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The contribution of adipose tissue from ovariectomized mice to colon cancer

by

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Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

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DEDICATION

To my parents for their constant encouragement and support as I pursue this goal, without them this would not be possible. To my family and friends for their unwavering support and continuous reassurances throughout the duration of this work.

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ABSTRACT

Obesity is considered a major public health concern worldwide due to the increased incidences of metabolic dysfunction and cancer risk. The obese state is, in part, attributable to the overconsumption of calorie-dense foods commonly seen in a standard Western diet, which aids in the progression of chronic, low-grade inflammation. The role of estrogen varies depending on menopausal status, where estrogen deficiency coupled with increased visceral fat associated with post-menopause leads to increased secretions of pro-inflammatory adipokines and cytokines. However, the presence of estrogen in a premenopausal state has been shown to attenuate the pro-inflammatory response, which has been demonstrated when estrogen replacement is administered to ovariectomized (OVX) mice. The purpose of this study was to determine if various pro-inflammatory cytokines secreted by adipose tissue influences cancer growth and if ovarian status affects this response. *Methods:* The study utilized a diet-induced model of obesity, where 8-week-old intact female, OVX female, and male mice were assigned to either a 40% high-fat diet (HFD) or a purified control low-fat diet (LFD) for 21 weeks. The ovaries of the OVX female group were removed in order to study the role of ovarian status in obesity and cancer initiation. To determine if pro-inflammatory cytokines associated with increases in body weight and fat mass influence cancer proliferation, adipose tissue-conditioned media (AT-CM) obtained from each

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dietary group was supplemented to MC38 colon cancer cells and a proliferation assay was performed. *Results:* An obese phenotype in mice fed a HFD was achieved and detectable increases in WBC, LYM, MON, and PLT count in the blood associated with obesity were identified. There were no diet effects discovered in the concentration of pro-inflammatory cytokines circulating in the plasma; however, a main effect of HFD ($p<0.05$) exhibited increased secretions of IL-1β and IL-6 from the AT-CM when comparing the intact and OVX female groups. No significant differences were discovered in the cell proliferation assay after treatment with AT-CM. *Conclusion:* HFD feedings resulted in significant increases in body weight and fat mass. Secreted levels of pro-inflammatory cytokines were detected in the AT-CM in the intact and OVX females. There was no difference detected in the cell proliferation assay after 24hrs.

KEYWORDS: Obesity, High-Fat Diet, Colon Cancer, Estrogen

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LIST OF ABBREVIATIONS

AT .... Adipose Tissue

AT-CM.. Adipose-Tissue Conditioned Media

BAT .... Brown Adipose Tissue

BMI.. Body Mass Index

CRC .... Colorectal Cancer

E2... 17β-estradiol

HCT... Hematocrit

HGB .... Hemoglobin

IGF .... Insulin-like Growth Factor

IL-1β... Interleukin-1beta

IL-6.. Interleukin-6

IR .... Insulin Resistance

LYM.. Lymphocyte

MC38 .... Murine carcinoma-38 colon cancer cell line

MCP-1... Monocyte Chemoattractant Protein 1

MON.. Monocyte

NAFLD .... Nonalcoholic fatty liver disease

NEU .... Neutrophil

OVX .... Ovariectomized

PLT .... Platelet

RBC .... Red Blood Cell

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T2D .... Type II Diabetes

TNF .... Tumor Necrosis Factor

WAT .... White Adipose Tissue

WBC.. White Blood Cell

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CHAPTER 1

REVIEW OF LITERATURE

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1.1 Colon cancer

Cancer is a major public health concern affecting individuals worldwide, with an expected 1.7 million new cancer cases to be diagnosed in 2019 in the United States. The second leading cause of death in the United States is cancer, preceded only by heart disease, with an estimated 1,660 deaths per day for 2019. Among the different types of cancer, colorectal cancer (CRC) is currently the third-most commonly diagnosed cancer after bladder and breast cancer. The rates for colon cancer are relatively equal among men and women where one in 22 men and one in 24 women will be diagnosed with colorectal cancer. In 2019, the total number of new cases of CRC is estimated to be 145,600, and the total estimated death for CRC is expected to be 51,020. The incidence trend, or the number of new cases, of colon cancer has been on the decline for several decades with a 3.7% annual decline among individuals 55 years or older. However, the overwhelming majority of CRC patients are 55 years or older, which masks the increasing incidence, 1.8% annually, in the younger age groups. In terms of mortality, the death rate declined by 2.7% per year in adults aged 55 years or older and increased by 1% per year in adults younger than 55 years old.[1]

The risk factors associated with CRC range from non-modifiable to modifiable by the individual. One of the biggest risk factors associated with CRC is age. Diagnosis of CRC increases after the age of 40 with more than 90% of CRC cases occurring in patients older than 50 years of age.[2] In addition to age, a personal history of Inflammatory Bowel Disease, including both ulcerative colitis

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and Crohn’s disease, as well as a history of adenomatous polyps both increase a person’s risk of developing CRC.[2] Genetic factors, inherited or *de novo* gene mutations, can increase the risk of a person developing CRC, with mutations occurring in the *APC* tumor suppressor gene often initiating cancer progression. Mutations in the *KRAS* oncogene increase polyp numbers and promote early carcinomas.[3] While some risk factors that lead to CRC development are uncontrollable by individuals, 55% of CRCs in the US are caused by various risk factors that can be changed by individuals. These risk factors include physical inactivity, obesity, long-term smoking, moderate to heavy alcohol consumption, high consumption of red or processed meat, and low intake of vegetables, fruits and whole-grain fiber. Vast epidemiological observations suggest that a major risk factor for the development of CRC in humans is obesity.[4] Independent predictors for the development of adenomatous polyps and malignant transformation to CRC are visceral adiposity and the metabolic syndrome.[3]

1.2 Obesity

Adipose tissue is a complex and highly active endocrine and metabolic organ. The functions of adipose tissue include thermic insulation, non-shivering thermogenesis, immune response, and regulated storage and release of energy. Obesity is characterized as excessive accumulation of adipose tissue, which is made up of adipocytes, and increased storage of fatty acids in the adipose tissue.[5, 6] The excess fat is stored by increasing the size and/or number of adipocytes and is seen in the majority of obese individuals.[7] There are three types of adipocytes in both humans and mice: white, brown and beige. White

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adipocytes permit fatty acid accumulation and secrete leptin whereas brown adipocytes play a role in heat production and express the protein Uncoupling protein-1 (UCP-1). These two distinct cell types organize into two specific tissues: white adipose tissue (WAT) and brown adipose tissue (BAT).[8] Beige fat, an intermediate-like type of fat, is inducible and shows thermogenic qualities when it appears in WAT, usually after cold exposure challenges. In rodents, brown and beige fat have the ability to limit fat gain that is usually caused by overeating.[9, 10]

WAT and BAT are contained in the body and located in fat depots, particularly the subcutaneous and abdominal compartments.[8] The WAT, which stores excess energy as triglycerides, can be further subdivided into two body compartments: the subcutaneous compartment that is localized under the skin and contains the subcutaneous adipose tissue (SAT) and the abdominal cavity, which contains visceral adipose tissue (VAT).[9] VAT is considered to be bioenergetically more active than SAT, and the adipocytes in VAT are also more lipolytically active.[11] An increase in WAT mass accelerates chronic inflammation as opposed to BAT.[12] With regards to BAT in humans, visceral depots of BAT can be found around the adrenals and other solid organs such as the heart and the kidneys.[77] The subcutaneous depots of BAT are located between the anterior neck muscles and the inter-and subscapular, axillary and clavicle regions.[78]

Important for the development of obesity is the manner in which the body regulates energy expenditure, energy intake, and energy storage or balance. An increase in the rate of obesity often reflects a state of positive energy balance.[13]

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Obesity is attributable to physical inactivity and the overconsumption of calorie-dense foods, particularity in developed nations that have adopted a predominantly Western diet composed of high-energy foods.[14, 15] The increased consumption of energy-dense foods high in saturated and trans fatty acids, processed starches and added sugars, commonly found in fast foods, has shown obesogenic effects.[16] According to the World Health Organization (WHO), worldwide obesity has nearly tripled since 1975, and in 2016 more than 1.9 billion adults were overweight.[17] The National Health and Nutrition Examination Survey (NHANES) conducted in 2015-2016 found the prevalence of obesity among adults in the United States to be 39.8% and 18.5% among youth. The same cross-sectional survey conducted by the Centers of Disease Control and Prevention (CDC) found the prevalence of obesity was higher among adults aged 40-59 compared to adults aged 20-39.[19] In order to clinically assess obesity in humans, the Body Mass Index (BMI) is a common screening tool. A BMI of >30 kg/m^2 is defined as obese, and a BMI of >40 kg/m^2 is classified as "extremely" obese.[19, 11] However, an ongoing criticism of BMI is that it is not able to discern between the different types of fat distribution.[16] The distribution of fat depots in the body differs between the sexes. In men, excess fat accumulation tends to be stored predominantly in the abdominal cavity as visceral fat whereas the fat reserves in women are primarily located subcutaneously.[7]

Obesity is considered a major public health concern worldwide due to the increased incidences of metabolic complications such as impaired glucose intake, insulin resistance, dyslipidemia (elevated plasma cholesterol,

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triglycerides, or low high-density lipoprotein cholesterol levels), type II diabetes (T2D), and cardiovascular dysfunction.[5, 19] These clustering of clinical findings, which include abdominal obesity, hyperglycemia, hypertension, and dyslipidemia are referred to as the metabolic syndrome (MetS). Discrepancies in the definition of MetS among different groups are a result of how each component is detected clinically. For example, insulin resistance (IR) is recognized by the WHO as a characteristic of the MetS but not by the National Cholesterol Education Program (NCEP): Adult Treatment Panel III (ATPIII).[20] An individual with MetS has a relative risk of approximately twofold for cardiovascular disease (CVD) and a five to seven fold increase for T2D.[20, 7] Furthermore, nonalcoholic fatty liver disease (NAFLD), or lipid accumulation in hepatocytes, is considered as the hepatic manifestation of the metabolic syndrome.[21,20] NAFLD comprises a spectrum of pathological changes in the liver beginning with simple hepatic steatosis (ranging from mild to severe) to nonalcoholic steatohepatitis (NASH) and often times will progress to cirrhosis of the liver, which increases the risk of hepatocellular carcinoma.[21] In addition to metabolic complications, obesity is a risk factor for various cancers including colorectal, liver, kidney, pancreas, gallbladder, esophageal, endometrial, multiple myeloma, thyroid, postmenopausal breast, and ovarian.[22]

