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Role of Estrogen in Regulating Diet-Induced Obesity in Females

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DEDICATION

I would be honored to dedicate this work to my almighty God, our creator, Allah. To my parents, Khaleel and Faiza, who give me all the support and values to be where I am today. To my lovely wife, Halah, my soulmate who encourages me, and she has been present every moment of this this journey. To my kids, Rawan, Reem, Mesk, Mohamd, and Khaleel, you are the best things that have happened to me in my life. To my brothers and sisters, your words gave me more power to keep going. To all the people who know me have given me advice, this work would not have been accomplished without you.

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ABSTRACT

Menopause puts a female at risk for several chronic diseases, including an increased risk of obesity. Estrogens are major sex hormones of females that dramatically decrease during menopause. Several murine studies have established the crucial role of estrogen and estrogen receptor alpha (ERα) on energy homeostasis, glucose metabolism, and insulin action. However, previous works have produced contradicting findings, hence, further work is needed to optimize better outcomes. Therefore, my dissertation focuses on optimizing and characterizing different estrogen-deficient models, as well as using novel double transgenic inducible and tissue-specific models that overexpress aromatase (estrogen producing enzyme) and estrogen receptor isomers alpha (ERα) in the skeletal muscle of female mice. Using the aromatase knock out (Arom KO) model, data analysis revealed that estrogen deficiency increases adiposity primarily via decreasing of physical activity rather than changes in food intake. Also, skeletal muscle ERα, protects female mice against obesity and metabolic dysregulation independent of the estrogen status. On the other hand, skeletal muscle aromatase overexpression limits adipose tissue inflammation, however, no impact on adiposity or metabolic dysregulation were observed. This work presents, for the first time, differing effects of estrogen manipulating models on the development of the obese phenotype in the females. Inducible transgenic model in this work can be utilized to overexpress/delete ERα in different metabolic tissues for better understanding estrogen action on metabolism.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Arom KO ..... Aromatase knock out

AT ..... Adipose tissue

AUC ..... Area under the curve

BMI ..... Body mass index

E2 ..... 17β-estradiol

EE ..... Energy Expenditure

ERα ..... Estrogen receptor alpha

ERβ ..... Estrogen receptor beta

ERE ..... Estrogen response element

FFAs ..... Free fatty acids

GTT ..... Glucose tolerance test

HFD ..... High-fat diet

HSA ..... Human skeletal actin

IR ..... Insulin resistance

ITT ..... Insulin tolerance test

LFD ..... Low-fat diet

Ovx ..... Ovariectomized

REE ..... Resting Energy Expenditure

rtTA ..... Reverse tetracycline-controlled transactivator

SkM ..... Skeletal muscle

T2D ..... Type-2 diabetes

Tet ..... Tetracycline response element promotor

CHAPTER ONE

Literature review

1.1 Definition and statistics:

Obesity is a complex multifactorial disease characterized as an abnormal excessive accumulation of fat that presents health risks[1-5]. Body mass index (BMI = kg of body weight/meters in height squared) is the most commonly used parameter to classify overweight (BMI $\geq$ 25) and obesity (BMI $\geq$ 30)[6]. Obesity is considered as a global pandemic illness as its rate has tripled since 1975. In the United States, the national center for health statistics (NCHS) reported that prevalence of obesity among American adults was 42.4% in 2017-2018[1, 7]. This percentage is expected to increase to 50% by 2030[8]. Strikingly enough, the prevalence percentages are increasing among younger ages recording 33.1% and 27.4% among children and adolescents, respectively [9].

1.2 Causes:

Obesity occurs when a sustained positive energy balance leads to increase in body weight which is majorly due to energy imbalance between calories consumed and calories expended [3, 10]. Globally, there has been an increase in the intake of high-fat and sugar-rich food and a decrease in physical activity due to a number of factors including environmental and social changes such as lifestyle, transportation, food processing, and

urban planning [11]. In summary, obesity is a multifactorial disease with a complex pathogenesis related to genetic[12], psychosocial[13], socioeconomic[14], and environmental[15] factors. Interestingly, except for the genetic factors, all other obesity risk factors can be prevented or treated [16].

1.3 Pathogenesis and complications of obesity:

A chronic imbalance between the energy ingested and energy expended overwhelms the body's metabolism process and leads to the storge of excess energy in the form of adipose tissue (AT). In response to a positive energy balance, adipocytes undergo process of hypertrophy and hyperplasia resulting body fat accumulation particularly in visceral adipose tissue (VAT) classified as white adipose tissue (WAT) [10]. Ultimately, obesity is a major risk factor for many pathological conditions and diseases such as, insulin resistance (IR), type 2 diabetes (T2D), cardiovascular disease (CVD), hypertension, inflammation, cancer, disability, and most recently more severe symptoms of COVID-19, all leading to an increased risk of mortality [17, 18].

1.3.1 Insulin resistance and T2D:

Insulin is a peptide hormone produced by beta cells of Langerhans islets of the pancreas that globally regulates glucose metabolism. It is the major anabolic hormone that responses to nutrient intake, as well as controls energy storage, mobilization, and utilization [19]. Not limited to glucose metabolism, insulin also controls lipid metabolism in different metabolic tissues including, but not limited to, liver, muscle, and AT [20]. For example, in

skeletal muscle, insulin promotes glucose uptake and storage. In liver, where glucose produced endogenously, insulin inhibits gluconeogenesis (production of glucose from non-carbohydrate source) and activates lipogenesis [19-23]. In WAT, insulin inhibits lipolysis and increases glucose transport and lipogenesis [24]. Obesity-induced IR is a pathological condition in which metabolic tissues response insufficiently to insulin, therefore higher levels of circulating insulin are required to achieve a normal glucose level [23]. The pancreatic β-cells have the capacity to increase insulin release in order to overcome the impaired efficacy of insulin action, which explains why most obese IR individuals do not develop hyperglycemia [20, 25, 26] . T2D is chronic metabolic disorder accounting 90% of diabetic cases which is characterized by persistent hyperglycemia due to impairment of insulin secretion and/or peripheral insulin resistance [22]. Fasting hyperglycemia that define T2D is primarily a consequence of inadequate insulin action on the metabolic tissues in insulin resistant individuals. T2D is strongly associated with obesity due to the ability of obesity to promote IR [27, 28]. Interestingly, gradual weight loss up to 16% of original body weight is sufficient to improve β-cells function and insulin sensitivity [27, 28]. Using the National Health Interview Survey data collected between 1997-2004, data analysis revealed that obesity, particularly in younger ages, substantially increases remaining lifetime diabetes risk in women recording 74% for >35 kg/m^2 BMI, compared to 12.2% for women with BMI of <18.5 kg/m^2 [29]. Furthermore, in a separate 3 -year follow-up study, the incidence of diabetes was effectively reduced by 58% in a group of obese women who successively reduced 7% of their body weight compared to control group. Also,

moderate, and sustained weight-loss has improved insulin action, in obese women, and reduced the need of diabetic medication [30].

