M., & Rosochacki, S. (2016). Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. *J Enzyme Inhib Med Chem*, *31*(sup1), 177-183. https://doi.org/10.3109/14756366.2016.1161620

Jewell, J. L., Kim, Y. C., Russell, R. C., Yu, F. X., Park, H. W., Plouffe, S. W., Tagliabracci, V. S., & Guan, K. L. (2015). Metabolism. Differential regulation of mTORC1 by leucine and glutamine. *Science*, *347*(6218), 194-198. https://doi.org/10.1126/science.1259472

Jiao, P., Chen, Q., Shah, S., Du, J., Tao, B., Tzameli, I., Yan, W., & Xu, H. (2009). Obesity-related upregulation of monocyte chemotactic factors in adipocytes: involvement of nuclear factor-kappaB and c-Jun NH2-terminal kinase pathways. *Diabetes*, *58*(1), 104-115. https://doi.org/10.2337/db07-1344

Jiralerspong, S., & Goodwin, P. J. (2016). Obesity and Breast Cancer Prognosis: Evidence, Challenges, and Opportunities. *J Clin Oncol*, *34*(35), 4203-4216. https://doi.org/10.1200/JCO.2016.68.4480

Jones, L. W., Viglianti, B. L., Tashjian, J. A., Kothadia, S. M., Keir, S. T., Freedland, S. J., Potter, M. Q., Moon, E. J., Schroeder, T., Herndon, J. E., 2nd, & Dewhirst, M. W. (2010). Effect of aerobic exercise on tumor physiology in an animal model of human breast cancer. *J Appl Physiol (1985)*, *108*(2), 343-348. https://doi.org/10.1152/japplphysiol.00424.2009

Jones, M. E., Schoemaker, M. J., Wright, L. B., Ashworth, A., & Swerdlow, A. J. (2017). Smoking and risk of breast cancer in the Generations Study cohort. *Breast Cancer Res*, *19*(1), 118. https://doi.org/10.1186/s13058-017-0908-4

Jones, S. B., Thomas, G. A., Hesselsweet, S. D., Alvarez-Reeves, M., Yu, H., & Irwin, M. L. (2013). Effect of exercise on markers of inflammation in breast cancer survivors: the Yale exercise and survivorship study. *Cancer Prev Res (Phila)*, *6*(2), 109-118. https://doi.org/10.1158/1940-6207.CAPR-12-0278

Juarez-Cruz, J. C., Zuniga-Eulogio, M. D., Olea-Flores, M., Castaneda-Saucedo, E., Mendoza-Catalan, M. A., Ortuno-Pineda, C., Moreno-Godinez, M. E., Villegas-Comonfort, S., Padilla-Benavides, T., & Navarro-Tito, N. (2019). Leptin induces cell migration and invasion in a FAK-Src-dependent manner in breast cancer cells. *Endocr Connect*, *8*(11), 1539-1552. https://doi.org/10.1530/EC-19-0442

Kaira, K., Sunose, Y., Arakawa, K., Ogawa, T., Sunaga, N., Shimizu, K., Tominaga, H., Oriuchi, N., Itoh, H., Nagamori, S., Kanai, Y., Segawa, A., Furuya, M., Mori, M., Oyama, T., & Takeyoshi, I. (2012). Prognostic significance of L-type amino-acid transporter 1 expression in surgically resected pancreatic cancer. *Br J Cancer*, *107*(4), 632-638. https://doi.org/10.1038/bjc.2012.310

Kanai, Y. (2022). Amino acid transporter LAT1 (SLC7A5) as a molecular target for cancer diagnosis and therapeutics. *Pharmacol Ther*, *230*, 107964. https://doi.org/10.1016/j.pharmthera.2021.107964

Kandasamy, K., Mohan, S. S., Raju, R., Keerthikumar, S., Kumar, G. S., Venugopal, A. K., Telikicherla, D., Navarro, J. D., Mathivanan, S., Pecquet, C., Gollapudi, S. K., Tattikota, S. G., Mohan, S., Padhukasahasram, H., Subbannayya, Y., Goel, R., Jacob, H. K., Zhong, J., Sekhar, R., . . . Pandey, A. (2010). NetPath: a public resource of curated signal transduction pathways. *Genome Biol*, *11*(1), R3. https://doi.org/10.1186/gb-2010-11-1-r3

Kapoor, N., Arora, S., & Kalra, S. (2021). Gender Disparities in People Living with Obesity - An Unchartered Territory. *J Midlife Health*, *12*(2), 103-107. https://doi.org/10.4103/jmh.jmh_48_21

Kazmi, A., Sattar, A., Hashim, R., Khan, S. P., Younus, M., & Khan, F. A. (2013). Serum leptin values in the healthy obese and non-obese subjects of Rawalpindi. *J Pak Med Assoc*, *63*(2), 245-248. https://www.ncbi.nlm.nih.gov/pubmed/23894904

Kelesidis, T., Kelesidis, I., Chou, S., & Mantzoros, C. S. (2010). Narrative review: the role of leptin in human physiology: emerging clinical applications. *Ann Intern Med*, *152*(2), 93-100. https://doi.org/10.7326/0003-4819-152-2-201001190-00008

Kelly, D. M., & Jones, T. H. (2015). Testosterone and obesity. *Obes Rev*, *16*(7), 581-606. https://doi.org/10.1111/obr.12282

Kenfield, S. A., Stampfer, M. J., Giovannucci, E., & Chan, J. M. (2011). Physical activity and survival after prostate cancer diagnosis in the health professionals follow-up study. *J Clin Oncol*, *29*(6), 726-732. https://doi.org/10.1200/JCO.2010.31.5226

Khabaz, M. N., Abdelrahman, A., Butt, N., Damnhory, L., Elshal, M., Aldahlawi, A. M., Ashoor, S., Al-Maghrabi, B., Dobson, P., Brown, B., Al-Sakkaf, K., Al-Qahtani, M., & Al-Maghrabi, J. (2017). Immunohistochemical staining of leptin is associated with grade, stage, lymph node involvement, recurrence, and hormone receptor phenotypes in breast cancer. *BMC Womens Health*, *17*(1), 105. https://doi.org/10.1186/s12905-017-0459-y

Khamzina, L., Veilleux, A., Bergeron, S., & Marette, A. (2005). Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. *Endocrinology*, *146*(3), 1473-1481. https://doi.org/10.1210/en.2004-0921

Khan, M. A., Jain, V. K., Rizwanullah, M., Ahmad, J., & Jain, K. (2019). PI3K/AKT/mTOR pathway inhibitors in triple-negative breast cancer: a review on

drug discovery and future challenges. *Drug Discov Today*, *24*(11), 2181-2191. https://doi.org/10.1016/j.drudis.2019.09.001

Khan, T., Muise, E. S., Iyengar, P., Wang, Z. V., Chandalia, M., Abate, N., Zhang, B. B., Bonaldo, P., Chua, S., & Scherer, P. E. (2009). Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. *Mol Cell Biol*, *29*(6), 1575-1591. https://doi.org/10.1128/MCB.01300-08

Kim, D. H., Sarbassov, D. D., Ali, S. M., Latek, R. R., Guntur, K. V., Erdjument-Bromage, H., Tempst, P., & Sabatini, D. M. (2003). GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol Cell*, *11*(4), 895-904. https://doi.org/10.1016/s1097-2765(03)00114-x

Kim, L. C., Cook, R. S., & Chen, J. (2017). mTORC1 and mTORC2 in cancer and the tumor microenvironment. *Oncogene*, *36*(16), 2191-2201. https://doi.org/10.1038/onc.2016.363

Kim, Y., & Kim, O. K. (2021). Potential Roles of Adipocyte Extracellular Vesicle-Derived miRNAs in Obesity-Mediated Insulin Resistance. *Adv Nutr*, *12*(2), 566-574. https://doi.org/10.1093/advances/nmaa105

Kimura, A., & Kishimoto, T. (2010). IL-6: regulator of Treg/Th17 balance. *Eur J Immunol*, *40*(7), 1830-1835. https://doi.org/10.1002/eji.201040391

King, H. W., Michael, M. Z., & Gleadle, J. M. (2012). Hypoxic enhancement of exosome release by breast cancer cells. *BMC Cancer*, *12*, 421. https://doi.org/10.1186/1471-2407-12-421

Kita, S., Maeda, N., & Shimomura, I. (2019). Interorgan communication by exosomes, adipose tissue, and adiponectin in metabolic syndrome. *J Clin Invest*, *129*(10), 4041-4049. https://doi.org/10.1172/JCI129193

Kleinendorst, L., Abawi, O., van der Kamp, H. J., Alders, M., Meijers-Heijboer, H. E. J., van Rossum, E. F. C., van den Akker, E. L. T., & van Haelst, M. M. (2020). Leptin receptor deficiency: a systematic literature review and prevalence estimation based on population genetics. *Eur J Endocrinol*, *182*(1), 47-56. https://doi.org/10.1530/EJE-19-0678

Klintman, M., Rosendahl, A. H., Randeris, B., Eriksson, M., Czene, K., Hall, P., & Borgquist, S. (2022). Postmenopausal overweight and breast cancer risk; results from the KARMA cohort. *Breast Cancer Res Treat*, *196*(1), 185-196. https://doi.org/10.1007/s10549-022-06664-7

Kobayashi, H., Ishii, Y., & Takayama, T. (2005). Expression of L-type amino acid transporter 1 (LAT1) in esophageal carcinoma. *J Surg Oncol*, *90*(4), 233-238. https://doi.org/10.1002/jso.20257

Kopan, R. (2012). Notch signaling. *Cold Spring Harb Perspect Biol*, *4*(10). https://doi.org/10.1101/cshperspect.a011213