Obesity is a state of chronic, low-grade inflammation.[3] Adipose tissue inflammation is recognized as a contributing factor to the metabolic dysfunctions seen in obesity as well as cancer.[23, 11] An excess of adipose tissue leads to increased levels of pro-inflammatory adipokines and cytokines, resulting in

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chronic low-grade inflammation due to the imbalance of the pro-inflammatory stimuli and the compensatory anti-inflammatory mediators.[24] Cytokines, cell signaling proteins, and adipokines are secreted from the adipose tissue. The adipokines, leptin and adiponectin, play a crucial role in adipose tissue inflammation. Leptin is a peptide hormone encoded by the *Ob* gene that is secreted mainly from adipose tissue and has a neuroendocrine role in food intake and satiety. In regards to obesity, leptin is directly correlated with fat mass; therefore, more leptin is secreted from the AT in overweight and obese individuals resulting in leptin resistance.[3] Additionally, obesity-related hyperleptinemia is partially responsible for promoting chronic-low grade inflammation.[76] In contrast, adiponectin acts to regulate the effects of leptin and aids in attenuating inflammation as well as cell proliferation. While leptin increases with body weight, adiponectin decreases with body weight, suggesting that low levels of adiponectin augment systemic and adipose inflammation as well as possibly increasing the risk of certain cancers.[25, 11, 3]

Not only do adipokines play a role in inflammation and increased cancer risk, but cytokines secreted by adipose tissue also have a significant impact in inflammation and cancer progression, especially Interleukin-6 (IL-6), tumor necrosis factor (TNF), IL-8, IL-1β, and monocyte chemoattractant protein 1 (MCP-1). These pro-inflammatory cytokines are directly influenced by the number of immune cells infiltrating the adipose tissue; therefore, an increase in fat mass due to an increase in body weight increases the amount of immune cells and cytokines.[24] Both IL-6 and TNFα are characterized as pro-tumorigenic

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cytokines due to their ability to influence various stages of cancer development including, initiation, promotion, progression, and metastasis.[26] An elevated plasma level of IL-6 correlates to poor prognosis and disease aggressiveness due to the ability of IL-6 to modulate the STAT pathway, which promotes cancer cell proliferation among others.[26,3] In addition, IL-6 has been found to be a powerful stimulator of CRC cell proliferation and growth.[26] The cytokine TNFα is produced during the on-set of an inflammatory response and is crucial for the continuation of chronic inflammation.[27] Furthermore, circulating TNFα levels are increased in obesity from secretions from adipose tissue and other immune cells. Similar to IL-6, TNFα has been linked to cellular transformations that are indicative of the hallmarks of cancer: cellular proliferation, invasion, angiogenesis and metastasis, but exerts its effects by activating the activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) signaling pathways.[26,28]

Another AT secreted cytokine that has been shown to be an important mediator of inflammatory reactions is IL-8.[29] IL-8 has previously been shown to promote cancer growth via an autocrine manner in human cancers including CRC.[30] The adipocytes in the cancer stroma have exhibited the ability to up-regulate the expression of IL-8, which exerts its effect on various signaling pathways that result in cell proliferation and other hallmarks of cancer.[29] MCP-1 (CCL2) is a member of the C-C chemokine family that binds to G protein coupled receptors. This binding regulates macrophage recruitment, particularly pro-inflammatory M1 macrophages in both adipose and tumor tissues.[31, 32] Studies

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have shown elevated adipose tissue MCP-1 in obese mice when compared to lean mice, demonstrating the role of MCP-1 in the enhancement of macrophage recruitment in obesity-associated AT.[11] Another pro-inflammatory cytokine secreted from adipose tissue is IL-1β. The production of IL-1β is stimulated by various factors that also increase with obesity such as hyperglycemia, cholesterol and free fatty acids (FFAs).[3]

A key player in metabolic dysregulation that enhances cancer risk is insulin. Insulin is a peptide hormone that is produced and secreted by the β cells in the pancreas in response to plasma glucose levels. Insulin is the main regulator of energy storage and stimulates glucose uptake by the muscles and adipose tissue when blood glucose levels increase. When plasma glucose levels are in excess, the liver and muscle store the additional glucose as glycogen. Insulin can induce fat storage, and in adipocytes will inhibit lipolysis while also inducing lipogenesis and fatty acid uptake. In overweight and obese individuals, there is an overproduction of insulin by the pancreatic β cells. This overproduction is the body's attempt to maintain homeostasis and prevent hyperglycemia when plasma glucose levels are high. When there is an increase in glucose production by the liver and a decrease in glucose uptake by the tissues of the body, mainly the insulin-resistant (IR) skeletal muscle, hyperinsulinemia occurs as a compensatory result.[33] In overweight individuals, the chronic hyperinsulinemia can increase insulin-like growth factors (IGF) while decreasing the expression of IGF-binding proteins in the liver, leading to estrogen and IGF-1 bioavailability.[34] These insulin-like growth factors have

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similar signaling pathways to that of insulin and have been found to be mutagenic in certain cancer cell lines.[35] Furthermore, epidemiological studies have shown that elevated levels of IGF-1 and insulin favor aggressive growth of various cancers including CRC, liver, pancreatic, endometrial and breast.[36] The signaling pathways that become stimulated by insulin are MAPK/ERK and PI3K/AKT, both of which promote tumor growth, migration and invasion.[37] An increasing amount of evidence has shown that metformin, a hyperinsulinemia drug used to treat T2D, can decrease incidence and mortality of pancreatic, hepatocellular, breast and CRC.[33] In summary, excess weight gain can result in increased levels of insulin and IGF-1, which may play a crucial role in the pathogenesis of many cancers.

While various cytokines, adipokines, and hormones have central roles in adipose tissue inflammation that lead to various metabolic dysfunctions and cancer, the immune system, particularly macrophages, is also a main player. As adipocytes increase in size due to increase in fat mass, some become apoptotic and are surrounded by macrophages. These macrophages form crown-like structures and are now considered a hallmark of adipose tissue inflammation.[38] This accumulation of macrophages in visceral adipose tissue enables chronic low-grade inflammation, which is associated with insulin resistance. In obesity, macrophages are characterized by their ability to polarize into pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages. The pro-inflammatory M1 macrophages invade the surrounding adipose tissue while the anti-inflammatory M2 macrophages activate immunosuppressive factors to promote

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an anti-inflammatory environment.[39, 40] With prolonged obesity, the M1 macrophages become the primary macrophage phenotype, leading to a pro-inflammatory environment that exacerbates the detrimental metabolic processes and increase the risk for various cancers.[41]

In summary, there are various factors that influence obesity; alterations in adipose tissue composition due to weight gain, decreased energy expenditure as a result of a sedentary life, a diet high in hydrogenated and saturated fats, and a chronic, low-grade inflammatory state that increases the secretions of pro-inflammatory cytokines, adipokines, and hormones. All of these factors must be considered when evaluating the adipose tissue and tumor microenvironment.

1.3 Estrogen

Estrogen, one of the primary sex hormones in women, is mainly produced by aromatase activity in placental and ovarian tissue in premenopausal women.[3] The main circulating estrogen hormone in premenopausal women is 17β-estradiol (E2). This hormone acts on distant target tissues as well as plays a role in normal menstrual cycles.[42] In postmenopausal women, the ovaries are no longer the main location of E2 production; therefore, circulating levels of E2 decrease and synthesis of estradiol needs to be carried out by other target tissues.[43] These extragonadal sites, such as the breast, bone, muscle, brain and adipose tissue, become the primary site of synthesis for estradiol, where it acts locally in an intracrine or paracrine manner to maintain tissue-specific functions.[44]

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The menopause transition begins with the onset of menstrual irregularities and ends with the last menstrual cycle.[45] After menopause, aromatase activity in the ovarian and placental tissues decreases, and the epidermis and adipose tissue play a more enhanced role in regulating systemic levels of estrogen.[3] The conversion of androgen to estradiol by aromatase in adipose tissue is the major source of circulating estradiol in postmenopausal women.[46] A decrease of aromatase activity in the ovaries along with increases in body mass associated with post-menopause can lead to greater amounts of pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) secreted from adipose tissue as well as IGF-1.[47] After menopause, a shift in fat distribution occurs where women accumulate more abdominal fat than subcutaneous fat.[48] This increase in intra-abdominal body fat can be seen in animal studies when the ovaries have been surgically removed. Several studies in rodents have demonstrated that OVX leads to increased adiposity, specifically abdominal and gonadal fat, where the gonadal fat is located near the reproductive organs.[45] Similar to OVX mice, aromatase knockout (ArKO) mice display increased abdominal fat accumulation in the gonadal and renal fat pads.[49] The OVX mice display decreased energy expenditure without changes in energy intake, resulting in adipose tissue inflammation, increased adipocyte hypertrophy, and fatty liver development.[50] It is important to note that hyperphagia does not completely account for the development of obesity and changes in metabolism after OVX.[51] Analogous to the OVX mice, female ArKO mice exhibit increased abdominal adiposity;

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however, evidence has shown that adiposity may be associated with reduced energy expenditure as a result of decreased physical activity.[52]

Taken together, estrogen deficiency in murine models as a result of OVX or aromatase knockout increases central adiposity.[45] It has been demonstrated that estradiol supplementation given to OVX and ArKO mice mitigates the effects associated with increased fat mass, specifically adipose tissue inflammation, insulin resistance, adipocyte hypertrophy, and liver steatosis.[53, 48] Overall, estrogen has been shown to contribute to the prevention of obesity-related metabolic syndromes and inflammation, in part, by regulating the production of adipokines and controlling insulin resistance.[19]

Not only does estrogen have a role in obesity, but it also has an impact on cancer initiation and tumor progression.[54] Studies have found a delayed development of adenomas and colon cancers in premenopausal women, suggesting a protective effect of female hormones.[55, 56] In a randomized clinical trial of postmenopausal women, the use of estrogen plus progestin, a form of progesterone that is commonly used in combination with estrogen, was associated with a statistically significant decrease in the incidence of CRC compared to postmenopausal women not on a hormonal therapy.[57] A study performed by *Yaker etc. al* demonstrated an increased susceptibility to insulin resistance, obesity and tumor growth in diet-induced obese female mice that underwent OVX surgery, positing a role of endogenous estrogens and diet.[54] However, not all cancers exhibit a decreased cancer incidence with the use of estrogen in post-menopausal women. Epidemiological evidence has

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demonstrated a positive relationship between estradiol and postmenopausal breast cancer risk, though there is conflicting data regarding the role of postmenopausal hormone use and high adult BMI.[58] For endometrial cancer, there is a stronger association between postmenopausal hormone use and high adult BMI and the risk of cancer development while the association is not as strong in ovarian cancer.[59, 60]

In conclusion, the amount and source of circulating estrogen changes after menopause. The role of estrogen varies during menopausal status, where estrogen deficiency along with increased visceral fat mass seen after menopause is associated with increased secretions of pro-inflammatory adipokines due to increases in fat mass. However, the presence of estrogen in a premenopausal state has been shown to attenuate the pro-inflammatory response, which has been demonstrated when estrogen replacement is administered to OVX mice. The interplay between estrogen and cancer remains an ongoing area of research due to estrogen's pro- and anti-tumorigenic effects. Hormone replacement therapy (estrogen and progesterone taken together) in postmenopausal women has been shown to decrease the incidence of CRC, whereas, a link between hormonal replacement therapies and a higher risk of breast cancer have been reported.