1.3.2 Cardiovascular diseases and hypertension:

Mortality rate of CVD has been reported worldwide to be increased in individuals who are overweight and obese, particularly with high abdominal adiposity[31]. Also, in general, with each incremental unit increase in BMI, the risk of coronary heart diseases increases 8%[32], and strong association has been reported between obesity and high blood pressure rates[33]. Interestingly, in most studies involving women [32-36], it has been confirmed that physical activity moderately attenuated but did not eliminated the adverse effect of obesity on CVD and hypertension which leads to the conclusion that both adiposity and physical inactivity are strong and independent predictors of death among women due to CVD[35, 36].

1.3.3 Adipose tissue inflammation:

Growing evidence suggests a critical role of energy imbalance in the development of low-grade inflammation[37, 38]. Several animal studies confirmed that AT inflammation is strongly and consistently characterized by infiltration and activation of proinflammatory macrophages (M1) [5]. Surrounding dead adipocytes, M1 macrophages form crown-like structures and produce proinflammatory chemokines and cytokines such as TNFα and IL-6, which in turn, can induce local and systemic inflammation as well as IR [5, 39-41]. Chronic and systemic inflammation is supported by clinical evidence as

levels of TNFα and IL-6 have been found to be elevated in obese and IR patients[42, 43]. Also, the obesity-induced AT expansion promotes a plethora of intrinsic signals (e.g., adipocyte death, hypoxia, mechanical stress) that can trigger an inflammatory response in AT [44, 45].

1.3.4 Obesity and cancer:

Epidemiological data has found that higher body fat is associated with increased risks of number of cancers[46]. Meta-analysis of many published clinical data in Europe and the United States has concluded that obesity is an associated risk factor in 39% of endometrial cancer cases, 11% of colon cancer, and 9% of postmenopausal breast cancer cases[47, 48]. Furthermore, proportion of deaths due to cancer was strongly attributed to obesity and overweight as high as 20 %, among adult women cancer patients [49].

1.4 Gender disparity in obesity

Even though the obesity rate has dramatically increased over the last three decades in both sexes, there is a sex difference among American adults according to most recent data from NHANES[1]. Clinical data suggests that women are slightly protected against obesity before the age of 60 compared to men. However, this partial protection is lost among the women over age of 60. Furthermore, postmenopausal women are prone to gain more weight than age-matched men. This same relationship has been established in murine models, as male mice are more vulnerable than females to the impact of diet-induced weight gain[50]. However, during estrogen deficiency, initiated either by ovariectomy or

genetic modulation, female mice exhibit high level of adiposity and weight gain compared to estrogen-sufficient females [51, 52]. Body weight is regulated by a complex interaction and feedback mechanisms of several hormones such as leptin, insulin, and estrogen. This hormones signaling to the brain and have to control food intake and energy expenditure to keep the adiposity levels static[53] Evidence suggests that sex disparity in obesity might be attributed to anti obese effects of estrogen in the females [54]. On average, a third of a woman's lifespan is during the postmenopausal stage in which circulating levels of estrogen gradually decrease. Therefore, hormonal replacement therapy was initiated for menopause-transitioning women to attenuate some of the negative physiological symptoms associated with menopause such as vasomotor and genitourinary symptoms[54]. Indeed, estrogens have been shown to have some beneficial effects on different body physiological processes such as metabolism, cardiovascular, bone, and brain health [54, 55].

1.7 Estrogens (formation, types, and actions)

Estrogens are the primary sex hormone in females synthesized from the aromatization of testosterone by aromatase, a cytochrome P450 enzyme located in the endoplasmic reticulum of estrogen-producing cell [56-58]. During pre-menopause, estrogens are mainly produced from ovaries and are released into circulation in large amounts in order to exert their effects on multiple target tissues through an endocrine manner[59]. However, estrogens are synthesized in extragonadal organs including the adrenal glands, brain, adipose tissue, and skin, mainly during postmenopausal stage[60]. There are four major estrogen types: 17β-estradiol (E2), Estrone (E1), Esteriol (E3), and

Estetriol (E4). The major physiological estrogen in females is E2 [61]. E1 is most dominant adipose tissue estrogen that is majorly synthesized in postmenopausal women[62]. E3 and E4 are less potent and found at low levels of circulating estrogens which are mainly produced by placenta during pregnancy. In normal physiological conditions, estrogen synthesis and secretion are regulated by the hypothalamus, pituitary, and gonad axis. Low levels of circulating E2 stimulates the hypothalamus to produce gonadotropin which in turn stimulates the pituitary gland to produce FSH and LH, which finally activate the ovaries to release more E2 into circulation[63]. However, during postmenopausal time, ovaries fail to produce E2, and the aromatization process will take place in extragonadal, in very low levels and act locally in a paracrine or intracrine manner[64, 65]. In addition to its roles in the reproductive system, E2 exhibits physiological action on other systems and organs, including but not limited to, metabolism, immunity, and the cardiovascular system[64]. Estrogen signaling is accomplished mainly through their specific nuclear receptors; estrogen receptor isomers alpha (ERα) and beta (ERβ). Acting upon E2 binding, estrogen receptor (ER)activation might go through either, genomic, or non-genomic mechanisms through following pathways: 1) Classical mechanism, in which ER acts as ligand-activated transcription factor, and exhibits tissue specificity in expression and function [66]. Upon binding to estrogen, homodimerization of cytoplasmic monomer ERs are activated and translocated into the nucleus and the E2-activated ER dimers bind directly to estrogen response elements (ERE) in target gene promotors. 2) Indirect DNA binding mechanism, in which E2-ER complex, independent to the ERE, indirectly interact with other DNA-bound transcription factors [67]. 3) Non-genomic mechanism, in which the E2-ER complex

can also act via extranuclear and membrane-associated receptors, within seconds to minutes, in congregate with other signaling molecules, such as G proteins, growth factor receptors and orphan G-protein coupled receptors[68, 69]. 4) Additionally, Ligand-independent genomic mechanism has been described recently, in which ER, independent to E2, are activated downstream of growth factors receptors stimulation, through the action of intracellular kinase enzyme cascades leading to phosphorylation of ER at ERE [66].