Koshi, H., Sano, T., Handa, T., Yanagawa, T., Saitou, K., Nagamori, S., Kanai, Y., Takagishi, K., & Oyama, T. (2015). L-type amino acid transporter-1 and CD98 expression in bone and soft tissue tumors. *Pathol Int*, *65*(9), 460-467. https://doi.org/10.1111/pin.12323

Kumar, P., & Aggarwal, R. (2016). An overview of triple-negative breast cancer. *Arch Gynecol Obstet*, *293*(2), 247-269. https://doi.org/10.1007/s00404-015-3859-y

Kumar, V., Kiran, S., Kumar, S., & Singh, U. P. (2022). Extracellular vesicles in obesity and its associated inflammation. *Int Rev Immunol*, *41*(1), 30-44. https://doi.org/10.1080/08830185.2021.1964497

Kunanopparat, A., Hirankarn, N., Issara-Amphorn, J., Tangkijvanich, P., & Sanpavat, A. (2021). The expression profile of Jagged1 and Delta-like 4 in hepatocellular carcinoma. *Asian Pac J Allergy Immunol*, *39*(1), 44-52. https://doi.org/10.12932/AP-040818-0388

Kusminski, C. M., Bickel, P. E., & Scherer, P. E. (2016). Targeting adipose tissue in the treatment of obesity-associated diabetes. *Nat Rev Drug Discov*, *15*(9), 639-660. https://doi.org/10.1038/nrd.2016.75

Kwan, H. Y., Chen, M., Xu, K., & Chen, B. (2021). The impact of obesity on adipocyte-derived extracellular vesicles. *Cell Mol Life Sci*, *78*(23), 7275-7288. https://doi.org/10.1007/s00018-021-03973-w

Landman, R. E., Puder, J. J., Xiao, E., Freda, P. U., Ferin, M., & Wardlaw, S. L. (2003). Endotoxin stimulates leptin in the human and nonhuman primate. *J Clin Endocrinol Metab*, *88*(3), 1285-1291. https://doi.org/10.1210/jc.2002-021393

Laplante, M., & Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell*, *149*(2), 274-293. https://doi.org/10.1016/j.cell.2012.03.017

Lazar, I., Clement, E., Dauvillier, S., Milhas, D., Ducoux-Petit, M., LeGonidec, S., Moro, C., Soldan, V., Dalle, S., Balor, S., Golzio, M., Burlet-Schiltz, O., Valet, P., Muller, C., & Nieto, L. (2016). Adipocyte Exosomes Promote Melanoma Aggressiveness through Fatty Acid Oxidation: A Novel Mechanism Linking Obesity and Cancer. *Cancer Res*, *76*(14), 4051-4057. https://doi.org/10.1158/0008-5472.CAN-16-0651

Lengyel, E., Makowski, L., DiGiovanni, J., & Kolonin, M. G. (2018). Cancer as a Matter of Fat: The Crosstalk between Adipose Tissue and Tumors. *Trends Cancer*, *4*(5), 374-384. https://doi.org/10.1016/j.trecan.2018.03.004

Levi, Z., Kark, J. D., Katz, L. H., Twig, G., Derazne, E., Tzur, D., Leibovici Weissman, Y., Leiba, A., Lipshiez, I., Keinan Boker, L., & Afek, A. (2017). Adolescent body mass index and risk of colon and rectal cancer in a cohort of 1.79 million Israeli men and women: A population-based study. *Cancer*, *123*(20), 4022-4030. https://doi.org/10.1002/cncr.30819

Lewandowska, A. M., Rudzki, M., Rudzki, S., Lewandowski, T., & Laskowska, B. (2019). Environmental risk factors for cancer - review paper. *Ann Agric Environ Med*, *26*(1), 1-7. https://doi.org/10.26444/aaem/94299

Li, Q., Zhang, J., Zhou, Y., & Qiao, L. (2012). Obesity and gastric cancer. *Front Biosci (Landmark Ed)*, *17*, 2383-2390. https://doi.org/10.2741/4059

Li, W., Zhang, X., Sang, H., Zhou, Y., Shang, C., Wang, Y., & Zhu, H. (2019). Effects of hyperglycemia on the progression of tumor diseases. *J Exp Clin Cancer Res*, *38*(1), 327. https://doi.org/10.1186/s13046-019-1309-6

Liang, Y., Cardoso, F. F., Parys, C., Cardoso, F. C., & Loor, J. J. (2021). Branched-Chain Amino Acid Supplementation Alters the Abundance of Mechanistic Target of Rapamycin and Insulin Signaling Proteins in Subcutaneous Adipose Explants from Lactating Holstein Cows. *Animals (Basel)*, *11*(9). https://doi.org/10.3390/ani11092714

Lipsey, C. C., Harbuzariu, A., Robey, R. W., Huff, L. M., Gottesman, M. M., & Gonzalez-Perez, R. R. (2020). Leptin Signaling Affects Survival and Chemoresistance of Estrogen Receptor Negative Breast Cancer. *Int J Mol Sci*, *21*(11). https://doi.org/10.3390/ijms21113794

Liu, J. F., Chen, P. C., Chang, T. M., & Hou, C. H. (2020). Monocyte Chemoattractant Protein-1 promotes cancer cell migration via c-Raf/MAPK/AP-1 pathway and MMP-9 production in osteosarcoma. *J Exp Clin Cancer Res*, *39*(1), 254. https://doi.org/10.1186/s13046-020-01756-y

Liu, M. L., Scalia, R., Mehta, J. L., & Williams, K. J. (2012). Cholesterol-induced membrane microvesicles as novel carriers of damage-associated molecular patterns: mechanisms of formation, action, and detoxification. *Arterioscler Thromb Vasc Biol*, *32*(9), 2113-2121. https://doi.org/10.1161/ATVBAHA.112.255471

Liu, P. H., Wu, K., Ng, K., Zauber, A. G., Nguyen, L. H., Song, M., He, X., Fuchs, C. S., Ogino, S., Willett, W. C., Chan, A. T., Giovannucci, E. L., & Cao, Y. (2019). Association of Obesity With Risk of Early-Onset Colorectal Cancer Among Women. *JAMA Oncol*, *5*(1), 37-44. https://doi.org/10.1001/jamaoncol.2018.4280

Liu, T., Zhang, L., Joo, D., & Sun, S. C. (2017). NF-kappaB signaling in inflammation. *Signal Transduct Target Ther*, *2*, 17023-. https://doi.org/10.1038/sigtrans.2017.23

Liu, X., Taftaf, R., Kawaguchi, M., Chang, Y. F., Chen, W., Entenberg, D., Zhang, Y., Gerratana, L., Huang, S., Patel, D. B., Tsui, E., Adorno-Cruz, V., Chirieleison, S. M., Cao, Y., Harney, A. S., Patel, S., Patsialou, A., Shen, Y., Avril, S., . . . Liu, H. (2019). Homophilic CD44 Interactions Mediate Tumor Cell Aggregation and Polyclonal Metastasis in Patient-Derived Breast Cancer Models. *Cancer Discov*, *9*(1), 96-113. https://doi.org/10.1158/2159-8290.CD-18-0065

Lobry, C., Oh, P., Mansour, M. R., Look, A. T., & Aifantis, I. (2014). Notch signaling: switching an oncogene to a tumor suppressor. *Blood*, *123*(16), 2451-2459. https://doi.org/10.1182/blood-2013-08-355818

Lu, J., Ye, X., Fan, F., Xia, L., Bhattacharya, R., Bellister, S., Tozzi, F., Sceusi, E., Zhou, Y., Tachibana, I., Maru, D. M., Hawke, D. H., Rak, J., Mani, S. A., Zweidler-McKay, P., & Ellis, L. M. (2013). Endothelial cells promote the colorectal cancer stem cell phenotype through a soluble form of Jagged-1. *Cancer Cell*, *23*(2), 171-185. https://doi.org/10.1016/j.ccr.2012.12.021

Lukasiewicz, S., Czeczelewski, M., Forma, A., Baj, J., Sitarz, R., & Stanislawek, A. (2021). Breast Cancer-Epidemiology, Risk Factors, Classification, Prognostic Markers, and Current Treatment Strategies-An Updated Review. *Cancers (Basel)*, *13*(17). https://doi.org/10.3390/cancers13174287

Lynch, C. J., Gern, B., Lloyd, C., Hutson, S. M., Eicher, R., & Vary, T. C. (2006). Leucine in food mediates some of the postprandial rise in plasma leptin concentrations. *Am J Physiol Endocrinol Metab*, *291*(3), E621-630. https://doi.org/10.1152/ajpendo.00462.2005

Lynch, C. J., Halle, B., Fujii, H., Vary, T. C., Wallin, R., Damuni, Z., & Hutson, S. M. (2003). Potential role of leucine metabolism in the leucine-signaling pathway involving mTOR. *Am J Physiol Endocrinol Metab*, *285*(4), E854-863. https://doi.org/10.1152/ajpendo.00153.2003

Ma, Y., Ren, Y., Dai, Z. J., Wu, C. J., Ji, Y. H., & Xu, J. (2017). IL-6, IL-8 and TNF-alpha levels correlate with disease stage in breast cancer patients. *Adv Clin Exp Med*, *26*(3), 421-426. https://doi.org/10.17219/acem/62120

Madani, R., Karastergiou, K., Ogston, N. C., Miheisi, N., Bhome, R., Haloob, N., Tan, G. D., Karpe, F., Malone-Lee, J., Hashemi, M., Jahangiri, M., & Mohamed-Ali, V. (2009). RANTES release by human adipose tissue in vivo and evidence for depot-specific differences. *Am J Physiol Endocrinol Metab*, *296*(6), E1262-1268. https://doi.org/10.1152/ajpendo.90511.2008