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CHAPTER 2

THE CONTRIBUTION OF ADIPOSE TISSUE FROM OVARIECTOMIZED MICE TO COLON CANCER

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2.1 Abstract

Obesity is considered a major public health concern worldwide due to the increased incidences of metabolic dysfunction and cancer risk. The obese state is, in part, attributable to the overconsumption of calorie-dense foods commonly seen in a standard Western diet, which aids in the progression of chronic, low-grade inflammation. The role of estrogen varies depending on menopausal status, where estrogen deficiency coupled with increased visceral fat associated with post-menopause leads to increased secretions of pro-inflammatory adipokines and cytokines. However, the presence of estrogen in a premenopausal state has been shown to attenuate the pro-inflammatory response, which has been demonstrated when estrogen replacement is administered to ovariectomized (OVX) mice. The purpose of this study was to determine if various pro-inflammatory cytokines secreted by adipose tissue influences cancer growth and if ovarian status affects this response. *Methods:* The study utilized a diet-induced model of obesity, where 8-week-old intact female, OVX female, and male mice were assigned to either a 40% high-fat diet (HFD) or a purified control low-fat diet (LFD) for 21 weeks. The ovaries of the OVX female group were removed in order to study the role of ovarian status in obesity and cancer initiation. To determine if pro-inflammatory cytokines associated with increases in body weight and fat mass influence cancer proliferation, adipose tissue-conditioned media (AT-CM) obtained from each dietary group was supplemented to MC38 colon cancer cells and a proliferation assay was performed. *Results:* An obese phenotype in mice fed a HFD was

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achieved and detectable increases in WBC, LYM, MON, and PLT count in the blood associated with obesity were identified. There were no diet effects discovered in the concentration of pro-inflammatory cytokines circulating in the plasma; however, a main effect of HFD ($p<0.05$) exhibited increased secretions of IL-1β and IL-6 from the AT-CM when comparing the intact and OVX female groups. No significant differences were discovered in the cell proliferation assay after treatment with AT-CM. *Conclusion:* HFD feedings resulted in significant increases in body weight and fat mass. Secreted levels of pro-inflammatory cytokines were detected in the AT-CM in the intact and OVX females. There was no difference detected in the cell proliferation assay after 24hrs.

KEYWORDS: Obesity, High-Fat Diet, Colon Cancer, Estrogen

2.2 Introduction

With the rates of obesity increasing since the 1970s, obesity has long been established as a major public health concern.[5, 17] The major cause of obesity can be attributable to a positive energy balance, where an increased consumption of energy-dense foods coupled with little physical activity results in an obese phenotype.[14, 15] The major characteristics of obesity are chronic, low-grade inflammation and metabolic dysfunction, specifically impaired glucose consumption, IR, dyslipidemia, T2D, and cardiovascular dysfunction.[3, 19] This inflammation in adipose tissue is recognized as a contributing factor to the metabolic dysfunctions seen in obesity as well as cancer.[23, 11] Adipose tissue inflammation is associated with altered levels of various pro-inflammatory adipokines and cytokines, including decreased levels of adiponectin and

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increased levels of leptin, IL-6, TNF, IL-1β, IL-8, and MCP-1. The increased levels of pro-inflammatory cytokines have been linked to various stages of cancer development.[26]

CRC is currently the third-most commonly diagnosed cancer in the US.[1] Epidemiological studies have suggested that a major risk factor for the development of CRC is obesity.[4] Additionally, a study examining the role of HFD feeding and CRC, by using the *APC*$^{min/+}$ mouse model, found increases in certain inflammatory mediators in the adipose tissue and tumor microenvironment, which was associated with an increase in the number of large polyps.[62] Increased fat mass coupled with ovarian hormones has been associated with cancer initiation and tumor progression.[54] Studies have found a delayed development of adenomas and CRCs in premenopausal women, suggesting a protective effect of female hormones.[55, 56] A study performed by *Yaker etc. al* demonstrated an increased susceptibility to insulin resistance, obesity and tumor growth in diet-induced obese female mice that underwent an ovariectomy, suggesting a role of endogenous estrogens and diet.[54] The role of estrogen varies depending on menopausal status, where estrogen deficiency coupled with increased visceral fat that occurs after menopause is associated with increased secretions of pro-inflammatory adipokines and cytokines. However, the presence of estrogen in a premenopausal state has been shown to attenuate the pro-inflammatory response, which has been demonstrated when estrogen replacement is administered to OVX mice.[50, 53]

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In order to further elucidate the associations between obesity and ovarian status and their influence on cancer proliferation, a diet-induced model of obesity was utilized in male and female mice. Furthermore, female mice were divided into two groups: intact females and OVX females. This was done to assess the role of ovarian status on adiposity and cancer initiation. The study was divided into two main components: 1) an *in vivo* model of obesity where 8 week old intact female, OVX female, and male mice were placed on a 40% HFD or a purified control LFD for 21 weeks after which plasma and adipose tissue was examined for pro-inflammatory cytokines and 2) an *in vitro* model of colon cancer in which cells were treated with adipose tissue-conditioned media obtained from each treatment group in order to assess cell proliferation in MC38 colon cancer cells. The purpose of this study was to determine if various pro-inflammatory cytokines secreted by adipose tissue influences cancer growth and if ovarian status, if at all, affects this response. We hypothesized that adipose tissue from HFD-fed mice will increase cell proliferation in MC38 cells and this will be further exacerbated in post-menopausal mice.

2.3 Methods

Animals

Male and female wild-type *C57BL/6* mice were purchased at four weeks of age from the Jackson Laboratories (Bar Harbor, ME) and cared for in the Department of Laboratory Animal Resources (DLAR) at the University of South Carolina. A total of 45 mice (n=6-9/group) were housed four to five mice per cage, maintained in a low-stress environment (22°C, 50% humidity, low noise) on

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a 12:12-h light-dark cycle. Food and water was provided *ad libitum*. Principles of laboratory animal care were followed, and the Institutional Animal Care and Usage Committee of the University of South Carolina approved all experiments.

Ovariectomy (OVX) surgery

In order to determine the effect of estrogen on obesity and cancer initiation, intact and OVX mice were used in this study. Ovariectomy (surgical removal of the ovaries) is a well-documented method to mimic the post-menopausal state in mice that have reached sexual maturity. Once removed, ovarian hormones (estrogen and progesterone) are no longer produced in the ovaries.[54, 61] At eight weeks of age, female mice underwent an OVX surgery (n=15). Briefly, mice were anesthetized with isoflurane and the dorsal mid-lumbar area was shaved and swabbed with iodine and alcohol. A 2cm dorsal midline skin incision was made halfway between the caudal edge of the ribcage and the base of the tail. A single incision of less than 1cm in length was created into the muscle wall on both the left and right sides, approximately 1cm lateral to the spine. The ovary and uterine horns, located in the gonadal fat pad under the dorsal muscle, were extracted through the incision with forceps. In order to excise the ovaries, the uterine horns were tied beneath the ovary with a 4-0 non-absorbable suture. The ovaries were cut and the uterine horns were placed back into the peritoneal cavity, where the muscle incisions were closed with 5-0 absorbable sutures. The incision was closed with wound clips, and the animals were examine for post-operative infection or discomfort for 72hours. Wound clips were removed seven days after surgery. In order to control for the possible

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effects of surgery on the parameters being studied, male mice (n=15) and intact females (n=15) underwent a sham surgery. The procedure for the sham surgery was identical to the ovariectomy procedure, except no tissue was excised from these mice.

Diets

At ten weeks of age, mice were randomly assigned to a control purified AIN-76A low-fat diet (LFD; 3.77 kcal/g) or a purified high-fat diet [HFD (40% of total kcal from fat); 4.57 kcal/g] designed to mimic the standard American diet[62] (BioServ, Frenchtown, NJ). The purified AIN-76A is a purified, balanced diet that is free of phytoestrogens. Dietary phytoestrogens, as found in regular chow diets in the form of soy, have been shown to influence food and water intake, anxiety-related behaviors, fat deposition, blood insulin, leptin and thyroid levels, and lipogenesis and lipolysis in rat adipocytes to name a few.[63] Intact female, OVX female and male mice were placed on either a LFD or HFD for 21 weeks.

Tissue Collection

Prior to necropsy, mice were fasted for five hours and blood samples were collected from the tip of the tail. Fasting blood glucose concentrations were determined in whole blood using a glucometer (Bayer Contour, Mishawaka, IN). After 21 weeks of dietary treatment, mice were euthanized via isoflurane inhalation for tissue collection. Whole blood was taken from the inferior vena cava and collected into K2 EDTA tubes (Franklin Lakes, NJ). Blood was analyzed for hematology using a VetScan HMT (Abaxis, Union City, CA). The

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remaining blood was spun at 1,600 rpm at 10°C for 10 minutes, and plasma was stored at -80°C. The gonadal, peri-renal, mesenteric and the dorsolumbar portion of the inguinal fat pads, as well as the spleen and uterus, were all removed and weighed. The left sides of the gonadal and inguinal fat pads were used for adipose tissue-conditioned media, and the right sides were immediately snap-frozen in liquid nitrogen and stored at -80°C.

Blood Profile

A complete blood profile was performed using the VetScan HMT (Abaxis, Union City, CA) to determine white blood cell (WBC), lymphocyte (LYM), monocyte (MON), neutrophil (NEU), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), and platelet (PLT) count. Whole blood was collected from the inferior vena cava at sacrifice and deposited in K2 EDTA microtubes where it was analyzed on the VetScan HMT according to manufacturer's instructions.

Adipose Tissue Conditioned Media

After mice were euthanized with isoflurane at sacrifice, the left side of the gonadal and the dorsolumbar portion of the inguinal fat pads were cut into 2-3mm^3 fragments and incubated in 100 ± 5mg/ml in Dulbeco's Modified Eagle Medium (DMEM) containing medium glucose (2.75 g/L), and 2% charcoal stripped Fetal Bovine Serum (FBS) for 24 hrs. After 24hrs, each tissue was washed with phosphate buffered saline (PBS) and re-incubated in the same medium conditions for an additional 24hrs at 37°C. Once incubated, the tissue explants were filtered through a 100µl nylon mesh strainer to remove larger

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contaminates, (VWR, Randor, PA) and the media for each sample was collected as AT-CM and stored at -20°C until further use. The AT-CM samples underwent an additional freeze/ thaw cycle in order to sterile filter the media using a 2μm pore sized sterile syringe filter.