1.8 Estrogen action on metabolism and metabolic organs

Estrogen plays a fundamental role in a plethora of physiological processes in many of our bodily systems such as the reproductive, cardiovascular, skeletal, and central nervous systems. Through their ERs, estrogens act on different organs, especially metabolically active organs, such as skeletal muscle, liver, adipose tissue, brain, and pancreas [61]. Several studies have been done in both human and murine models to establish the crucial roles of estrogen in metabolism and energy homeostasis. In humans, a strong correlation has been observed between E2 and glucose and lipid metabolism, insulin resistance and metabolism. As many metabolic dysregulation conditions were observed among women suffering from menstrual dysregulation and E2 level fluctuation in the human body[70-73]. As soon as women enter menopause, the level of E2 dramatically reduces (on average 80% loss of their estrogens during 1st year of menopause). This E2 reduction is accompanied with increase of food intake and reduced physical activity leading weight gain and obesity-induced complications [74-76]. In animal studies, using different models, the effect of estrogen on different tissues and organs has been extensively

investigated [77-81]. The outcomes revealed that, in the absence of estrogen or its receptors, female mice are more prone to weight gain and are metabolically impaired [51, 52, 65, 81-85]. Furthermore, it is well established that estrogen regulates insulin action in premenopausal women as they exhibit enhanced insulin sensitivity compared to age-matched men normalized to lean mass[64, 85]. However, postmenopausal women and ovariectomized mice both exhibit a significant decrease in insulin sensitivity accompanied with an increase in fat mass and elevations in circulating inflammatory markers[86-90]. Estrogen action(s) on different organs and tissues involved in metabolism can be summarized below:

1.8.1 Central nervous system:

The hypothalamus is an area in central nervous system that controls food intake, body weight homeostasis, and energy expenditure [91]. ERs are highly expressed in different regions of the hypothalamus including ventromedial, arcuate, and paraventricular (PVN) nuclei where estrogen and ERs directly and indirectly control the activity of these regions[2, 92, 93].

1.8.2 Adipose tissue

In adipose tissue, both animal models and human studies have shown that E2 prevents visceral fat accumulation by decreasing fatty acid synthesis and lipogenesis and increases insulin receptors in adipocytes [94-97]. Removal of gonadal hormones disrupts several steps involved in glucose and lipid metabolisms in adipose tissue, many of them

reversed by E2 replacement [93, 94, 98-100]. For instance, in mice, ovariectomy increases triglyceride lipase signaling, resulting in elevation of non-esterified fatty acids and glycerol, which are attenuated by E2 replacement[101].

1.8.3 Skeletal muscle

Skeletal muscle is considered the major organ of glucose disposal in humans, as approximately 75-80% of glucose disposal occurs in skeletal muscle. A meta-analysis of data from patients receiving hormone replacement therapy (HRT) have confirmed the beneficial effects of E2 in muscle physiology and metabolism[102, 103]. Also, clinical data reported that cycling women are protected against lipid-induced insulin resistance relative to estrogen-deficient women and men[88, 104] In rat and mice, estrogen deficiency impairs exercise-stimulated glucose disposal and E2 restoration maintains insulin action and glucose tolerance in female rodents. Global ERα deletion, as well as skeletal muscle ERα deficiency have been shown to impair metabolism and elicit insulin resistance [105, 106]. The mechanistic function of ERα in regulating skeletal muscle insulin action remains unknown. Some studies suggest that ERα regulates Glut4, the main insulin-induced glucose transporter in muscle [107-109]. However, other reports have found no changes of Glut4 mRNA expression in skeletal muscle of ERαKO mice [66, 95].

1.8.4 Liver:

Several studies indicate that liver steatosis incidence is higher in men than women[110]. Data collected from an estrogen deficient mouse model showed that

estrogen-deficient males, but not females, develop insulin resistance due to hepatic steatosis[111]. Also, in the liver of ERαKO mice, there is an increase in glucose production and genes involved in hepatic lipid synthesis [112]. However, a direct role and involvement of ERα in liver are not clear yet[64, 85].

1.9 Preclinical models of estrogen deficiency:

In order to better understand the role of estrogen in female metabolism, many murine preclinical models have been generated to simulate estrogen action and levels in women during peri-and postmenopausal stages. Coupled with obesity-induced models, many estrogen-deficient models have been established. There are two major routes to establish estrogen-deficient status: 1) genetic modulation or 2) surgical intervention. For the genetic-based models, the aromatase knock out (Arom KO) mouse is the most model has been used for more than 25 years [82]. In the Arom KO model, CYP19A gene, which encodes aromatase, is mutated, therefore the mouse cannot convert testosterone to estrogen. After three months of free access to low fat diet, Arom KO females gain more body weight and accumulate more adipose tissue than their WT littermates , and exogenous E2 reverse this phenotype [52]. An alternative approach in genetic modulation is alteration of ER action by global deletion of ERα (αERKO), ERβ (βERKO), or both together (αβERKO) [84, 113]. Lacking both ERs or ERα alone, but not ERβ, causes female mice to gain more body weight and have higher fasting insulin and glucose levels than WT controls [85, 105, 114]. Also, αERKO mice show impairments in hepatic insulin sensitivity accompanied with upregulation lipogenic genes[96, 112]. Recently, more advanced tissue

specific- ERα KO mouse models were successfully generated such as skeletal muscle ERα KO (MERKO) and adipose tissue-specific ERα KO (FERKO)[106, 115]. Both studies concluded that tissue specific ERα deletion impacts glucose homeostasis and promotes insulin resistance due to impairment of insulin signal transduction, like the phenotype observed in αERKO model [66, 114]. In summary, tissue-specific ERα deletion approach may provide more specific targeted therapeutic plans to combat global obesity-related complications in females. Another model is a surgical menopause by removal of ovaries, during puberty, in a procedure called ovariectomy[116]. After the surgery, the endogenous ovarian estrogen production ceases and circulating level is suddenly decreased, yet extragonadal estrogen actions cannot be eliminated [117]. As the common procedure in preclinical field, ovariectomized mice have been extensively used to evaluate specific effects of gonadal hormone deprivation and subsequent exogenous hormone treatment on the central and peripheral systems controlling metabolism[54, 77, 80, 118].