Mao, Z., & Zhang, W. (2018). Role of mTOR in Glucose and Lipid Metabolism. *Int J Mol Sci*, *19*(7). https://doi.org/10.3390/ijms19072043

Marengo, A., Rosso, C., & Bugianesi, E. (2016). Liver Cancer: Connections with Obesity, Fatty Liver, and Cirrhosis. *Annu Rev Med*, *67*, 103-117. https://doi.org/10.1146/annurev-med-090514-013832

Masui, K., Cavenee, W. K., & Mischel, P. S. (2014). mTORC2 in the center of cancer metabolic reprogramming. *Trends Endocrinol Metab*, *25*(7), 364-373. https://doi.org/10.1016/j.tem.2014.04.002

Mathew, R., Karantza-Wadsworth, V., & White, E. (2007). Role of autophagy in cancer. *Nat Rev Cancer*, *7*(12), 961-967. https://doi.org/10.1038/nrc2254

Matos, A., Marinho-Dias, J., Ramalheira, S., Oliveira, M. J., Bicho, M., & Ribeiro, R. (2016). Mechanisms underlying the association between obesity and Hodgkin lymphoma. *Tumour Biol*, *37*(10), 13005-13016. https://doi.org/10.1007/s13277-016-5198-4

Matrone, A., Ferrari, F., Santini, F., & Elisei, R. (2020). Obesity as a risk factor for thyroid cancer. *Curr Opin Endocrinol Diabetes Obes*, *27*(5), 358-363. https://doi.org/10.1097/MED.0000000000000556

McMahon, G., Weir, M. R., Li, X. C., & Mandelbrot, D. A. (2011). The evolving role of mTOR inhibition in transplantation tolerance. *J Am Soc Nephrol*, *22*(3), 408-415. https://doi.org/10.1681/ASN.2010040351

Meng, D., Yang, Q., Wang, H., Melick, C. H., Navlani, R., Frank, A. R., & Jewell, J. L. (2020). Glutamine and asparagine activate mTORC1 independently of Rag GTPases. *J Biol Chem*, *295*(10), 2890-2899. https://doi.org/10.1074/jbc.AC119.011578

Meurette, O., & Mehlen, P. (2018). Notch Signaling in the Tumor Microenvironment. *Cancer Cell*, *34*(4), 536-548. https://doi.org/10.1016/j.ccell.2018.07.009

Meyerhardt, J. A., Giovannucci, E. L., Holmes, M. D., Chan, A. T., Chan, J. A., Colditz, G. A., & Fuchs, C. S. (2006). Physical activity and survival after colorectal cancer diagnosis. *J Clin Oncol*, *24*(22), 3527-3534. https://doi.org/10.1200/JCO.2006.06.0855

Millan, O., Jimenez, O., Fortuna, V., Barcelo, J. J., & Brunet, M. (2006). Role of FK778 alone or in combination with tacrolimus or mTOR inhibitors as an immunomodulator of immunofunctions: in vitro evaluation of T cell proliferation and the expression of lymphocyte surface antigens. *Int J Immunopathol Pharmacol*, *19*(2), 317-330. https://www.ncbi.nlm.nih.gov/pubmed/16831299

Mitsdoerffer, M., Lee, Y., Jager, A., Kim, H. J., Korn, T., Kolls, J. K., Cantor, H., Bettelli, E., & Kuchroo, V. K. (2010). Proinflammatory T helper type 17 cells are effective B-cell helpers. *Proc Natl Acad Sci U S A*, *107*(32), 14292-14297. https://doi.org/10.1073/pnas.1009234107

Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D. R., Miles, J. M., Yudkin, J. S., Klein, S., & Coppack, S. W. (1997). Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab*, *82*(12), 4196-4200. https://doi.org/10.1210/jcem.82.12.4450

Mondal, S., Adhikari, N., Banerjee, S., Amin, S. A., & Jha, T. (2020). Matrix metalloproteinase-9 (MMP-9) and its inhibitors in cancer: A minireview. *Eur J Med Chem*, *194*, 112260. https://doi.org/10.1016/j.ejmech.2020.112260

Monteiro, R., & Azevedo, I. (2010). Chronic inflammation in obesity and the metabolic syndrome. *Mediators Inflamm*, *2010*. https://doi.org/10.1155/2010/289645

Moore, S. C., Lee, I. M., Weiderpass, E., Campbell, P. T., Sampson, J. N., Kitahara, C. M., Keadle, S. K., Arem, H., Berrington de Gonzalez, A., Hartge, P., Adami, H. O., Blair, C. K., Borch, K. B., Boyd, E., Check, D. P., Fournier, A., Freedman, N. D., Gunter, M., Johannson, M., . . . Patel, A. V. (2016). Association of Leisure-Time Physical Activity With Risk of 26 Types of Cancer in 1.44 Million Adults. *JAMA Intern Med*, *176*(6), 816-825. https://doi.org/10.1001/jamainternmed.2016.1548

Morales-Martinez, M., Lichtenstein, A., & Vega, M. I. (2021). Function of Deptor and its roles in hematological malignancies. *Aging (Albany NY)*, *13*(1), 1528-1564. https://doi.org/10.18632/aging.202462

Morris, G. J., Naidu, S., Topham, A. K., Guiles, F., Xu, Y., McCue, P., Schwartz, G. F., Park, P. K., Rosenberg, A. L., Brill, K., & Mitchell, E. P. (2007). Differences in breast carcinoma characteristics in newly diagnosed African-American and Caucasian patients: a single-institution compilation compared with the National Cancer Institute's Surveillance, Epidemiology, and End Results database. *Cancer*, *110*(4), 876-884. https://doi.org/10.1002/cncr.22836

Mossmann, D., Park, S., & Hall, M. N. (2018). mTOR signalling and cellular metabolism are mutual determinants in cancer. *Nat Rev Cancer*, *18*(12), 744-757. https://doi.org/10.1038/s41568-018-0074-8

Mowad, R., Chu, Q. D., Li, B. D., Burton, G. V., Ampil, F. L., & Kim, R. H. (2013). Does obesity have an effect on outcomes in triple-negative breast cancer? *J Surg Res*, *184*(1), 253-259. https://doi.org/10.1016/j.jss.2013.05.037

Munsell, M. F., Sprague, B. L., Berry, D. A., Chisholm, G., & Trentham-Dietz, A. (2014). Body mass index and breast cancer risk according to postmenopausal estrogen-progestin use and hormone receptor status. *Epidemiol Rev*, *36*, 114-136. https://doi.org/10.1093/epirev/mxt010

Munzberg, H., & Morrison, C. D. (2015). Structure, production and signaling of leptin. *Metabolism*, *64*(1), 13-23. https://doi.org/10.1016/j.metabol.2014.09.010

Naik, A., Monjazeb, A. M., & Decock, J. (2019). The Obesity Paradox in Cancer, Tumor Immunology, and Immunotherapy: Potential Therapeutic Implications in Triple

Negative Breast Cancer. *Front Immunol*, *10*, 1940. https://doi.org/10.3389/fimmu.2019.01940

Nakashima, S. (2002). Protein kinase C alpha (PKC alpha): regulation and biological function. *J Biochem*, *132*(5), 669-675. https://doi.org/10.1093/oxfordjournals.jbchem.a003272

Nepal, S., Kim, M. J., Hong, J. T., Kim, S. H., Sohn, D. H., Lee, S. H., Song, K., Choi, D. Y., Lee, E. S., & Park, P. H. (2015). Autophagy induction by leptin contributes to suppression of apoptosis in cancer cells and xenograft model: involvement of p53/FoxO3A axis. *Oncotarget*, *6*(9), 7166-7181. https://doi.org/10.18632/oncotarget.3347

Netland, I. A., Forde, H. E., Sleire, L., Leiss, L., Rahman, M. A., Skeie, B. S., Gjerde, C. H., Enger, P. O., & Goplen, D. (2016). Dactolisib (NVP-BEZ235) toxicity in murine brain tumour models. *BMC Cancer*, *16*, 657. https://doi.org/10.1186/s12885-016-2712-4

Nettle, D., Andrews, C., & Bateson, M. (2017). Food insecurity as a driver of obesity in humans: The insurance hypothesis. *Behav Brain Sci*, *40*, e105. https://doi.org/10.1017/S0140525X16000947

Neuhouser, M. L., Aragaki, A. K., Prentice, R. L., Manson, J. E., Chlebowski, R., Carty, C. L., Ochs-Balcom, H. M., Thomson, C. A., Caan, B. J., Tinker, L. F., Urrutia, R. P., Knudtson, J., & Anderson, G. L. (2015). Overweight, Obesity, and Postmenopausal Invasive Breast Cancer Risk: A Secondary Analysis of the Women's Health Initiative Randomized Clinical Trials. *JAMA Oncol*, *1*(5), 611-621. https://doi.org/10.1001/jamaoncol.2015.1546

Newman, L. A., & Kaljee, L. M. (2017). Health Disparities and Triple-Negative Breast Cancer in African American Women: A Review. *JAMA Surg*, *152*(5), 485-493. https://doi.org/10.1001/jamasurg.2017.0005

Nojima, H., Tokunaga, C., Eguchi, S., Oshiro, N., Hidayat, S., Yoshino, K., Hara, K., Tanaka, N., Avruch, J., & Yonezawa, K. (2003). The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. *J Biol Chem*, *278*(18), 15461-15464. https://doi.org/10.1074/jbc.C200665200

Norton, K. A., Popel, A. S., & Pandey, N. B. (2015). Heterogeneity of chemokine cell-surface receptor expression in triple-negative breast cancer. *Am J Cancer Res*, *5*(4), 1295-1307. https://www.ncbi.nlm.nih.gov/pubmed/26101698