Cell culture

The murine carcinoma-38 (MC38) colon cancer cell line (Kerafast Inc., Boston, MA) was derived from C57BL/6 mice. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (4.5g/L), 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin. Cells were incubated at 37°C with 5% CO_2.

Cell Counting Kit-8 (CCK-8) proliferation assay

To determine the proliferation of MC38 cancer cells in response to AT-CM obtained from intact female, and OVX female and male mice fed a low or high-fat diet, cell proliferation was determined using the CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, cells were split into a separate flask that differed from the maintenance flask and grown in no phenol red Dulbecco's Modified Eagle Medium (DMEM) containing low glucose (1000mg/L), 10% charcoal-stripped fetal bovine serum (FBS) and 1% penicillin/ streptomycin. Cells were grown until 70-80% confluency was achieved. Cells were seeded into 96-well plates (5,000 cells/well) in the same culture medium and incubated overnight (14-15hrs) at 37°C with 5% CO_2 before being serum starved (DMEM with no phenol red, low glucose, no FBS, 1% penicillin/ streptomycin) for 4 hours.

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Following the serum starve, the media was aspirated and cells were treated with a 1:2 dilution of AT-CM and culture medium (DMEM containing no phenol-red, medium glucose (2.75g/L), 2% charcoal stripped FBS, and 1% penicillin/ streptomycin) and incubated for 24 hours. Each group (six groups total, n=5=8/group) was tested in duplicate in two replicate wells. CCK-8 solution (10µl) was added to each well and incubated for another three hours at 37°C. Following the 3hr incubation, the absorption was measured at a wavelength of 450nm using a microplate reader (Molecular Devices, LLC, San Jose, CA). Cell proliferation studies were repeated four times.

Circulating and secreted cytokine analysis

Plasma and adipose tissue-conditioned media samples were analyzed using the Bio-Plex 200 Reader to measure the concentration of the circulating and secreted cytokine levels of: IL-1β, IL-6, IL-10, GM-CSF, IFN-γ, MCP-1, RANTES, KC and TNFα (Express Custom Assay kit, BioRad Inc., Hercules, CA). Plasma samples and AT-CM were diluted 1:4 and probed for cytokines according to manufacturer's instructions. AT-CM samples were not analyzed for the cytokine KC.

Statistical Analysis

All data were analyzed using commercially available software: Prism 8 (GraphPad Software, La Jolla, CA). Body weight, body composition, fasting blood glucose, circulating and secreted cytokines were analyzed using a two-way ANOVA followed by Newman-Keuls post-hoc analysis to determine differences

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between diet (LFD vs. HFD) and ovarian status (intact vs. OVX) or sex (intact females vs males). A one-way ANOVA followed by a Newman-Keuls post-hoc test was performed to determine differences in cell proliferation. All data was assessed for outliers prior to running a one or two-way ANOVA. Data are presented as mean ± SEM, and the significance was set with an alpha value of $p < 0.05$.

2.4 Results

HFD consumption for 21 weeks leads to an obese phenotype.

In order to verify that HFD feeding resulted in an obese phenotype, body weights were measured throughout the study and fat pad weights were determined at euthanasia. Mice consuming the HFD had significantly heavier body weights ($p<0.05$) compared to the LFD control mice. On average, HFD male mice had a body weight of 49g followed by HFD OVX (45g) and finally HFD females (36g) (Fig 2.1a). Fasting blood glucose was measured after 21 weeks of diet feeding. A main effect of HFD ($p<0.05$) was evident by the elevated fasting blood glucose levels (Fig 2.1b). Fat depots were measured to assess changes in body composition. The visceral fat depot (peri-renal (kidney), gonadal, and mesentery) weights displayed a main effect of diet (HFD) and ovarian status (OVX) ($p<0.05$) and were significantly increased in the HFD-fed mice relative to the LFD-fed control mice ($p<0.05$) (Fig 2.1c-e). An interaction between diet (HFD) and ovarian status (intact and OVX) was apparent by the increased peri-renal and mesentery fat depot weights (Fig 2.1c, e). A main effect of diet (HFD) and ovarian status (intact and OVX) contributed to increased spleen weight (Fig 2.1f). Finally,

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uterine weight was measured to verify that the OVX surgery was successful. As expected, OVX females had significantly decreased uterine weights compared to intact females ($p<0.05$), resulting in a main effect of ovarian status (Fig 2.1g).

In addition to the main comparison of intact females and OVX females, interactions were assessed between intact females and males. In regards to spleen weight, a main effect of HFD indicated increased spleen weight ($p<0.05$) (Fig 2.2a). A main effect of diet (HFD) was evident by the increased fasting blood glucose levels ($p<0.05$) (Fig 2.2b). Main effects of diet (HFD) and sex (intact females and males) as well as interactions identified increased peri-renal, gonadal, and mesenteric fat depots weights (Fig 2.2c-e). A main effect of diet (HFD) and sex (intact females and males) ($p<0.05$) for increased peri-renal fat pad weight were observed (Fig 2.2c). An overall interaction and a main effect of diet (HFD) ($p<0.05$) were discovered in the gonadal fat (Fig 2.2d). A main effect of diet (HFD) and sex (intact females and males) as well an interaction ($p<0.05$) signified increased mesentery fat weight (Fig 2.2e).

Diet and Sex Differences in intact females, OVX females and males affects blood profile.

After performing a blood panel in the intact females and OVX females, there was a main effect of ovarian status (OVX) for WBC, LYM, and HCT count (Fig 2.3a,b,g). Additionally, there was a main effect of diet (HFD) signifying increased PLT count ($P<0.05$) (Fig 2.3h). No main effects of diet or ovarian status was determined in MON, NEU, RBC, and HGB count (Fig 2.3c-f).

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Next, we investigated the blood panel between intact females and males. A main effect of diet (HFD) and sex (males) in addition to an interaction ($p<0.05$) indicated an increase in WBC and LYM counts (Fig 2.4a,b). We found a main effect of diet (HFD) ($p<0.05$) for increased MON count (Fig 2.4c). A main effect of sex (males) and an interaction ($p<0.05$) displayed an increased NEU count (Fig 2.4d). Additionally, there was an interaction found for the HGB count ($p<0.05$) (Fig 2.4f). No main effect of diet or sex was found for RBC, HCT and PLT count between intact females and males (Fig 2.4e,g,h).

Circulating pro-inflammatory cytokines in the plasma had an impact on ovarian status and sex, but surprisingly had no effect on diet.

Since pro-inflammatory cytokines are increased in obesity due to increases in fat mass, we sought to determine if pro-inflammatory cytokines were elevated in the plasma of intact female, OVX female, and male mice fed a 40% HFD. We found main effects of ovarian status (OVX) depicting decreased circulating levels of the cytokines IL-1β, IL-6, IFN-γ, GM-CSF, MCP-1 and TNFα ($p<0.05$) (Fig 2.5a-f); however, no main effects of ovarian status (OVX) for the cytokines KC, IL-10 and RANTES were detected (Fig 2.5g-i). Contrary to what we expected, there were no main effect of diet (LFD or HFD) identified between intact and OVX females.

Similar to intact females and OVX females, there were main effects of sex; however, there were no main effects of diet. Main effects of sex displayed a significant decrease ($p<0.05$) in all nine cytokines in male mice: IL-1β, IL-6, IFN-γ, GM-CSF, MCP-1, TNFα, KC, IL-10, and RANTES (Fig 2.6a-i). Overall, the

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levels of circulating cytokines had a decreasing trend in the male mice compared to the intact female mice among the circulating cytokines measured.

Pro-inflammatory cytokines secreted from adipose tissue-conditioned media resulted in diet and ovarian status effects but no sex effect.

In an obese setting, pro-inflammatory cytokines are secreted from the adipose tissue; therefore, various pro-inflammatory cytokines (IL-1β, IL-6, IFN-γ, GM-CSF, MCP-1, TNFα and RANTES) and the anti-inflammatory cytokine IL-10 were measured in the adipose tissue-conditioned media collected from mice fed either a LFD or HFD. A main effect of diet (HFD) was evident by the increased secreted levels of the pro-inflammatory cytokines IL-1β and IL-6 (Fig 2.7a,b); however a main effect of diet displayed decreases in the secretion of IFN-γ in the HFD fed mice (Fig 2.7c). In addition to a main of effect of diet in IL-1β, a main effect of ovarian status (OVX) indicated increased secretion of IL-1β in OVX mice ($p<0.05$) (Fig 2.7a). Interestingly, a main effect of ovarian status in the OVX mice displayed decreased concentration of MCP-1 compared to intact females (Fig 2.7e). There was no significant difference in the concentration of the cytokines GM-CSF, IL-10 and RANTES secreted from the AT-CM (Fig 2.7d,f, g). The concentration of secreted levels of the cytokine TNFα was not detected.

When the same cytokines were assessed in the intact females and males, a main effect of diet (HFD) in the decreased concentration of IFN-γ secreted from the adipose tissue was identified (Figure 2.8c). There was no significant difference in the concentration of the cytokines IL-1β, IL-6, GM-CSF, MCP-1, IL-10 and RANTES secreted from the adipose tissue (Fig 2.8 a-b, d-g). While the interaction between

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intact females and males did not reach significance ($p=0.06$) in the concentration of IL-1β, it is worth mentioning that there were elevated levels in the HFD male group ($p=0.057$) (Fig 2.8a). Similar to intact and OVX females, the concentration of secreted levels of the cytokine TNFα was not detected.

No difference in the proliferation of MC38 cells when treated with adipose tissue-conditioned media from intact female, OVX female, and male mice fed either a low or high-fat diet.

Since pro-inflammatory cytokines secreted from adipose tissue play a role in cancer proliferation, we hypothesized that the AT-CM isolated from HFD-fed groups would exhibit an increase in cell proliferation of MC38 cells compared to the LFD-fed groups and that this would be further exacerbated in the HFD OVX female group. However, when MC38 cells were treated with AT-CM taken from each group and assessed for proliferation, there was no significance difference between the groups (Fig 2.9).

2.5 Discussion

Since the majority of individuals become overweight due to the consumption of a poor diet rich in energy-dense foods, we used a diet-induced model of obesity to measure body weight and body composition changes associated with an obese state. A growing body of literature has exhibited differences in adiposity between pre-and post-menopausal women, suggesting women after menopause are at greater risk for obesity, which is associated with an increased cancer risk. Therefore, we were interested in assessing the influence of ovarian status on pro-inflammatory cytokines and cancer growth in

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an obesity model. We were further interested in sex differences in these outcomes in an obesity model.