1.10 Gaps in the field and limitations

Previous work using preclinical models has already established the fact that estrogen action is a crucial factor to combat diet-induced obesity [51, 105]. However, the exact mechanism(s) by which estrogen acts is still under investigation with many contradicting findings. Addressing some of limitations in previous works may uncover novel mechanistic pathways leading to discover therapeutic potential targets to combat obesity in women. For instance, in the Arom KO model, the major contingency is those animals were born with no estrogens from birth. Therefore, neglecting confounding

developmental factors of estrogen action could have significantly affected the interpretation of the results [66]. Also, it has already confirmed the importance of using a purified diet in obesity-related research. However, it's noticeable that in previous works on Arom KO models, only chow or soy-free diets were utilized [51, 52, 83, 119, 120]. Chow diet contains non-negligible amounts of phyto-estrogens which have an estrogenic effect in Arom KO mice. Specifically, phyto-estrogens affect the serum gonadotropin levels which in turn offset the ovaries development in those mice[121]. On the other hand, purified diet, instead, is a fundamental factor to optimize clinically relevant obesity-related research. Furthermore, up to date, there is no study has addressed the initiation mechanism that drives obesity phenotype in Arom KO. Yet, deep analysis of metabolic and behavioral assessments has not been conducted on this model. Addressing all previous concerns in Arom KO would generate more relevant data to better characterize this model for more realistic and applicable outcomes. In previous studies using ERα-ablation models, contradicting findings have been published. Spangenburg and his colleagues successively deleted ERα from skeletal muscle of adult mice upon treating their inducible transgenic mouse model (ERα KO^{ism}) with tamoxifen[122]. They concluded that muscle ERα is not necessary to enhance insulin sensitivity and does not protect female mice against diet-induced obesity. Their finding contradicted Hevener and her colleagues, in MERKO mouse model (deletion of muscle ER from birth)[106]. Deep analysis in ERα KO^{ism} data, it's noticeable that the authors utilized only double transgenic mice that harbor both ERα f/f allele and human skeletal actin (HSA)-driven mER-Cre-mER transgenes. In which, the authors were obligated to treat only the experimental groups with tamoxifen in order to

activate Cre-induced ERα deletion from skeletal muscle. Even though the authors utilized a washout period of 10 weeks, the direct or indirect impact of tamoxifen on skeletal muscle physiology and metabolism cannot be excluded. Philip Scherer's group found that tamoxifen by itself can impact adipose tissue physiology which may indirectly affect skeletal muscle metabolism[123]. Also, the effect of tamoxifen on body weight and food consumption has already been established [76] Therefore, administration of tamoxifen only to the experimental groups has raised another fundamental concern. Furthermore, to our knowledge, no other study has shown the global effect of gain of function of skeletal muscle ERα. Gain of function approach may be considered as a legitimate approach in order to better understanding role of estrogen to combat obesity in women. Also, novel mechanistic pathways could be uncovered to develop potentially safe and targeted therapeutic plans.

1.11 Objectives

The objectives of this dissertation are reasoned to fill the knowledge gaps in the field which are listed:

1) Comparing two murine E2-defecient models, utilizing mor clinically relevant and purified diets, for better characterization the role of E2 in diet-induced obesity in females.
2) Determine the therapeutic potential of skeletal muscle aromatase overexpression to mitigate obesity-induced metabolic dysfunction in females of differing estrogen status
3) Determine the therapeutic potential of skeletal muscle ERα overexpression to mitigate obesity-induced metabolic dysfunction in females of differing estrogen status

CHAPTER TWO

Estrogen plays a crucial role in regulating diet-induced obesity and metabolic dysfunction in females

2.1 Introduction:

Arom KO, as a global estrogen ablation mouse model, has been utilized for almost three decades to investigate the role of estrogen in metabolism and obesity[82]. The generation of this model has provided additional insight into the role of estrogens in regulating female physiology. The females in this model exhibit several metabolic dysregulations including glucose and lipid metabolic impairments, insulin resistance, hyperleptinemia, as well as the accumulation of excess intra-abdominal fat [52, 83, 124]. These phenotypes were reversable upon exogenous E2 administration, which confirms the crucial roles of estrogen in energy homeostasis[79].

However, several limitations were noticed in previous works which include 1) low sample size. 2) diet formula and composition problems 3) lacking thorough behavioral as well as adipose tissue inflammation assessments. Therefore, this current study is designed to address previous limitations to better understand of estrogen in regulating of obesity and energy homeostasis in females. Also, adding ovariectomized mice (Ovx) in this study, as another estrogen-deficient model, empowers our data analysis to investigate involvement

of extragonadal estrogen in regulating diet-induced obesity in females. Regarding of diet formula issues, all previous reports utilized either regular chow or soy-free chow diets in which phytoestrogens effect is not controlled. The effect of phytoestrogens on the pituitary and gonadal phenotypes is already established as it increases uterine and ovaries size, and these effects vary according to the diet source of phytoestrogens [121]. To eliminate the estrogenic effects of phytoestrogens, this study utilized two different purified diets to assess role of estrogen on females at different levels of fat content. Importantly, high fat diet (HFD) utilized in this study mimics the standardized American western high-fat diet composition as described in our previous studies [125-131]. Our findings revealed that estrogen regulates body weight and fat accumulation, primarily via physical activity and food intake, as well as increases adipose tissue inflammation. Using high resolution indirect colorimetric metabolic cages empower this study observation, specifically in food intake assessment. In independent analysis, Ovx mice show partial protection against diet-induced metabolic disorders including glucose intolerance and insulin resistance when compared to WT controls. However, adipose tissue inflammation was exacerbated in both estrogen deficient models compared to WT controls.