Nosalski, R., & Guzik, T. J. (2017). Perivascular adipose tissue inflammation in vascular disease. *Br J Pharmacol*, *174*(20), 3496-3513. https://doi.org/10.1111/bph.13705

Novikov, N. M., Zolotaryova, S. Y., Gautreau, A. M., & Denisov, E. V. (2021). Mutational drivers of cancer cell migration and invasion. *Br J Cancer*, *124*(1), 102-114. https://doi.org/10.1038/s41416-020-01149-0

O'Conor, C. J., Chen, T., Gonzalez, I., Cao, D., & Peng, Y. (2018). Cancer stem cells in triple-negative breast cancer: a potential target and prognostic marker. *Biomark Med*, *12*(7), 813-820. https://doi.org/10.2217/bmm-2017-0398

Obradovic, M., Sudar-Milovanovic, E., Soskic, S., Essack, M., Arya, S., Stewart, A. J., Gojobori, T., & Isenovic, E. R. (2021). Leptin and Obesity: Role and Clinical Implication. *Front Endocrinol (Lausanne)*, *12*, 585887. https://doi.org/10.3389/fendo.2021.585887

Oh, W. J., & Jacinto, E. (2011). mTOR complex 2 signaling and functions. *Cell Cycle*, *10*(14), 2305-2316. https://doi.org/10.4161/cc.10.14.16586

Ohkame, H., Masuda, H., Ishii, Y., & Kanai, Y. (2001). Expression of L-type amino acid transporter 1 (LAT1) and 4F2 heavy chain (4F2hc) in liver tumor lesions of rat models. *J Surg Oncol*, *78*(4), 265-271; discussion 271-262. https://doi.org/10.1002/jso.1165

Oikonomou, E. K., & Antoniades, C. (2019). The role of adipose tissue in cardiovascular health and disease. *Nat Rev Cardiol*, *16*(2), 83-99. https://doi.org/10.1038/s41569-018-0097-6

Okano, N., Naruge, D., Kawai, K., Kobayashi, T., Nagashima, F., Endou, H., & Furuse, J. (2020). First-in-human phase I study of JPH203, an L-type amino acid transporter 1 inhibitor, in patients with advanced solid tumors. *Invest New Drugs*, *38*(5), 1495-1506. https://doi.org/10.1007/s10637-020-00924-3

Ostrowski, M., Carmo, N. B., Krumeich, S., Fanget, I., Raposo, G., Savina, A., Moita, C. F., Schauer, K., Hume, A. N., Freitas, R. P., Goud, B., Benaroch, P., Hacohen, N., Fukuda, M., Desnos, C., Seabra, M. C., Darchen, F., Amigorena, S., Moita, L.

F., & Thery, C. (2010). Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol*, *12*(1), 19-30; sup pp 11-13. https://doi.org/10.1038/ncb2000

Otake, S., Takeda, H., Fujishima, S., Fukui, T., Orii, T., Sato, T., Sasaki, Y., Nishise, S., & Kawata, S. (2010). Decreased levels of plasma adiponectin associated with increased risk of colorectal cancer. *World J Gastroenterol*, *16*(10), 1252-1257. https://doi.org/10.3748/wjg.v16.i10.1252

Otvos, L., Jr., Kovalszky, I., Scolaro, L., Sztodola, A., Olah, J., Cassone, M., Knappe, D., Hoffmann, R., Lovas, S., Hatfield, M. P., Beko, G., Zhang, S., Wade, J. D., & Surmacz, E. (2011). Peptide-based leptin receptor antagonists for cancer treatment and appetite regulation. *Biopolymers*, *96*(2), 117-125. https://doi.org/10.1002/bip.21377

Pages, C., Simon, M. F., Valet, P., & Saulnier-Blache, J. S. (2001). Lysophosphatidic acid synthesis and release. *Prostaglandins Other Lipid Mediat*, *64*(1-4), 1-10. https://doi.org/10.1016/s0090-6980(01)00110-1

Palomeras, S., Ruiz-Martinez, S., & Puig, T. (2018). Targeting Breast Cancer Stem Cells to Overcome Treatment Resistance. *Molecules*, *23*(9). https://doi.org/10.3390/molecules23092193

Pan, J., Fan, Z., Wang, Z., Dai, Q., Xiang, Z., Yuan, F., Yan, M., Zhu, Z., Liu, B., & Li, C. (2019). CD36 mediates palmitate acid-induced metastasis of gastric cancer via AKT/GSK-3beta/beta-catenin pathway. *J Exp Clin Cancer Res*, *38*(1), 52. https://doi.org/10.1186/s13046-019-1049-7

Panigoro, S. S., Kurnia, D., Kurnia, A., Haryono, S. J., & Albar, Z. A. (2020). ALDH1 Cancer Stem Cell Marker as a Prognostic Factor in Triple-Negative Breast Cancer. *Int J Surg Oncol*, *2020*, 7863243. https://doi.org/10.1155/2020/7863243

Paranjape, T., Heneghan, H., Lindner, R., Keane, F. K., Hoffman, A., Hollestelle, A., Dorairaj, J., Geyda, K., Pelletier, C., Nallur, S., Martens, J. W., Hooning, M. J., Kerin, M., Zelterman, D., Zhu, Y., Tuck, D., Harris, L., Miller, N., Slack, F., & Weidhaas, J. (2011). A 3'-untranslated region KRAS variant and triple-negative breast cancer: a case-control and genetic analysis. *Lancet Oncol*, *12*(4), 377-386. https://doi.org/10.1016/S1470-2045(11)70044-4

Pardo, M., Roca-Rivada, A., Seoane, L. M., & Casanueva, F. F. (2012). Obesidomics: contribution of adipose tissue secretome analysis to obesity research. *Endocrine*, *41*(3), 374-383. https://doi.org/10.1007/s12020-012-9617-z

Park, H. K., & Ahima, R. S. (2014). Leptin signaling. *F1000Prime Rep*, *6*, 73. https://doi.org/10.12703/P6-73

Park, J., Morley, T. S., Kim, M., Clegg, D. J., & Scherer, P. E. (2014). Obesity and cancer--mechanisms underlying tumour progression and recurrence. *Nat Rev Endocrinol*, *10*(8), 455-465. https://doi.org/10.1038/nrendo.2014.94

Parlee, S. D., Lentz, S. I., Mori, H., & MacDougald, O. A. (2014). Quantifying size and number of adipocytes in adipose tissue. *Methods Enzymol*, *537*, 93-122. https://doi.org/10.1016/B978-0-12-411619-1.00006-9

Paz-Filho, G., Lim, E. L., Wong, M. L., & Licinio, J. (2011). Associations between adipokines and obesity-related cancer. *Front Biosci (Landmark Ed)*, *16*(5), 1634-1650. https://doi.org/10.2741/3810

Pearce, E. N. (2012). Thyroid hormone and obesity. *Curr Opin Endocrinol Diabetes Obes*, *19*(5), 408-413. https://doi.org/10.1097/MED.0b013e328355cd6c

Pedrosa, A. R., Trindade, A., Carvalho, C., Graca, J., Carvalho, S., Peleteiro, M. C., Adams, R. H., & Duarte, A. (2015). Endothelial Jagged1 promotes solid tumor growth through both pro-angiogenic and angiocrine functions. *Oncotarget*, *6*(27), 24404-24423. https://doi.org/10.18632/oncotarget.4380

Perez-Perez, A., Sanchez-Jimenez, F., Vilarino-Garcia, T., & Sanchez-Margalet, V. (2020). Role of Leptin in Inflammation and Vice Versa. *Int J Mol Sci*, *21*(16). https://doi.org/10.3390/ijms21165887

Pi-Sunyer, F. X. (2002). The obesity epidemic: pathophysiology and consequences of obesity. *Obes Res*, *10 Suppl 2*, 97S-104S. https://doi.org/10.1038/oby.2002.202

Pickup, M. W., Mouw, J. K., & Weaver, V. M. (2014). The extracellular matrix modulates the hallmarks of cancer. *EMBO Rep*, *15*(12), 1243-1253. https://doi.org/10.15252/embr.201439246

Pierobon, M., & Frankenfeld, C. L. (2013). Obesity as a risk factor for triple-negative breast cancers: a systematic review and meta-analysis. *Breast Cancer Res Treat*, *137*(1), 307-314. https://doi.org/10.1007/s10549-012-2339-3

Pires, B. R., Mencalha, A. L., Ferreira, G. M., de Souza, W. F., Morgado-Diaz, J. A., Maia, A. M., Correa, S., & Abdelhay, E. S. (2017). NF-kappaB Is Involved in the Regulation of EMT Genes in Breast Cancer Cells. *PLoS One*, *12*(1), e0169622. https://doi.org/10.1371/journal.pone.0169622

Piwarski, S. A., Thompson, C., Chaudhry, A. R., Denvir, J., Primerano, D. A., Fan, J., & Salisbury, T. B. (2020). The putative endogenous AHR ligand ITE reduces JAG1 and associated NOTCH1 signaling in triple negative breast cancer cells. *Biochem Pharmacol*, *174*, 113845. https://doi.org/10.1016/j.bcp.2020.113845

Pothuraju, R., Rachagani, S., Junker, W. M., Chaudhary, S., Saraswathi, V., Kaur, S., & Batra, S. K. (2018). Pancreatic cancer associated with obesity and diabetes: an alternative approach for its targeting. *J Exp Clin Cancer Res*, *37*(1), 319. https://doi.org/10.1186/s13046-018-0963-4