In agreement with previous studies, the mice assigned to a 40% HFD exhibited higher body weights compared to the control LFD mice, proving that our HFD significantly increased body weight.[40, 62, 68] As expected, HFD elevated resting blood glucose, which has been demonstrated to produce complications with glucose tolerance.[68] These findings are consistent with hyperglycemia, a key component of metabolic dysfunction. Previous studies have shown that increased fasting blood glucose is associated with glucose intolerance and insulin resistance; however, we did not perform glucose and insulin tolerance tests and interpretations need to be made cautiously.[62] Since an increase in body weight is directly related to increased adiposity, particularly abdominal fat, mice fed a HFD sufficiently displayed significant increases in the visceral adipose depots; peri-renal, gonadal and mesentery.

Various clinical and experimental studies have found differences in WBC, LYM, and HCT counts with ovarian status, which is consistent with the findings from our study.[66, 71] Since associations between obesity and elevated platelet count in females with chronic inflammation have been posited, it was of no surprise that a diet effect was found between intact and OVX female mice.[69] Studies have reported differences between sex and diet in some cells in the blood. There were significantly elevated levels of WBC counts in HFD male mice compared to HFD female mice. Elevated WBC counts are associated with insulin resistance and MetS and are related to the occurrence of NAFLD, which may

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explain the high counts in male mice, even though IR was not directly measured.[64, 65] In an obese state, anti-inflammatory T cells, Tregs and T_H2 cells, decline while pro-inflammatory T cells, T_H1 and CD8+, increase.[3] While the blood panel is unable to differentiate between T cells, B cells and natural killer (NK) cells that comprise lymphocytes, significant increases in LYM count in HFD male mice compared to LFD male mice may suggest an increase in pro-inflammatory T cells. Additionally, studies have found a sex effect in NEU and HGB count between males and females as well as increases in NEU count in an obese setting, which is consistent with our findings.[67, 68, 70] Monocytes differentiate into macrophages, which are known to be present in obesity and infiltrate adipose tissue. Unsurprisingly, our study found a diet effect in MON count.

After an obese phenotype was established and a blood panel performed, the first major component of the study was to determine if circulating pro-inflammatory cytokines were present in the blood and secreted from adipose tissue after 21 weeks of HFD feeding. Since adipose tissue increases with obesity, we wanted to determine which cytokines were affected by diet and/or ovarian status; therefore, a multiplex was performed to quantify the concentration of the cytokines IL-1β, IL-6, IFN-γ, GM-CSF, MCP-1, TNFα, KC, RANTES and IL-10 present in the plasma. A main effect of ovarian status (OVX females) and sex (males) was discovered, but surprisingly a main effect of diet was undetected. A possible explanation for no effect of diet could be a plateau in cytokine concentration after pro-longed high-fat diet feeding. Consistent with our data, a study conducted by Guan *et. al.* found no difference in the same cytokines

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measured in our study after male mice were fed a 45% high-fat diet for 20 weeks.[73] Additionally, another study reported increased levels of IL-6 in mice fed a 60% HFD after three days on a HFD diet; however, after one week, levels remained unchanged for the duration of the 16 weeks of HFD feeding, which was the same for plasma levels of TNFα and IL-1β.[74] In a study that sought to explore the interplay between diet-induced obesity, ovarian status, and tumor growth in MC38 cells, the researchers found no significant difference in serum MCP-1, IL-6 and TNFα between control LFD mice and obese mice, which is consistent with our data that found no main effect of diet.[54] Taken together, it is plausible that after 21 weeks of HFD feeding, circulating plasma cytokines remained unaltered. It is possible that changes in pro-inflammatory cytokines occurred between mice fed a low or high-fat diet at an earlier time point that was missed when measured at the conclusion of the study. In regards to the findings of main effect of ovarian status displayed in intact and OVX females, the lower levels of cytokine concentrations in OVX mice may in part be due to lower levels of estrogen production in these mice. The ability to measure low E2 levels in the plasma has consistently been a problem in biomedical research.[75] While estrogen levels were not directly measured in this study, it is possible that the assay performed was not sensitive enough to detect any potential differences in E2 levels that may have been present in the OVX mice compared to the intact females.

The increased secreted levels of IL-1β and IL-6 in the adipose tissue of HFD fed mice support the current literature that as fat mass increases adipose tissue directly secretes pro-inflammatory cytokines independent of cancer.

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Based on the concentration of secreted pro-inflammatory cytokines in the AT-CM, the final part of the study sought to explore the role between adiposity, ovarian status and its role in cancer proliferation. Using an *in vitro* model of colon cancer, we were unable to detect differences in cancer cell proliferation when treated with AT-CM for 24-hours. A potential explanation for the lack of proliferation may be that the time point selected was not long enough to see changes in cell proliferation; therefore, extending the assay past 24 hours to 48 and 72 hours may result in differences in colon cancer cell proliferation. Additionally, the AT-CM samples were subjected to repeated freeze/thaw cycles after secreted cytokine concentrations were measured; therefore, possible degradation of secreted cytokines may be responsible for the unchanged difference in the proliferation assay.

In summary, the current study was able to demonstrate that HFD fed mice display significantly higher body weights compared to the LFD fed mice. The diet composition used in this study was unique in that it was designed to mimic a standard American diet where the fat sources where diverse and not reliant on a large percentage of any particular fat source. The implementation of a 40% HFD in the study altered body composition, which resulted in higher fat pad weights of the gonadal, peri-renal (kidney), mesenteric fat depots of high-fat diet fed mice. Additionally, fasting blood glucose levels were significantly increased in the HFD-fed groups as well. When adipose tissue-conditioned media was analyzed for pro-inflammatory cytokine concentration, there was an increase in the secreted concentrations of IL-1β and IL-6 in the HFD mice, suggesting that pro-

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inflammatory cytokines are secreted from the adipose tissue of obese mice. There was no difference in cell proliferation between intact and female mice fed a HFD and ovarian status had no effect on this response. However, it is plausible that the 24-hour time point was not sufficient enough to detect differences in cell proliferation.

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2.6 Figures

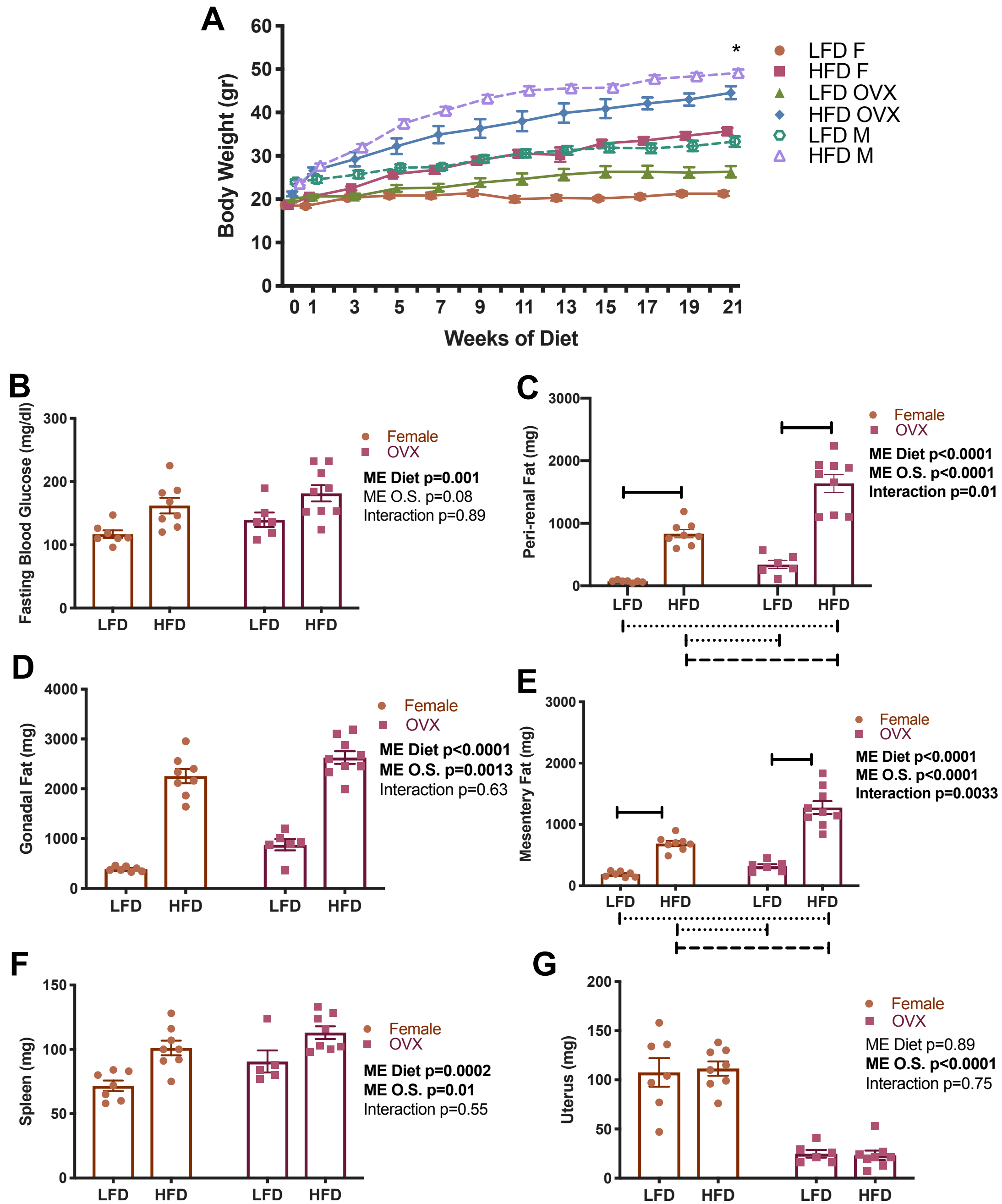

Figure 2.1. Body composition after 21 wks of dietary treatment of high-fat diet (HFD) or low-fat diet (LFD) feeding in intact and OVX mice. Body weight data displays weight gain in intact females, OVX females and males (A). Fasting blood glucose (B), peri-renal (C), gonadal (D), and mesentery (E) fat depot weight, spleen weight (F) and uterine weight (G) in intact and OVX females after 21 weeks of dietary treatment. * Represents a significant difference ($p<0.05$) in body weight between HFD and LFD fed mice. The solid black line represents differences in main effect of diet; the dashed black line represents main effects of

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ovarian status; and the dotted black line represent an interaction. Lines represent a significant difference (p <0.05). Values are mean ± SE; n=6-9 mice per group.