2.2 Materials and method:

Mice: Heterozygous females and males mice on a C57Bl6 background were bred and their homozygous aromatase null (Arom KO) as well as the wildtype littermate siblings were used for the following experiments. For genotyping, following primers were used: AromWTf; 5’-CGT GGG CAG GTG ATC AGT TTA CCA TGT CCT AAT CTT CAC-3’,

AromWTr: 5'-TCT TCT GAG GCC AAA TAG CGC AAG ATG TTC -3', and AromKOr: 5'-CTG CTA AAG CGC ATG CTC CAG ACT GCC TTG- 3'. For the Ovx group, bilateral ovariectomy was done to animals at the 9th week of age and the study was started one week later. The sample size for each experimental group is presented in the figure legend of each figure. Mice were housed, 3-5/cage, maintained on 12:12-h light-dark cycle in a low stress environment (22 C, 50% humidity, low noise), and given food and water as libitum. All methods were in accordance with the American Association for Laboratory Animal Science, and the Institutional Animal Care and Usage Committee of the University of South Carolina approved all experiments.

Diet: At 10 weeks of age, animals were randomly assigned to 1 of 5 groups: WT-LFD, Arom KO-LFD, WT-HFD, Arom KO-HFD, and Ovx-HFD (n= 15-25/group). The LFD utilized in this study was an open-source, purified AIN-76A diet (3.77 kcal/g) that contained 12.2% of fat, 19% protein, and 68.8 of carbohydrate. The HFD was a custom-made purified HFD (4.57 kcal/g) consisted of 47%, 40%, and 13% of total calories from carbohydrates, fat, and protein, respectively, with saturated fat making up 12% of total calories in order to mimic the standard American diet (Bio-Serv, Frenchtown, NJ).

Body weight and body composition

Body weight was monitored on a weekly basis throughout the study. Body composition was assessed after 16 weeks of diet to use lean mass as the basis for the dose of glucose and insulin administration for glucose and insulin tolerance tests, respectively.

For this procedure, mice were briefly anesthetized via isoflurane inhalation, and lean mass, fat mass, and percent body fat were assessed by dual-energy X-ray absorptiometry (Lunar PIXImus).

Indirect Calorimetry and Behavioral Phenotyping

In two separate runs, WT vs Arom KO and WT vs Ovx mice were analyzed. Promethion multichannel continuous measurement indirect calorimetry system (Sable international, Las Vegas, Nevada) was utilized to measure physical activity and food intake [132]. Mice at 12 -13 weeks of age were singly housed in metabolic cages on LFD for 3 weeks at 22C^{o} on a 12-h light and 12-h dark cycle. After they were acclimated for 24hrs, data was collected accordingly. Physical activity was measured as (all meters) assessment of cage activity including gross and fine movements. All data were analyzed using an ANCOVA with lean mass as a covariate utilizing the MMPC Statistical Analysis Page (https://www.mmpc.org/shared/regression.aspx) [133].

Metabolic assessments

Fasting blood glucose and insulin levels were assessed after 12 and 16 weeks of dietary treatment. After a 5-hour fast, blood samples were collected from the tip of the tail. A glucometer (Bayer Contour, Mishawaka, IN) was used to determine blood glucose concentrations in whole blood. Collected blood was centrifuged at 4,000 rpm for 10 minutes at 4°C. Plasma insulin concentrations were analyzed according to the manufacturer's instructions using a mouse insulin ELISA kit (Mercodia, Winston Salem,

NC). Glucose and insulin tolerances tests (GTTs and ITTs, respectively) were performed after 17 and 18 weeks of dietary treatment, respectively. For these procedures, mice were fasted for 5 hours, and glucose or insulin was administered intraperitoneally at 2 g/kg or 0.75 U/kg lean mass, respectively. A glucometer (Bayer Contour) was used to measure blood glucose concentrations (tail sampling) intermittently over a 2-h period (0, 15, 30, 60, 90, and 120 minutes) for GTTs and intermittently over a 1-h period (0, 15, 30, 45, and 60 minutes) for ITTs. Area under the curve (AUC) was calculated using the trapezoidal rule. Blood was collected from the tip of the tail during the GTT (0, 15, 30, and 60 minutes) to assess the insulin response to the GTT for a subset of mice from each group. Fasting serum was collected (using non-heparinized capillary tubes) for free fatty acid (FFA) analysis at the 0- and 30-min time points of the ITT to assess insulin's ability to inhibit lipolysis. FFAs were analyzed using a commercially available kit according to the manufacturer's instructions (Wako Diagnostics, Richmond, VA).

Tissue collection

After 18 weeks of dietary treatment, mice were euthanized via isoflurane inhalation for tissue collection. Gonadal, mesenteric, and perirenal fat pads and the liver were removed, weighed, and immediately snap-frozen in liquid nitrogen and stored at −80°C.

Quantitative real-time PCR

An EZNA Total RNA Kit (Omega Bio-Tek, Norcross, GA) was used to isolate RNA from gonadal adipose tissue.QRT-PCR. Bio-Rad reverse transcription reagents and

probe assays (Bio-Rad, Hercules, CA) were used to reverse transcribe and analyze the expression of the following genes in adipose tissue: EMR1, CD11c, CD206, MCP-1, TNFα, and IL-10, Potential reference genes (HPRT, 18s, GAPDH, β-Actin, HMBS, TBP, H2AFV, and B2M) were analyzed for stability using Qbase+software (Biogazelle, Ghent, Belgium) for each tissue analyzed. The optimal number of reference genes was determined by Qbase+, and the geometric mean of these genes was used as the normalization factor for each analysis: gonadal adipose tissue (GAPDH, B2M, and H2AFV). Gene expression was quantified using the ΔΔCT method and Qbase+software[134].

Statistical analysis

Data were analyzed using commercially available statistical software: Prism 9 (GraphPad Software, La Jolla, CA) and SigmaStat (Systat Software, San Jose, CA). For Arom KO study, a two-way ANOVA (diet x genotype) followed by a Tukey post hoc multiple comparisons analysis were used for most outcomes analyzed. For Ovx mice, a two-tailed student's t test was used when only two groups were compared (Ovx mice vs Arom KO) and (Ovx vs WT intact). Any statistical test that did not pass the equal-variance test (Bartlett's test for equal variances) was transformed accordingly and then reanalyzed.