Prakash, O., Hossain, F., Danos, D., Lassak, A., Scribner, R., & Miele, L. (2020). Racial Disparities in Triple Negative Breast Cancer: A Review of the Role of Biologic and Non-biologic Factors. *Front Public Health*, *8*, 576964. https://doi.org/10.3389/fpubh.2020.576964

Procaccini, C., De Rosa, V., Galgani, M., Abanni, L., Cali, G., Porcellini, A., Carbone, F., Fontana, S., Horvath, T. L., La Cava, A., & Matarese, G. (2010). An oscillatory switch in mTOR kinase activity sets regulatory T cell responsiveness. *Immunity*, *33*(6), 929-941. https://doi.org/10.1016/j.immuni.2010.11.024

Protani, M., Coory, M., & Martin, J. H. (2010). Effect of obesity on survival of women with breast cancer: systematic review and meta-analysis. *Breast Cancer Res Treat*, *123*(3), 627-635. https://doi.org/10.1007/s10549-010-0990-0

Reedijk, M., Odorcic, S., Chang, L., Zhang, H., Miller, N., McCready, D. R., Lockwood, G., & Egan, S. E. (2005). High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Res*, *65*(18), 8530-8537. https://doi.org/10.1158/0008-5472.CAN-05-1069

Reedijk, M., Pinnaduwage, D., Dickson, B. C., Mulligan, A. M., Zhang, H., Bull, S. B., O'Malley, F. P., Egan, S. E., & Andrulis, I. L. (2008). JAG1 expression is

associated with a basal phenotype and recurrence in lymph node-negative breast cancer. *Breast Cancer Res Treat*, *111*(3), 439-448. https://doi.org/10.1007/s10549-007-9805-3

Riching, K. M., Cox, B. L., Salick, M. R., Pehlke, C., Riching, A. S., Ponik, S. M., Bass, B. R., Crone, W. C., Jiang, Y., Weaver, A. M., Eliceiri, K. W., & Keely, P. J. (2014). 3D collagen alignment limits protrusions to enhance breast cancer cell persistence. *Biophys J*, *107*(11), 2546-2558. https://doi.org/10.1016/j.bpj.2014.10.035

Rivas, D. A., Yaspelkis, B. B., 3rd, Hawley, J. A., & Lessard, S. J. (2009). Lipid-induced mTOR activation in rat skeletal muscle reversed by exercise and 5'-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside. *J Endocrinol*, *202*(3), 441-451. https://doi.org/10.1677/JOE-09-0202

Robado de Lope, L., Alcibar, O. L., Amor Lopez, A., Hergueta-Redondo, M., & Peinado, H. (2018). Tumour-adipose tissue crosstalk: fuelling tumour metastasis by extracellular vesicles. *Philos Trans R Soc Lond B Biol Sci*, *373*(1737). https://doi.org/10.1098/rstb.2016.0485

Rogers, R., Eagle, T. F., Sheetz, A., Woodward, A., Leibowitz, R., Song, M., Sylvester, R., Corriveau, N., Kline-Rogers, E., Jiang, Q., Jackson, E. A., & Eagle, K. A. (2015). The Relationship between Childhood Obesity, Low Socioeconomic Status, and Race/Ethnicity: Lessons from Massachusetts. *Child Obes*, *11*(6), 691-695. https://doi.org/10.1089/chi.2015.0029

Rosilio, C., Nebout, M., Imbert, V., Griessinger, E., Neffati, Z., Benadiba, J., Hagenbeek, T., Spits, H., Reverso, J., Ambrosetti, D., Michiels, J. F., Bailly-Maitre, B., Endou, H., Wempe, M. F., & Peyron, J. F. (2015). L-type amino-acid transporter 1 (LAT1): a therapeutic target supporting growth and survival of T-cell lymphoblastic lymphoma/T-cell acute lymphoblastic leukemia. *Leukemia*, *29*(6), 1253-1266. https://doi.org/10.1038/leu.2014.338

Roulin, D., Cerantola, Y., Dormond-Meuwly, A., Demartines, N., & Dormond, O. (2010). Targeting mTORC2 inhibits colon cancer cell proliferation in vitro and tumor formation in vivo. *Mol Cancer*, *9*, 57. https://doi.org/10.1186/1476-4598-9-57

Roytblat, L., Rachinsky, M., Fisher, A., Greemberg, L., Shapira, Y., Douvdevani, A., & Gelman, S. (2000). Raised interleukin-6 levels in obese patients. *Obes Res*, *8*(9), 673-675. https://doi.org/10.1038/oby.2000.86

Rozengurt, E., Soares, H. P., & Sinnet-Smith, J. (2014). Suppression of feedback loops mediated by PI3K/mTOR induces multiple overactivation of compensatory pathways: an unintended consequence leading to drug resistance. *Mol Cancer Ther*, *13*(11), 2477-2488. https://doi.org/10.1158/1535-7163.MCT-14-0330

Ruiz-Ojeda, F. J., Mendez-Gutierrez, A., Aguilera, C. M., & Plaza-Diaz, J. (2019). Extracellular Matrix Remodeling of Adipose Tissue in Obesity and Metabolic Diseases. *Int J Mol Sci*, *20*(19). https://doi.org/10.3390/ijms20194888

Sabatini, D. M. (2017). Twenty-five years of mTOR: Uncovering the link from nutrients to growth. *Proc Natl Acad Sci U S A*, *114*(45), 11818-11825. https://doi.org/10.1073/pnas.1716173114

Sabol, R. A., Bowles, A. C., Cote, A., Wise, R., O'Donnell, B., Matossian, M. D., Hossain, F. M., Burks, H. E., Del Valle, L., Miele, L., Collins-Burow, B. M., Burow, M. E., & Bunnell, B. A. (2019). Leptin produced by obesity-altered adipose stem cells promotes metastasis but not tumorigenesis of triple-negative breast cancer in orthotopic xenograft and patient-derived xenograft models. *Breast Cancer Res*, *21*(1), 67. https://doi.org/10.1186/s13058-019-1153-9

Safaei, R., Larson, B. J., Cheng, T. C., Gibson, M. A., Otani, S., Naerdemann, W., & Howell, S. B. (2005). Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. *Mol Cancer Ther*, *4*(10), 1595-1604. https://doi.org/10.1158/1535-7163.MCT-05-0102

Saito, Y., Li, L., Coyaud, E., Luna, A., Sander, C., Raught, B., Asara, J. M., Brown, M., & Muthuswamy, S. K. (2019). LLGL2 rescues nutrient stress by promoting leucine uptake in ER(+) breast cancer. *Nature*, *569*(7755), 275-279. https://doi.org/10.1038/s41586-019-1126-2

Saito, Y., & Soga, T. (2021). Amino acid transporters as emerging therapeutic targets in cancer. *Cancer Sci*, *112*(8), 2958-2965. https://doi.org/10.1111/cas.15006

Salisbury, T. B., & Arthur, S. (2018). The Regulation and Function of the L-Type Amino Acid Transporter 1 (LAT1) in Cancer. *Int J Mol Sci*, *19*(8). https://doi.org/10.3390/ijms19082373

Sanchez-Iranzo, H., Halavatyi, A., & Diz-Munoz, A. (2022). Strength of interactions in the Notch gene regulatory network determines patterning and fate in the notochord. *Elife*, *11*. https://doi.org/10.7554/eLife.75429

Sanchez-Jimenez, F., Perez-Perez, A., de la Cruz-Merino, L., & Sanchez-Margalet, V. (2019). Obesity and Breast Cancer: Role of Leptin. *Front Oncol*, *9*, 596. https://doi.org/10.3389/fonc.2019.00596

Sanguesa, G., Roglans, N., Baena, M., Velazquez, A. M., Laguna, J. C., & Alegret, M. (2019). mTOR is a Key Protein Involved in the Metabolic Effects of Simple Sugars. *Int J Mol Sci*, *20*(5). https://doi.org/10.3390/ijms20051117

Sano, S., Izumi, Y., Yamaguchi, T., Yamazaki, T., Tanaka, M., Shiota, M., Osada-Oka, M., Nakamura, Y., Wei, M., Wanibuchi, H., Iwao, H., & Yoshiyama, M. (2014). Lipid synthesis is promoted by hypoxic adipocyte-derived exosomes in 3T3-L1 cells. *Biochem Biophys Res Commun*, *445*(2), 327-333. https://doi.org/10.1016/j.bbrc.2014.01.183

Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P., & Sabatini, D. M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol*, *14*(14), 1296-1302. https://doi.org/10.1016/j.cub.2004.06.054

Sato, M., Harada-Shoji, N., Toyohara, T., Soga, T., Itoh, M., Miyashita, M., Tada, H., Amari, M., Anzai, N., Furumoto, S., Abe, T., Suzuki, T., Ishida, T., & Sasano, H. (2021). L-type amino acid transporter 1 is associated with chemoresistance in breast cancer via the promotion of amino acid metabolism. *Sci Rep*, *11*(1), 589. https://doi.org/10.1038/s41598-020-80668-5

Saxton, R. A., & Sabatini, D. M. (2017a). mTOR Signaling in Growth, Metabolism, and Disease. *Cell*, *169*(2), 361-371. https://doi.org/10.1016/j.cell.2017.03.035

Saxton, R. A., & Sabatini, D. M. (2017b). mTOR Signaling in Growth, Metabolism, and Disease. *Cell*, *168*(6), 960-976. https://doi.org/10.1016/j.cell.2017.02.004

Scalise, M., Galluccio, M., Console, L., Pochini, L., & Indiveri, C. (2018). The Human SLC7A5 (LAT1): The Intriguing Histidine/Large Neutral Amino Acid Transporter and Its Relevance to Human Health. *Front Chem*, *6*, 243. https://doi.org/10.3389/fchem.2018.00243