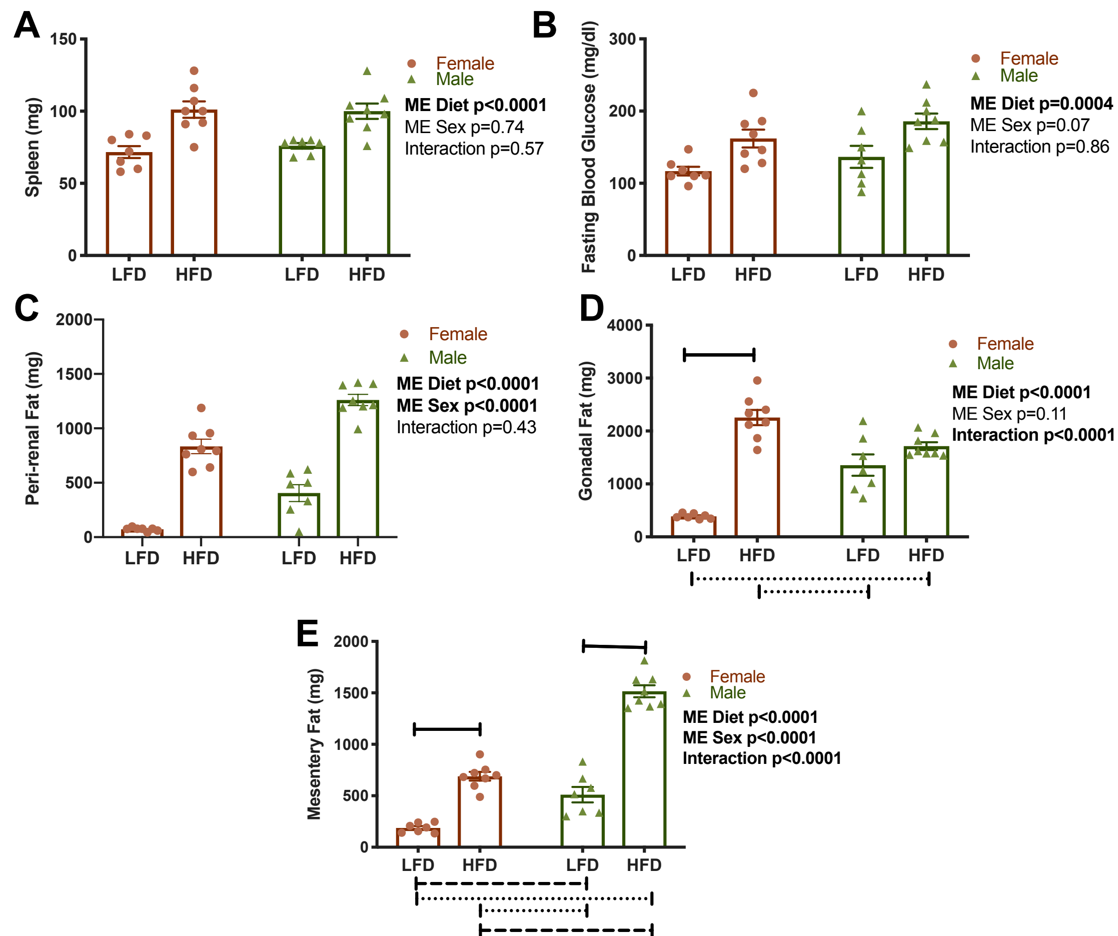

Figure 2.2. Body composition after 21 wks of dietary treatment of high-fat diet (HFD) or control low-fat diet (LFD) feeding in intact female and male mice. Spleen weight (A), fasting blood glucose (B), and peri-renal (C), gonadal (D), and mesentery (E) fat depot after 21 weeks of dietary treatment. The solid black line represents differences in main effect of diet; the dashed black line represents main effects of ovarian status; and the dotted black line represent an interaction. Lines represent a significant difference (p <0.05). Values are mean ± SE; n=6-9 mice per group.

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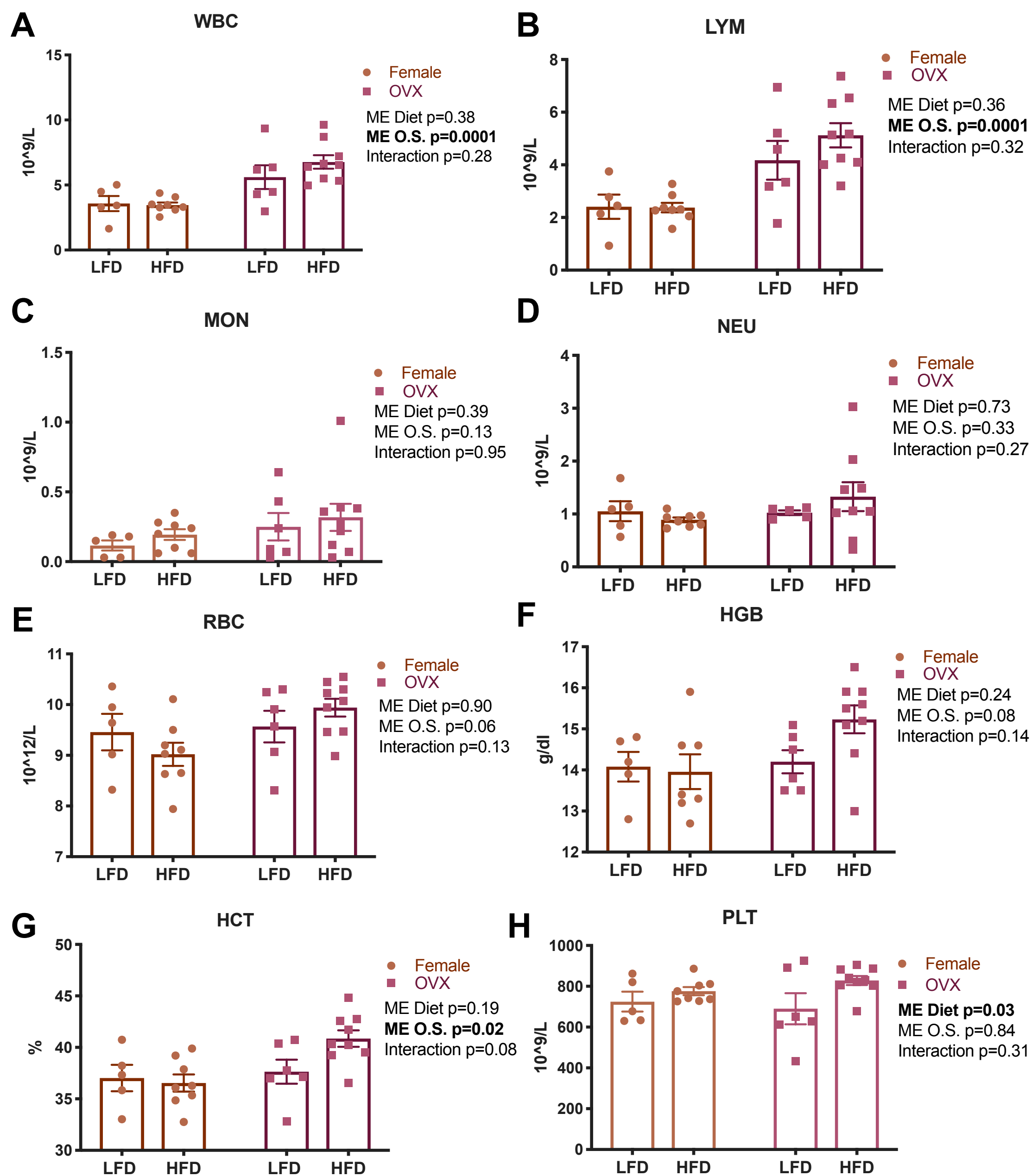

Figure 2.3. High-fat diet feeding and ovarian status alters blood profile in intact and OVX female mice. (A) White blood cells (WBC), (B) Lymphocytes (LYM), (C) Monocytes (MON), (D) Neutrophils (NEU), (E) Red Blood Cells (RBC), (F) Hemoglobin (HGB), (G) Hematocrit (HCT) and (H) Platelets (PLT) count. Values are mean ± SE; n=6-9 mice per group.

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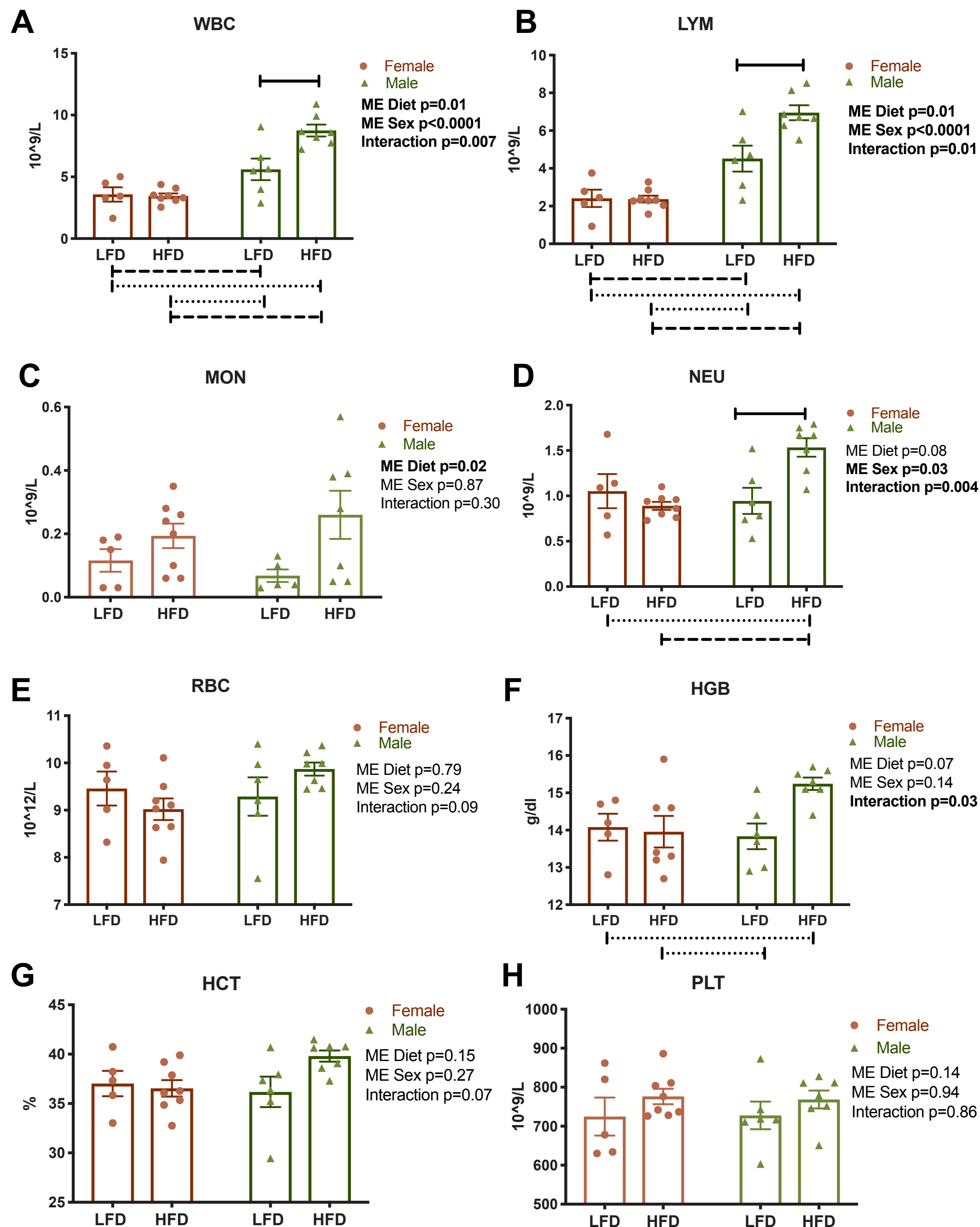