2.3 Results

2.3.1 Global estrogen deficiency increases body weight gain and fat accumulation in females independent of diet

To assess body weight gain, all animal groups were weekly monitored, and body weight was registered accordingly. Our collected data showed that there was main effect of HFD and Arom KO to increase body weight, lean mass, and fat mass when compared to their corresponding littermate controls. Post-hoc analysis showed that Arom KO mice gained more body weight, and accumulated more fat mass than WT littermate controls, independent to the fed-diet ($P < .05$). Interestingly, Ovx mice on HFD gained body weight as much as Arom KO mice, however, they significantly accumulated less fat mass and lean mass than Arom KO, and they were significantly higher than WT intact mice ($P < .05$) figure (1A-C).

2.3.2 Estrogen deficiency promotes more visceral and hepatic fat accumulation

Fat distribution analysis revealed that there was a main effect of Arom KO and HFD to increase total visceral fat, liver weight, and hepatic lipid accumulation when compared to their counterpart controls (figure 2.2A,B,C). Post-hoc analysis showed that Arom KO significantly increased total visceral fat and liver weight, independent to the diet, compared to their controls ($P < .05$). However, Arom KO increased hepatic lipid accumulation only in HFD compared to the corresponding controls ($P < .05$) (figure 2.2

B). Also, our data analysis showed that Ovx mice accumulated more visceral fat and increased liver weight when compared with WT controls ($P < .05$). Furthermore, Ovx mice significantly accumulated less visceral fat than Arom KO ($P < .05$). On the other hand, there was no differences in hepatic lipid accumulation in Ovx mice when independently compared to WT controls and Arom KO (figure 2.2 C).

2.3.3 Uterus weight is significantly decreased after estrogen depletion

Our study reported that Arom KO and HFD exhibited main effect to significantly decreasing of uterus weight compared to their corresponding controls. Post-hoc analysis showed that Arom KO mice have smaller uterus than littermate controls independent to the diet ($P < .05$) (figure 2.2 D). Even though they have similar uterus size as Arom KO mice, Ovx females showed significant decreasing of uterus size when compared to WT controls ($P < .05$) (figure 2.2 D).

2.3.4 Estrogen deficiency significantly decreases physical activity and increases food intake, but not in Ovx mice

Physical activity and food intake were assessed and analyzed among different animal groups fed with LFD. Our data analysis revealed that Arom KO females registered significantly less physical activity compared to WT animals ($P < .05$). Same observation was noticed, in separate comparison, as Ovx mice showed significant decreasing in physical activity compared to sham group ($P < .05$) (figure 2.3A). On the other hand, food

intake was significantly increased in Arom KO females ($P < .05$), but not in Ovx mice, when compared to their controls separately (figure 2.3B).

2.3.5 Aromatase deletion impairs glucose metabolism and increases insulin resistance in female mice independent to fed diet

Fasting blood glucose levels were significantly higher in Arom KO groups (genotype was the main effect) when compared to their WT littermate controls. Furthermore, Ovx mice reported similar levels of fasting glucose compared to WT controls, however, they were significantly lower than Arom KO females ($P < .05$) (figure 2.4A). Regarding of fasting insulin levels, Arom KO and HFD were the main effects to increase fasting insulin levels compared to their corresponding controls ($P < .05$). Post-hoc analysis showed that Arom KO females on HFD have reported the highest level of insulin among studied groups (figure 2.4B). Regarding of the Ovx mice, they registered same levels of fasting glucose and insulin as WT controls, however, they reported significantly lower outcomes than Arom Ko females (figure 2.4 A,B). Regarding of GTT results, our data analysis revealed that Aroma KO and HFD were the main effects to impair glucose metabolism in female mice ($P < .05$). Post-hoc analysis showed that Arom KO females, independent to the diet, had higher AUC values of glucose metabolism when compared to their littermate controls ($P < .05$) (figure 2.4C). Similar relationship was reported regarding to the insulin response to the GTT, as Arom KO on both diets reported the highest values of insulin-AUC ($P < .05$) (figure 2.4D). Additionally, Ovx animals reported same glucose metabolism levels when compared to Arom KO females and WT littermate controls (figure

2.4C). Despite Ovx mice reported significantly lower levels of insulin response compared to Arom KO females ($P < .05$), still they were matching the levels of WT controls (figure 2.4 D). For the FFAs blood concentration, there were no differences between the animal groups in Arom KO comparisons in the baseline and 30min timepoint of ITT. Furthermore, Ovx mice exhibited higher FFAs levels in both timepoints only when compared to the Arom KO animals ($P < .05$) (figure 2.4F). However, when assess ΔFFAs levels between two timepoints, there was no differences among all tested groups (figure 2.4F).

2.3.6 Estrogen deficiency aggravates adipose tissue inflammation

To assess adipose tissue inflammation, gene expression of macrophage and inflammatory markers were quantified. Our data analysis reported that Arom KO and HFD exhibited main effect to increase gene expression of EMR1 (total macrophage marker), CD11c (M1 Macrophage marker), and MCP-1 when compared to their littermate controls ($P < .05$). Post-hoc analysis revealed that Arom KO females on HFD increased significantly the gene expression of proinflammatory markers ($P < .05$) (figure 2.4A,B,D). In the Ovx mice, data analysis reported higher levels of adipose tissue EMR1, CD11c, and MCP-1 genes when compared to the WT controls ($P < .05$), however, they showed similar level of EMR1, but less of CD11c ($P < .05$), when compared to Arom KO females (figure 2.4A-B). Regarding of gene expression of adipose tissue CD206 (M2 macrophage marker), main effect of HFD to increase gene expression compared to LFD groups ($P < .05$). Also, Ovx mice were registered similar levels of CD206 as WT and Arom KO females (Figure 4C).