Schalm, S. S., & Blenis, J. (2002). Identification of a conserved motif required for mTOR signaling. *Curr Biol*, *12*(8), 632-639. https://doi.org/10.1016/s0960-9822(02)00762-5

Schlich, R., Willems, M., Greulich, S., Ruppe, F., Knoefel, W. T., Ouwens, D. M., Maxhera, B., Lichtenberg, A., Eckel, J., & Sell, H. (2013). VEGF in the crosstalk between human adipocytes and smooth muscle cells: depot-specific release from visceral and perivascular adipose tissue. *Mediators Inflamm*, *2013*, 982458. https://doi.org/10.1155/2013/982458

Schmid, P., Zaiss, M., Harper-Wynne, C., Ferreira, M., Dubey, S., Chan, S., Makris, A., Nemsadze, G., Brunt, A. M., Kuemmel, S., Ruiz, I., Perello, A., Kendall, A., Brown, J., Kristeleit, H., Conibear, J., Saura, C., Grenier, J., Mahr, K., . . . Cortes, J. (2019). Fulvestrant Plus Vistusertib vs Fulvestrant Plus Everolimus vs Fulvestrant Alone for Women With Hormone Receptor-Positive Metastatic Breast Cancer: The MANTA Phase 2 Randomized Clinical Trial. *JAMA Oncol*, *5*(11), 1556-1564. https://doi.org/10.1001/jamaoncol.2019.2526

Schorey, J. S., Cheng, Y., Singh, P. P., & Smith, V. L. (2015). Exosomes and other extracellular vesicles in host-pathogen interactions. *EMBO Rep*, *16*(1), 24-43. https://doi.org/10.15252/embr.201439363

Segawa, A., Nagamori, S., Kanai, Y., Masawa, N., & Oyama, T. (2013). L-type amino acid transporter 1 expression is highly correlated with Gleason score in prostate cancer. *Mol Clin Oncol*, *1*(2), 274-280. https://doi.org/10.3892/mco.2012.54

She, P., Van Horn, C., Reid, T., Hutson, S. M., Cooney, R. N., & Lynch, C. J. (2007). Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. *Am J Physiol Endocrinol Metab*, *293*(6), E1552-1563. https://doi.org/10.1152/ajpendo.00134.2007

Sheldon, H., Heikamp, E., Turley, H., Dragovic, R., Thomas, P., Oon, C. E., Leek, R., Edelmann, M., Kessler, B., Sainson, R. C., Sargent, I., Li, J. L., & Harris, A. L. (2010). New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes. *Blood*, *116*(13), 2385-2394. https://doi.org/10.1182/blood-2009-08-239228

Shen, Y., Schmidt, B. U. S., Kubitschke, H., Morawetz, E. W., Wolf, B., Kas, J. A., & Losert, W. (2020). Detecting heterogeneity in and between breast cancer cell lines. *Cancer Converg*, *4*(1), 1. https://doi.org/10.1186/s41236-020-0010-1

Shigeyama, Y., Kobayashi, T., Kido, Y., Hashimoto, N., Asahara, S., Matsuda, T., Takeda, A., Inoue, T., Shibutani, Y., Koyanagi, M., Uchida, T., Inoue, M., Hino, O., Kasuga, M., & Noda, T. (2008). Biphasic response of pancreatic beta-cell

mass to ablation of tuberous sclerosis complex 2 in mice. *Mol Cell Biol*, *28*(9), 2971-2979. https://doi.org/10.1128/MCB.01695-07

Shimizu, A., Kaira, K., Kato, M., Yasuda, M., Takahashi, A., Tominaga, H., Oriuchi, N., Nagamori, S., Kanai, Y., Oyama, T., Asao, T., & Ishikawa, O. (2015). Prognostic significance of L-type amino acid transporter 1 (LAT1) expression in cutaneous melanoma. *Melanoma Res*, *25*(5), 399-405. https://doi.org/10.1097/CMR.0000000000000181

Sidhu, S., Parikh, T., & Burman, K. D. (2000). Endocrine Changes in Obesity. In K. R. Feingold, B. Anawalt, A. Boyce, G. Chrousos, W. W. de Herder, K. Dhatariya, K. Dungan, J. M. Hershman, J. Hofland, S. Kalra, G. Kaltsas, C. Koch, P. Kopp, M. Korbonits, C. S. Kovacs, W. Kuohung, B. Laferrere, M. Levy, E. A. McGee, R. McLachlan, J. E. Morley, M. New, J. Purnell, R. Sahay, F. Singer, M. A. Sperling, C. A. Stratakis, D. L. Trence, & D. P. Wilson (Eds.), *Endotext*. https://www.ncbi.nlm.nih.gov/pubmed/25905281

Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. (2021). Cancer Statistics, 2021. *CA Cancer J Clin*, *71*(1), 7-33. https://doi.org/10.3322/caac.21654

Singh, G., Akcakanat, A., Sharma, C., Luyimbazi, D., Naff, K. A., & Meric-Bernstam, F. (2011). The effect of leucine restriction on Akt/mTOR signaling in breast cancer cell lines in vitro and in vivo. *Nutr Cancer*, *63*(2), 264-271. https://doi.org/10.1080/01635581.2011.523504

Singh, N., & Ecker, G. F. (2018). Insights into the Structure, Function, and Ligand Discovery of the Large Neutral Amino Acid Transporter 1, LAT1. *Int J Mol Sci*, *19*(5). https://doi.org/10.3390/ijms19051278

Sizemore, G., & Rudisill, T. M. (2021). Triple Negative Breast Cancer in an Appalachian Region: Exponential Tumor Grade Increase with Age of Diagnosis. *J Appalach Health*, *3*(3), 97-109. https://doi.org/10.13023/jah.0303.08

Skurk, T., Alberti-Huber, C., Herder, C., & Hauner, H. (2007). Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab*, *92*(3), 1023-1033. https://doi.org/10.1210/jc.2006-1055

Song, W., Jackson, K., & McGuire, P. G. (2000). Degradation of type IV collagen by matrix metalloproteinases is an important step in the epithelial-mesenchymal transformation of the endocardial cushions. *Dev Biol*, *227*(2), 606-617. https://doi.org/10.1006/dbio.2000.9919

Sonoshita, M., Aoki, M., Fuwa, H., Aoki, K., Hosogi, H., Sakai, Y., Hashida, H., Takabayashi, A., Sasaki, M., Robine, S., Itoh, K., Yoshioka, K., Kakizaki, F., Kitamura, T., Oshima, M., & Taketo, M. M. (2011). Suppression of colon cancer metastasis by Aes through inhibition of Notch signaling. *Cancer Cell*, *19*(1), 125-137. https://doi.org/10.1016/j.ccr.2010.11.008

Stahl, A. L., Johansson, K., Mossberg, M., Kahn, R., & Karpman, D. (2019). Exosomes and microvesicles in normal physiology, pathophysiology, and renal diseases. *Pediatr Nephrol*, *34*(1), 11-30. https://doi.org/10.1007/s00467-017-3816-z

Stattin, P., Lukanova, A., Biessy, C., Soderberg, S., Palmqvist, R., Kaaks, R., Olsson, T., & Jellum, E. (2004). Obesity and colon cancer: does leptin provide a link? *Int J Cancer*, *109*(1), 149-152. https://doi.org/10.1002/ijc.11668

Sugiyama, M., Oki, E., Nakaji, Y., Tsutsumi, S., Ono, N., Nakanishi, R., Sugiyama, M., Nakashima, Y., Sonoda, H., Ohgaki, K., Yamashita, N., Saeki, H., Okano, S., Kitao, H., Morita, M., Oda, Y., & Maehara, Y. (2016). High expression of the Notch ligand Jagged-1 is associated with poor prognosis after surgery for colorectal cancer. *Cancer Sci*, *107*(11), 1705-1716. https://doi.org/10.1111/cas.13075

Sun, H., Zou, J., Chen, L., Zu, X., Wen, G., & Zhong, J. (2017). Triple-negative breast cancer and its association with obesity. *Mol Clin Oncol*, *7*(6), 935-942. https://doi.org/10.3892/mco.2017.1429

Sundaram, S., Johnson, A. R., & Makowski, L. (2013). Obesity, metabolism and the microenvironment: Links to cancer. *J Carcinog*, *12*, 19. https://doi.org/10.4103/1477-3163.119606

Tai, Y. L., Chen, K. C., Hsieh, J. T., & Shen, T. L. (2018). Exosomes in cancer development and clinical applications. *Cancer Sci*, *109*(8), 2364-2374. https://doi.org/10.1111/cas.13697

Takahara, T., Amemiya, Y., Sugiyama, R., Maki, M., & Shibata, H. (2020). Amino acid-dependent control of mTORC1 signaling: a variety of regulatory modes. *J Biomed Sci*, *27*(1), 87. https://doi.org/10.1186/s12929-020-00679-2

Taniguchi, K., & Karin, M. (2018). NF-kappaB, inflammation, immunity and cancer: coming of age. *Nat Rev Immunol*, *18*(5), 309-324. https://doi.org/10.1038/nri.2017.142

Tartibian, B., FitzGerald, L. Z., Azadpour, N., & Maleki, B. H. (2015). A randomized controlled study examining the effect of exercise on inflammatory cytokine levels in post-menopausal women. *Post Reprod Health*, *21*(1), 9-15. https://doi.org/10.1177/2053369114565708

Tartibian, B., Hajizadeh Maleki, B., Kanaley, J., & Sadeghi, K. (2011). Long-term aerobic exercise and omega-3 supplementation modulate osteoporosis through inflammatory mechanisms in post-menopausal women: a randomized, repeated measures study. *Nutr Metab (Lond)*, *8*, 71. https://doi.org/10.1186/1743-7075-8-71