Figure 2.4. High-fat diet feeding and sex alters blood profile in intact female and male mice. (A) White blood cells (WBC), (B) Lymphocytes (LYM), (C) Monocytes (MON), (D) Neutrophils (NEU), (E) Red Blood Cells (RBC), (F) Hemoglobin (HGB), (G) Hematocrit (HCT) and (H) Platelets (PLT) count. The solid black line represents differences in main effect of diet; the dashed black line represents main effects of ovarian status; and the dotted black line represent an interaction.

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Lines represent a significant difference (p <0.05). Values are mean ± SE; n=6-9 mice per group.

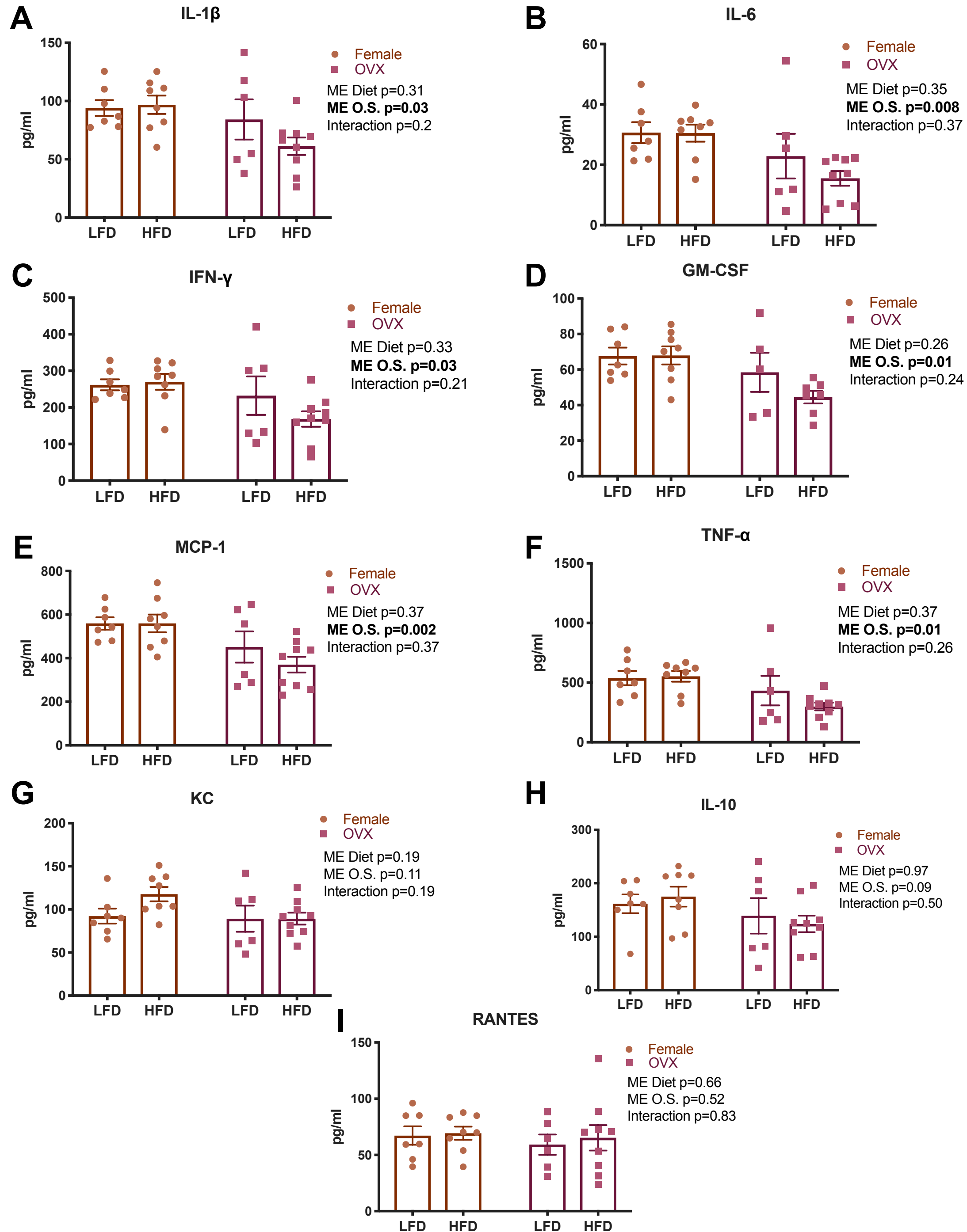

Figure 2.5. Ovarian status in intact and OVX female mice alters pro-inflammatory cytokine concentration instead of diet composition. The concentration of

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circulating cytokines (A) IL-1β, (B) IL-6, (C) IFN-γ, (D) GM-CSF, (E) MCP-1, (F) TNFα, (G) KC, (H) IL-10 and (I) RANTES is shown. Values are mean ± SE; n=6-9 mice per group.

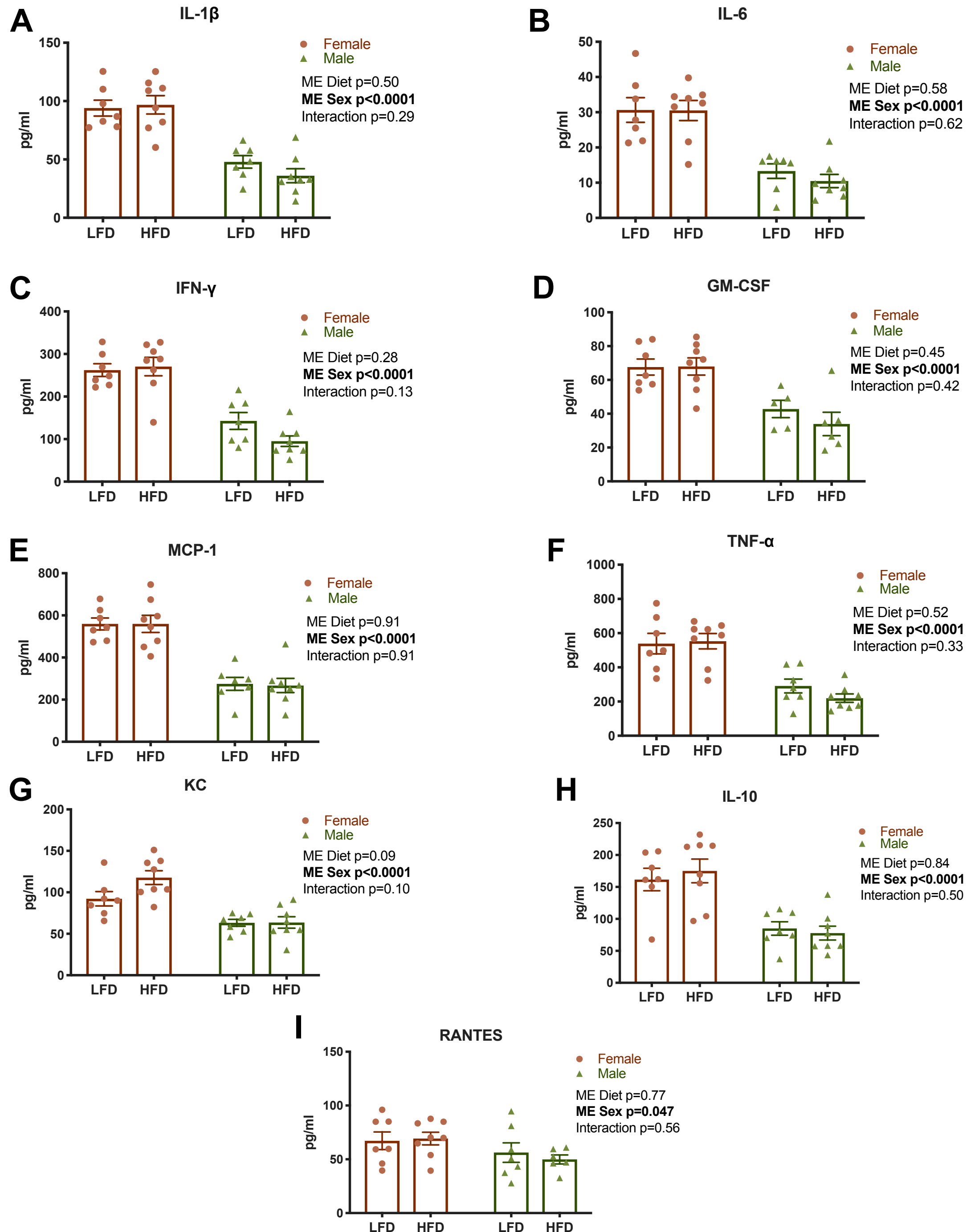

Figure 2.6. Sex difference in intact female and male mice alters pro-inflammatory cytokine concentration instead of diet composition. The concentration of circulating cytokines (A) IL-1β, (B) IL-6, (C) IFN-γ, (D) GM-CSF, (E) MCP-1, (F)

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TNFα, (G) KC, (H) IL-10 and (I) RANTES is shown. Values are mean ± SE; n=6-9 mice per group.