2.4 Discussions

The Arom KO model is considered as a valuable tool to study the physiological role of estrogen in adiposity, energy homoeostasis and metabolic process. This model has been investigated for almost 3 decades in which several publications reported the influence role of estrogen deletion on glucose and lipid metabolism, insulin sensitivity, and body fat accumulation. However, several limitations were observed in previous works. Surprisingly, all previous work used either chow diet or soy-free diet, in which phytoestrogens are not optimized and batch-effect cannot be eradicated. It's already established the effects of phytoestrogen on the gonadal phenotypes of Arom KO, as reported by Britt et al, who showed that ovaries and uteri sizes of Arom KO females were significantly increased when they were on soy+ diet compared to Arom KO on soy free diet[121]. It's imperative to optimize diet quality in obesity-related works to exclude unrelated and not specific factors that might affect potential observed metabolism-related phenotypes. In current study we utilized, for first time with Arom KO model, two purified diets, LFD, and HFD. Additionally, our customized HFD diet is mimicking western diet to reproduce similar obese phenotype level occurred in human body physiology. Also, our work presented for first time two different estrogen-deficient models combined with different diet formulas. Also, high input behavioral and metabolic assessments system was utilized for first time for better characterization the phenotype of E2-difiecient murine models. Regarding to body weight alteration and fat deposition, Arom KO females gained significantly more weight, accumulated more fat, and had higher lean mass compared to WT littermate controls independent to fed diet. It is obvious that estrogen action is a

dominating factor that control body weight and body fat deposition in females regardless to the diet formula. Our findings were supported by previous work conducted by Jones et al. where they found that Arom KO females were heavier and accumulated more fat than WT controls, however, they showed lower lean mass levels in Arom KO animals compared to their WT counterparts. Regarding of lean mass results, this discrepancy between our study and others may be due to the age of animals when being assessed for body composition, 25 weeks in our study vs 15 weeks in other study. Also, it might be due to different diet purity and formula that have been used in previous study, in which chow diet was used, thus phytoestrogen effects cannot be eliminated. Remarkably, Ovx mice registered similar weight gain, but lower total fat mass and lean mass, compared to Arom KO females. Even though extragonadal estrogen biosynthesis in Ovx mice, but not in Arom KO females, decreased fat deposition and lean mass, still has no power to mitigate body weight gain in females. Our data agreed with previous works in which Arom KO females gained significantly more weight and accumulated more fat deposition[52, 79, 135]. Regarding to metabolic and behavioral assessment, our data analysis confirmed that estrogen deficiency drives obesity majorly by impacting physical activity and food intake. Previous works showed that the adiposity is driven only by physical activity and Arom KO mice did not show hyperphagic phenotype [51, 136]. Using high-resolution real-time indirect calorimetric system to assess behavioral and metabolism in our study augmented the data analysis to accurately describe Arom KO phenotypes. Additionally, putting this model for 3weeks in such a sophisticated metabolic enabled us to adjust our data based on the lean mass regardless of body weight differences between studied groups. Regarding of

glucose utilizing and insulin action, our data suggested that Arom KO mice have hyperglycemia, hyperinsulinemia, and have glucose intolerance and insulin resistance. However, Ovx mice were metabolically better than Arom KO as they showed no significant difference in glucose metabolism and insulin action when compared to WT controls. Our findings agreed with previous works performed by different scientist groups, who showed impairment of glucose metabolism and insulin action in Arom KO mice [52, 82, 135]. Additionally, our study was capable to show positive metabolic effects of extragonadal estrogen biosynthesis in Oxv mice, compared to Arom KO, which might be a promising approach to augment such an alternative physiological process to mitigate obesity-induced metabolic dysfunction in postmenopausal women. Regarding to adipose tissue inflammation, macrophage infiltration and proinflammatory markers were higher in Arom KO compared to their controls. Our findings agreed with previous work conducted by Sinderen et al when they showed that MCP-1 gene expression and CD45 positive cells were significantly increased in Arom KO adipose tissue compared to WT[137].It is worth to notice that even though they exhibited higher levels of pro-inflammatory markers, still Arom KO adipose tissue metabolically was not impaired as FFAs analysis showed, no significant difference of ΔFFAs between animal groups. Therefore, our data support the hypothesis of obesity is driving the inflammation not the other way. We already established in another work that deletion of pro-inflammatory cytokine, macrophage derived TNFα, had no effect on impeding the development of obesity[125]. Also, another manuscript by Bader et al showed that macrophage depletion using clodronate liposomes doesn't rescue

obesity-induced metabolic dysfunction[138]. In conclusion, in this study we successively characterized two different E2-deficient models, Arom KO and Ovx side by side, using two different purified diet formulas. Our findings confirmed previous observations that Arom KO females are heavier and accumulate higher fat mass. However, we found, for the first time, that Arom KO has a hyperphagic phenotype in addition to dominant physical inactivity phenotype. We described adipose tissue inflammation in Arom KO females, and this inflammation was driven by estrogen deficiency. Our work has already established the importance of utilizing a purified diet in obesity- related studies, specifically in interrogation the role of estrogen in metabolism.