Tesmer, L. A., Lundy, S. K., Sarkar, S., & Fox, D. A. (2008). Th17 cells in human disease. *Immunol Rev*, *223*, 87-113. https://doi.org/10.1111/j.1600-065X.2008.00628.x

Thomou, T., Mori, M. A., Dreyfuss, J. M., Konishi, M., Sakaguchi, M., Wolfrum, C., Rao, T. N., Winnay, J. N., Garcia-Martin, R., Grinspoon, S. K., Gorden, P., & Kahn, C. R. (2017). Adipose-derived circulating miRNAs regulate gene expression in other tissues. *Nature*, *542*(7642), 450-455. https://doi.org/10.1038/nature21365

Thompson, C., Rahman, M. M., Singh, S., Arthur, S., Sierra-Bakhshi, C., Russell, R., Denning, K., Sundaram, U., & Salisbury, T. (2021). The Adipose Tissue-Derived Secretome (ADS) in Obesity Uniquely Induces L-Type Amino Acid Transporter 1 (LAT1) and mTOR Signaling in Estrogen-Receptor-Positive Breast Cancer Cells. *Int J Mol Sci*, *22*(13). https://doi.org/10.3390/ijms22136706

Tomblin, J. K., Arthur, S., Primerano, D. A., Chaudhry, A. R., Fan, J., Denvir, J., & Salisbury, T. B. (2016). Aryl hydrocarbon receptor (AHR) regulation of L-Type Amino Acid Transporter 1 (LAT-1) expression in MCF-7 and MDA-MB-231 breast cancer cells. *Biochem Pharmacol*, *106*, 94-103. https://doi.org/10.1016/j.bcp.2016.02.020

Toyoda, M., Kaira, K., Ohshima, Y., Ishioka, N. S., Shino, M., Sakakura, K., Takayasu, Y., Takahashi, K., Tominaga, H., Oriuchi, N., Nagamori, S., Kanai, Y., Oyama, T., & Chikamatsu, K. (2014). Prognostic significance of amino-acid transporter expression (LAT1, ASCT2, and xCT) in surgically resected tongue cancer. *Br J Cancer*, *110*(10), 2506-2513. https://doi.org/10.1038/bjc.2014.178

Tremblay, F., Brule, S., Hee Um, S., Li, Y., Masuda, K., Roden, M., Sun, X. J., Krebs, M., Polakiewicz, R. D., Thomas, G., & Marette, A. (2007). Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance.

Proc Natl Acad Sci U S A, *104*(35), 14056-14061. https://doi.org/10.1073/pnas.0706517104

Trivers, K. F., Lund, M. J., Porter, P. L., Liff, J. M., Flagg, E. W., Coates, R. J., & Eley, J. W. (2009). The epidemiology of triple-negative breast cancer, including race. *Cancer Causes Control*, *20*(7), 1071-1082. https://doi.org/10.1007/s10552-009-9331-1

Ugai, T., Sasamoto, N., Lee, H. Y., Ando, M., Song, M., Tamimi, R. M., Kawachi, I., Campbell, P. T., Giovannucci, E. L., Weiderpass, E., Rebbeck, T. R., & Ogino, S. (2022). Is early-onset cancer an emerging global epidemic? Current evidence and future implications. *Nat Rev Clin Oncol*, *19*(10), 656-673. https://doi.org/10.1038/s41571-022-00672-8

Um, S. H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P. R., Kozma, S. C., Auwerx, J., & Thomas, G. (2004). Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature*, *431*(7005), 200-205. https://doi.org/10.1038/nature02866

Unamuno, X., Gomez-Ambrosi, J., Rodriguez, A., Becerril, S., Fruhbeck, G., & Catalan, V. (2018). Adipokine dysregulation and adipose tissue inflammation in human obesity. *Eur J Clin Invest*, *48*(9), e12997. https://doi.org/10.1111/eci.12997

Vachher, M., Arora, K., Burman, A., & Kumar, B. (2020). NAMPT, GRN, and SERPINE1 signature as predictor of disease progression and survival in gliomas. *J Cell Biochem*, *121*(4), 3010-3023. https://doi.org/10.1002/jcb.29560

Valladares, M., Corsini, G., & Romero, C. (2014). [Association between obesity and ovarian cancer]. *Rev Med Chil*, *142*(5), 593-598. https://doi.org/10.4067/S0034-98872014000500007 (Asociacion entre obesidad y cancer de ovario.)

Van Cutsem, E., Kohne, C. H., Hitre, E., Zaluski, J., Chang Chien, C. R., Makhson, A., D'Haens, G., Pinter, T., Lim, R., Bodoky, G., Roh, J. K., Folprecht, G., Ruff, P., Stroh, C., Tejpar, S., Schlichting, M., Nippgen, J., & Rougier, P. (2009). Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med*, *360*(14), 1408-1417. https://doi.org/10.1056/NEJMoa0805019

Vellai, T. (2021). How the amino acid leucine activates the key cell-growth regulator mTOR. *Nature*, *596*(7871), 192-194. https://doi.org/10.1038/d41586-021-01943-7

Vona-Davis, L., & Rose, D. P. (2007). Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression. *Endocr Relat Cancer*, *14*(2), 189-206. https://doi.org/10.1677/ERC-06-0068

Vona-Davis, L., Rose, D. P., Hazard, H., Howard-McNatt, M., Adkins, F., Partin, J., & Hobbs, G. (2008). Triple-negative breast cancer and obesity in a rural Appalachian population. *Cancer Epidemiol Biomarkers Prev*, *17*(12), 3319-3324. https://doi.org/10.1158/1055-9965.EPI-08-0544

Voss, M. H., Gordon, M. S., Mita, M., Rini, B., Makker, V., Macarulla, T., Smith, D. C., Cervantes, A., Puzanov, I., Pili, R., Wang, D., Jalal, S., Pant, S., Patel, M. R., Neuwirth, R. L., Enke, A., Shou, Y., Sedarati, F., Faller, D. V., & Burris, H. A., 3rd. (2020). Phase 1 study of mTORC1/2 inhibitor sapanisertib (TAK-228) in advanced solid tumours, with an expansion phase in renal, endometrial or bladder cancer. *Br J Cancer*, *123*(11), 1590-1598. https://doi.org/10.1038/s41416-020-01041-x

Wang, D., Chen, J., Chen, H., Duan, Z., Xu, Q., Wei, M., Wang, L., & Zhong, M. (2012). Leptin regulates proliferation and apoptosis of colorectal carcinoma through PI3K/Akt/mTOR signalling pathway. *J Biosci*, *37*(1), 91-101. https://doi.org/10.1007/s12038-011-9172-4

Wang, H., Wang, L., Song, Y., Wang, S., Huang, X., Xuan, Q., Kang, X., & Zhang, Q. (2017). CD44(+)/CD24(-) phenotype predicts a poor prognosis in triple-negative breast cancer. *Oncol Lett*, *14*(5), 5890-5898. https://doi.org/10.3892/ol.2017.6959

Wang, Q., & Holst, J. (2015). L-type amino acid transport and cancer: targeting the mTORC1 pathway to inhibit neoplasia. *Am J Cancer Res*, *5*(4), 1281-1294. https://www.ncbi.nlm.nih.gov/pubmed/26101697

Wang, T., & He, C. (2018). Pro-inflammatory cytokines: The link between obesity and osteoarthritis. *Cytokine Growth Factor Rev*, *44*, 38-50. https://doi.org/10.1016/j.cytogfr.2018.10.002

Wang, X. W., & Zhang, Y. J. (2014). Targeting mTOR network in colorectal cancer therapy. *World J Gastroenterol*, *20*(15), 4178-4188. https://doi.org/10.3748/wjg.v20.i15.4178

Wolf, G. (2004). Insulin resistance and obesity: resistin, a hormone secreted by adipose tissue. *Nutr Rev*, *62*(10), 389-394. https://doi.org/10.1111/j.1753-4887.2004.tb00009.x

Wolfson, R. L., Chantranupong, L., Saxton, R. A., Shen, K., Scaria, S. M., Cantor, J. R., & Sabatini, D. M. (2016). Sestrin2 is a leucine sensor for the mTORC1 pathway. *Science*, *351*(6268), 43-48. https://doi.org/10.1126/science.aab2674

Wolin, K. Y., Carson, K., & Colditz, G. A. (2010). Obesity and cancer. *Oncologist*, *15*(6), 556-565. https://doi.org/10.1634/theoncologist.2009-0285

Wueest, S., & Konrad, D. (2018). The role of adipocyte-specific IL-6-type cytokine signaling in FFA and leptin release. *Adipocyte*, *7*(3), 226-228. https://doi.org/10.1080/21623945.2018.1493901

Xie, J., Wang, X., & Proud, C. G. (2016). mTOR inhibitors in cancer therapy. *F1000Res*, *5*. https://doi.org/10.12688/f1000research.9207.1

Xie, X. L., Kakehashi, A., Wei, M., Yamano, S., Takeshita, M., Yunoki, T., & Wanibuchi, H. (2013). L-Leucine and L-isoleucine enhance growth of BBN-induced urothelial tumors in the rat bladder by modulating expression of amino acid transporters and tumorigenesis-associated genes. *Food Chem Toxicol*, *59*, 137-144. https://doi.org/10.1016/j.fct.2013.05.044

Xiu, M. X., Liu, Y. M., & Kuang, B. H. (2020). The oncogenic role of Jagged1/Notch signaling in cancer. *Biomed Pharmacother*, *129*, 110416. https://doi.org/10.1016/j.biopha.2020.110416