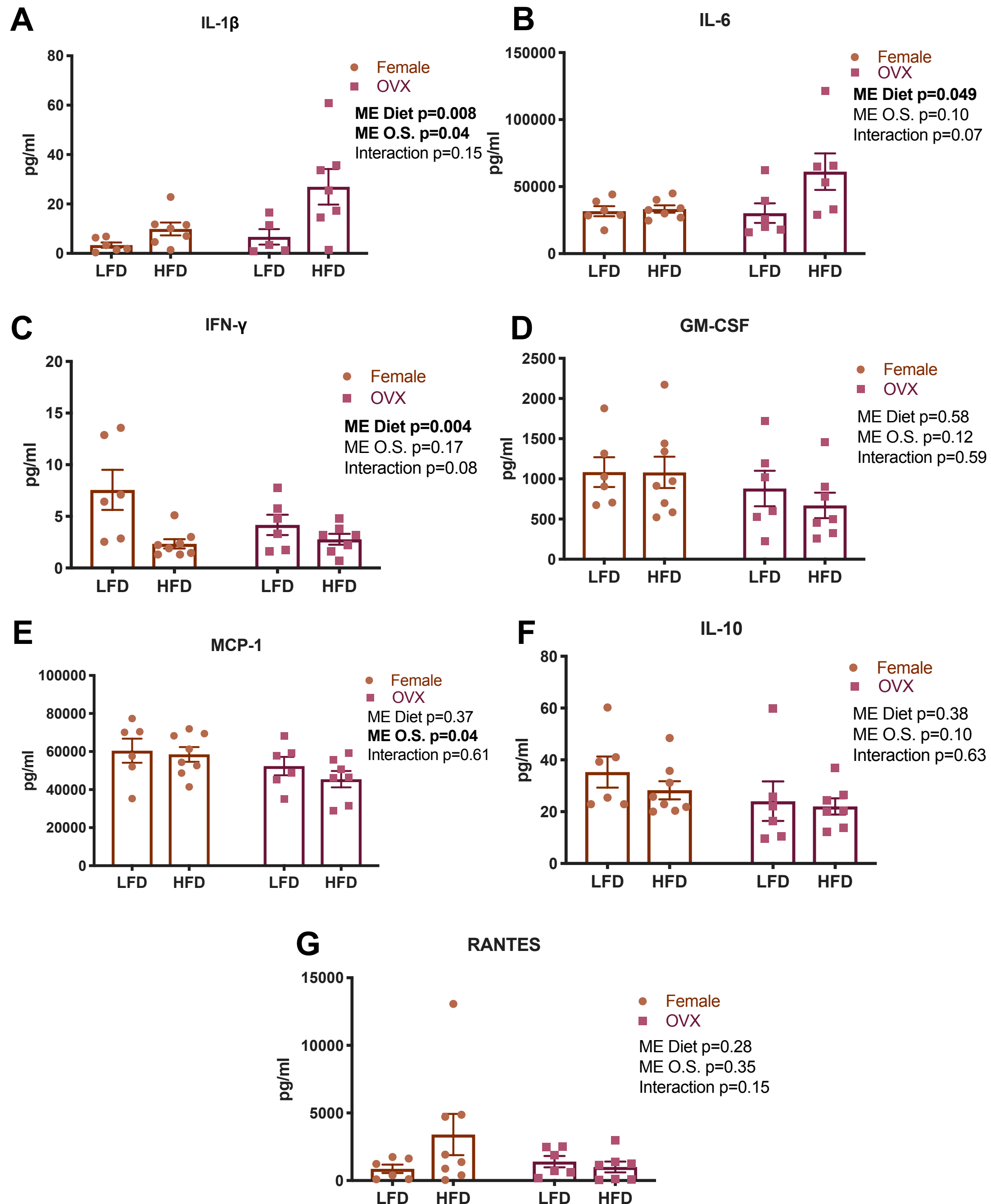

Figure 2.7. Diet composition and ovarian status in intact and OVX female mice alters the pro-inflammatory cytokine concentration secreted from adipose tissue-conditioned media. The concentration of circulating cytokines (A) IL-1β, (B) IL-6,

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(C) IFN-γ, (D) GM-CSF, (E) MCP-1, (F) IL-10 and (G) RANTES is shown. Values are mean ± SE; n=6-9 mice per group.

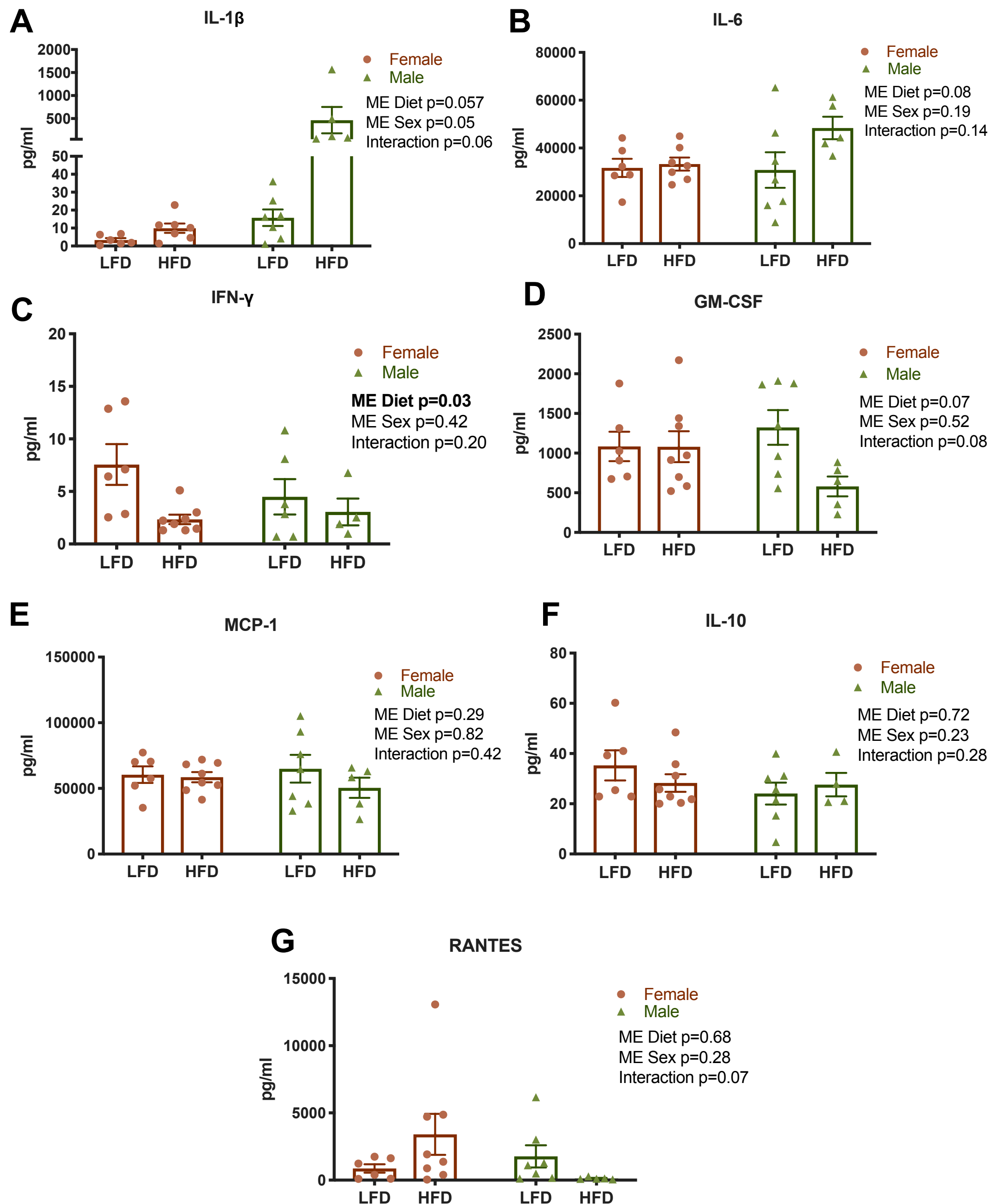

Figure 2.8. Diet composition in intact female and male mice alters the of pro-inflammatory cytokine concentration secreted from adipose-tissue conditioned media. The concentration of circulating cytokines (A) IL-1β, (B) IL-6, (C) IFN-γ, (D) GM-CSF, (E) MCP-1, (F) IL-10 and (G) RANTES is shown. Values are mean ± SE; n=6-9 mice per group.

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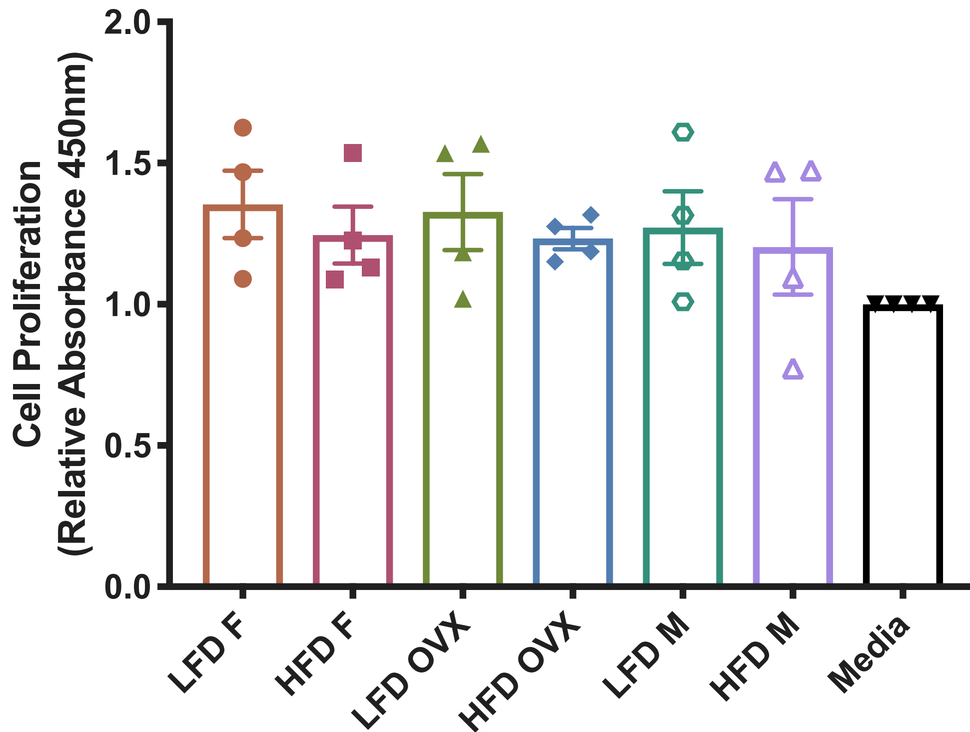

Figure 2.9. Pro-inflammatory cytokine secretion from adipose tissue-conditioned media had no effect on cell proliferation of colon MC38 cancer cells. No significant difference ($p<0.05$) was detected between the six groups (LFD F, HFD F, LFD OVX, HFD OVX, LFD M, and HFD M) after a 3-hour incubation of CCK-8.

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