Xu, H., Tian, Y., Yuan, X., Wu, H., Liu, Q., Pestell, R. G., & Wu, K. (2015). The role of CD44 in epithelial-mesenchymal transition and cancer development. *Onco Targets Ther*, *8*, 3783-3792. https://doi.org/10.2147/OTT.S95470

Xu, Y. X. Z., & Mishra, S. (2018). Obesity-Linked Cancers: Current Knowledge, Challenges and Limitations in Mechanistic Studies and Rodent Models. *Cancers (Basel)*, *10*(12). https://doi.org/10.3390/cancers10120523

Yang, G., Murashige, D. S., Humphrey, S. J., & James, D. E. (2015). A Positive Feedback Loop between Akt and mTORC2 via SIN1 Phosphorylation. *Cell Rep*, *12*(6), 937-943. https://doi.org/10.1016/j.celrep.2015.07.016

Yang, X. R., Chang-Claude, J., Goode, E. L., Couch, F. J., Nevanlinna, H., Milne, R. L., Gaudet, M., Schmidt, M. K., Broeks, A., Cox, A., Fasching, P. A., Hein, R., Spurdle, A. B., Blows, F., Driver, K., Flesch-Janys, D., Heinz, J., Sinn, P., Vrieling, A., . . . Garcia-Closas, M. (2011). Associations of breast cancer risk

factors with tumor subtypes: a pooled analysis from the Breast Cancer Association Consortium studies. *J Natl Cancer Inst*, *103*(3), 250-263. https://doi.org/10.1093/jnci/djq526

Yeh, T. S., Wu, C. W., Hsu, K. W., Liao, W. J., Yang, M. C., Li, A. F., Wang, A. M., Kuo, M. L., & Chi, C. W. (2009). The activated Notch1 signal pathway is associated with gastric cancer progression through cyclooxygenase-2. *Cancer Res*, *69*(12), 5039-5048. https://doi.org/10.1158/0008-5472.CAN-08-4021

Yi, Z., Ma, F., Liu, B., Guan, X., Li, L., Li, C., Qian, H., & Xu, B. (2019). Everolimus in hormone receptor-positive metastatic breast cancer: PIK3CA mutation H1047R was a potential efficacy biomarker in a retrospective study. *BMC Cancer*, *19*(1), 442. https://doi.org/10.1186/s12885-019-5668-3

Yin, L., Duan, J. J., Bian, X. W., & Yu, S. C. (2020). Triple-negative breast cancer molecular subtyping and treatment progress. *Breast Cancer Res*, *22*(1), 61. https://doi.org/10.1186/s13058-020-01296-5

Yip, C. K., Murata, K., Walz, T., Sabatini, D. M., & Kang, S. A. (2010). Structure of the human mTOR complex I and its implications for rapamycin inhibition. *Mol Cell*, *38*(5), 768-774. https://doi.org/10.1016/j.molcel.2010.05.017

Yokoi, A., & Ochiya, T. (2021). Exosomes and extracellular vesicles: Rethinking the essential values in cancer biology. *Semin Cancer Biol*, *74*, 79-91. https://doi.org/10.1016/j.semcancer.2021.03.032

Yoon, M. S. (2017). The Role of Mammalian Target of Rapamycin (mTOR) in Insulin Signaling. *Nutrients*, *9*(11). https://doi.org/10.3390/nu9111176

Yuan, H. X., & Guan, K. L. (2015). The SIN1-PH Domain Connects mTORC2 to PI3K. *Cancer Discov*, *5*(11), 1127-1129. https://doi.org/10.1158/2159-8290.CD-15-1125

Yuan, T., Rafizadeh, S., Gorrepati, K. D., Lupse, B., Oberholzer, J., Maedler, K., & Ardestani, A. (2017). Reciprocal regulation of mTOR complexes in pancreatic islets from humans with type 2 diabetes. *Diabetologia*, *60*(4), 668-678. https://doi.org/10.1007/s00125-016-4188-9

Yue, M., Jiang, J., Gao, P., Liu, H., & Qing, G. (2017). Oncogenic MYC Activates a Feedforward Regulatory Loop Promoting Essential Amino Acid Metabolism and

Tumorigenesis. *Cell Rep*, *21*(13), 3819-3832. https://doi.org/10.1016/j.celrep.2017.12.002

Zatterale, F., Longo, M., Naderi, J., Raciti, G. A., Desiderio, A., Miele, C., & Beguinot, F. (2019). Chronic Adipose Tissue Inflammation Linking Obesity to Insulin Resistance and Type 2 Diabetes. *Front Physiol*, *10*, 1607. https://doi.org/10.3389/fphys.2019.01607

Zeng, H., & Chi, H. (2013). The interplay between regulatory T cells and metabolism in immune regulation. *Oncoimmunology*, *2*(11), e26586. https://doi.org/10.4161/onci.26586

Zhang, J., Xu, Y., Li, D., Fu, L., Zhang, X., Bao, Y., & Zheng, L. (2020). Review of the Correlation of LAT1 With Diseases: Mechanism and Treatment. *Front Chem*, *8*, 564809. https://doi.org/10.3389/fchem.2020.564809

Zhang, L., & Yu, D. (2019). Exosomes in cancer development, metastasis, and immunity. *Biochim Biophys Acta Rev Cancer*, *1871*(2), 455-468. https://doi.org/10.1016/j.bbcan.2019.04.004

Zhang, Q., Deng, T., Zhang, H., Zuo, D., Zhu, Q., Bai, M., Liu, R., Ning, T., Zhang, L., Yu, Z., Zhang, H., & Ba, Y. (2022). Adipocyte-Derived Exosomal MTTP Suppresses Ferroptosis and Promotes Chemoresistance in Colorectal Cancer. *Adv Sci (Weinh)*, *9*(28), e2203357. https://doi.org/10.1002/advs.202203357

Zhang, X., Powell, K., & Li, L. (2020). Breast Cancer Stem Cells: Biomarkers, Identification and Isolation Methods, Regulating Mechanisms, Cellular Origin, and Beyond. *Cancers (Basel)*, *12*(12). https://doi.org/10.3390/cancers12123765

Zhao, C., Wu, M., Zeng, N., Xiong, M., Hu, W., Lv, W., Yi, Y., Zhang, Q., & Wu, Y. (2020). Cancer-associated adipocytes: emerging supporters in breast cancer. *J Exp Clin Cancer Res*, *39*(1), 156. https://doi.org/10.1186/s13046-020-01666-z

Zhao, D., Jiang, M., Zhang, X., & Hou, H. (2020). The role of RICTOR amplification in targeted therapy and drug resistance. *Mol Med*, *26*(1), 20. https://doi.org/10.1186/s10020-020-0146-6

Zhao, Y., Wang, L., & Pan, J. (2015). The role of L-type amino acid transporter 1 in human tumors. *Intractable Rare Dis Res*, *4*(4), 165-169. https://doi.org/10.5582/irdr.2015.01024

Zhou, H. Y., & Huang, S. L. (2012). Current development of the second generation of mTOR inhibitors as anticancer agents. *Chin J Cancer*, *31*(1), 8-18. https://doi.org/10.5732/cjc.011.10281

Zhou, L., Sheng, D., Wang, D., Ma, W., Deng, Q., Deng, L., & Liu, S. (2019). Identification of cancer-type specific expression patterns for active aldehyde dehydrogenase (ALDH) isoforms in ALDEFLUOR assay. *Cell Biol Toxicol*, *35*(2), 161-177. https://doi.org/10.1007/s10565-018-9444-y

Zhou, M., Shao, J., Wu, C. Y., Shu, L., Dong, W., Liu, Y., Chen, M., Wynn, R. M., Wang, J., Wang, J., Gui, W. J., Qi, X., Lusis, A. J., Li, Z., Wang, W., Ning, G., Yang, X., Chuang, D. T., Wang, Y., & Sun, H. (2019). Targeting BCAA Catabolism to Treat Obesity-Associated Insulin Resistance. *Diabetes*, *68*(9), 1730-1746. https://doi.org/10.2337/db18-0927

Zhou, Y., & Rui, L. (2013). Leptin signaling and leptin resistance. *Front Med*, *7*(2), 207-222. https://doi.org/10.1007/s11684-013-0263-5

Zinzalla, V., Stracka, D., Oppliger, W., & Hall, M. N. (2011). Activation of mTORC2 by association with the ribosome. *Cell*, *144*(5), 757-768. https://doi.org/10.1016/j.cell.2011.02.014

Zoncu, R., Efeyan, A., & Sabatini, D. M. (2011). mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol*, *12*(1), 21-35. https://doi.org/10.1038/nrm3025

Zou, Z., Tao, T., Li, H., & Zhu, X. (2020). mTOR signaling pathway and mTOR inhibitors in cancer: progress and challenges. *Cell Biosci*, *10*, 31. https://doi.org/10.1186/s13578-020-00396-1

Zukiewicz-Sobczak, W., Wroblewska, P., Zwolinski, J., Chmielewska-Badora, J., Adamczuk, P., Krasowska, E., Zagorski, J., Oniszczuk, A., Piatek, J., & Silny, W. (2014). Obesity and poverty paradox in developed countries. *Ann Agric Environ Med*, *21*(3), 590-594. https://doi.org/10.5604/12321966.1120608

Appendix A: IRB Approval Letter

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

January 11, 2023

Cora E. Miracle
1684 Wiltshire Blvd
Huntington, WV 25701

Dear Cora:

This letter is in response to the submitted thesis abstract entitled *"High Body Mass Index changes Peri-Tumor Adipose Tissue which in turn promotes Triple Negative Breast Cancer."* After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making t/his determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction, it is not considered human subject research. If there are any changes to the abstract, you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director