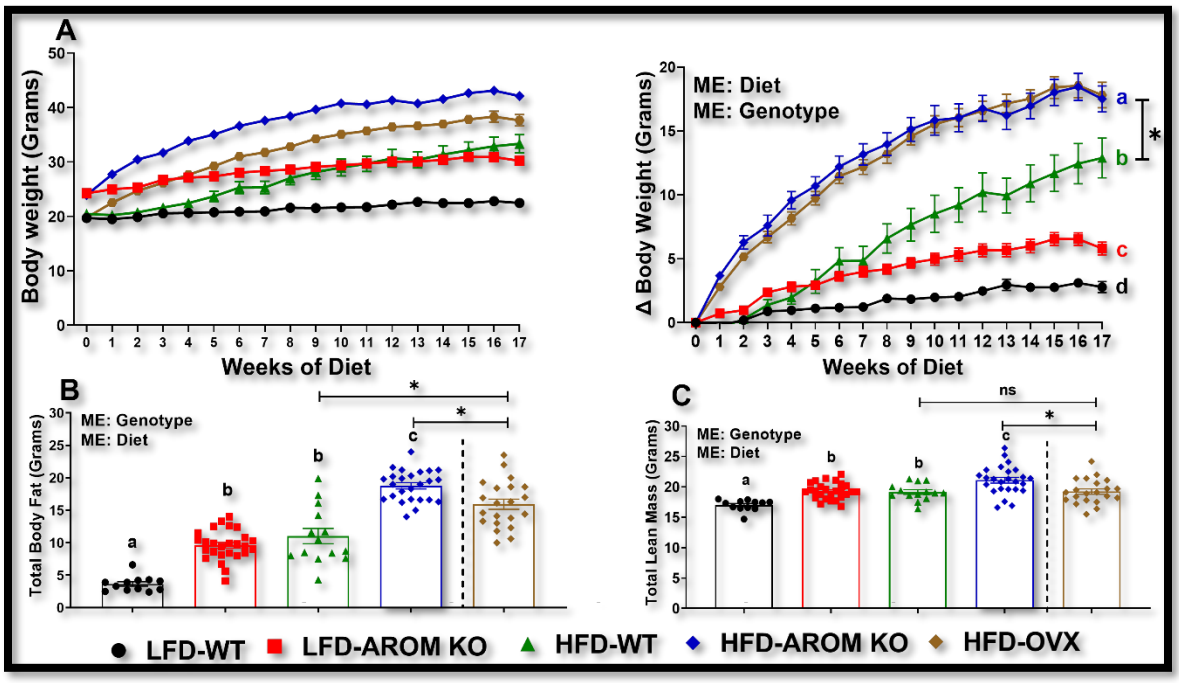

Figure 2.1 Arom KO females increase body weight gain and accumulate more fat, however, Ovx mice gain body weight as much as Arom KO females. Arom KO females, WT littermates, and Ovx mice were either on LFD or HFD for 17 weeks (n=25/group). A, body weight. B, Δ body weight. B and C total body fat mass and lean mass (analyzed by DEXA). Data are presented as mean ±SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA was used for Arom KO comparisons. ME= Main effect. A two-tailed student's *t* used for Ovx mice comparisons (* = $P<.05$).

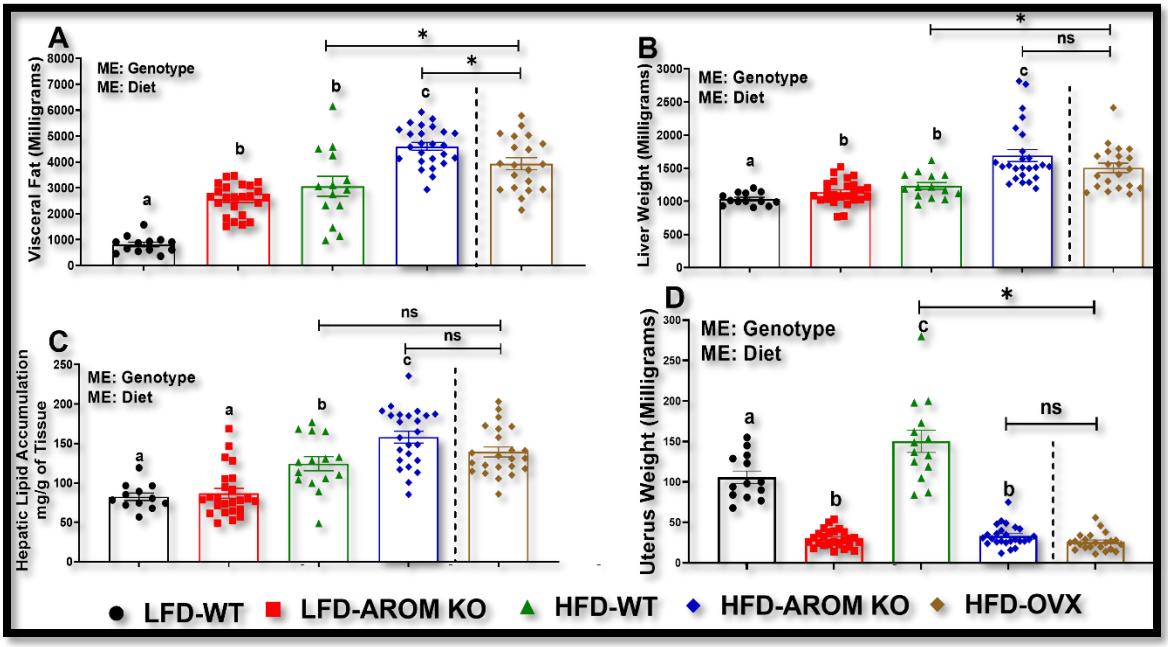

Figure 2.2 Fat deposition and organ weights in different estrogen-deficient models. Wet weights at euthanized time after 17 weeks on either LFD or HFD, A, total visceral weight in milligram. B and C, liver weight and hepatic lipid accumulation. D, uterus weight in mg Data is presented as mean ±SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA was used for Arom KO comparisons. ME= Main effect. A two-tailed student's *t* test used for Ovx mice comparisons (* = $P<.05$, ns= not significant).

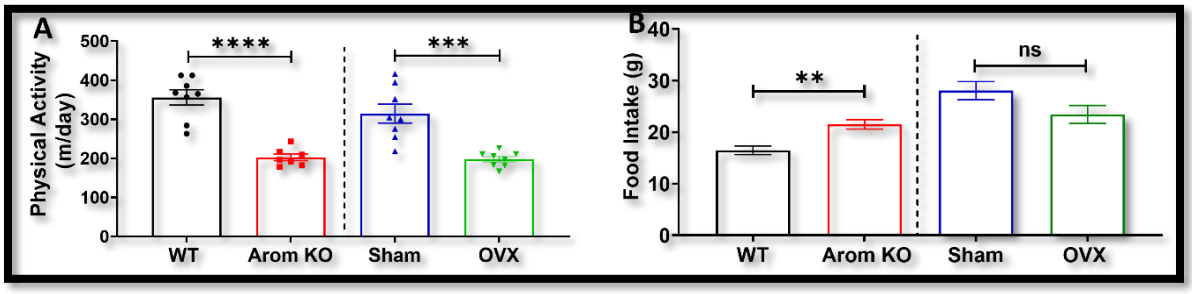

Figure 2.3 Metabolic and behavioral assessments. 12-13 weeks old females on LFD were housed in metabolic cages for 3 weeks (n= 8-9/group). In two separate runs (Arom KO vs WT) and (sham vs Ovx mice), A, physical activity (m/day) and B, Food intake (g) were assessed. Data are presented as mean ±SEM. A two-tailed student's *t* test used for each run independently. (** = *P<.001*, ****=*P<.0001*).

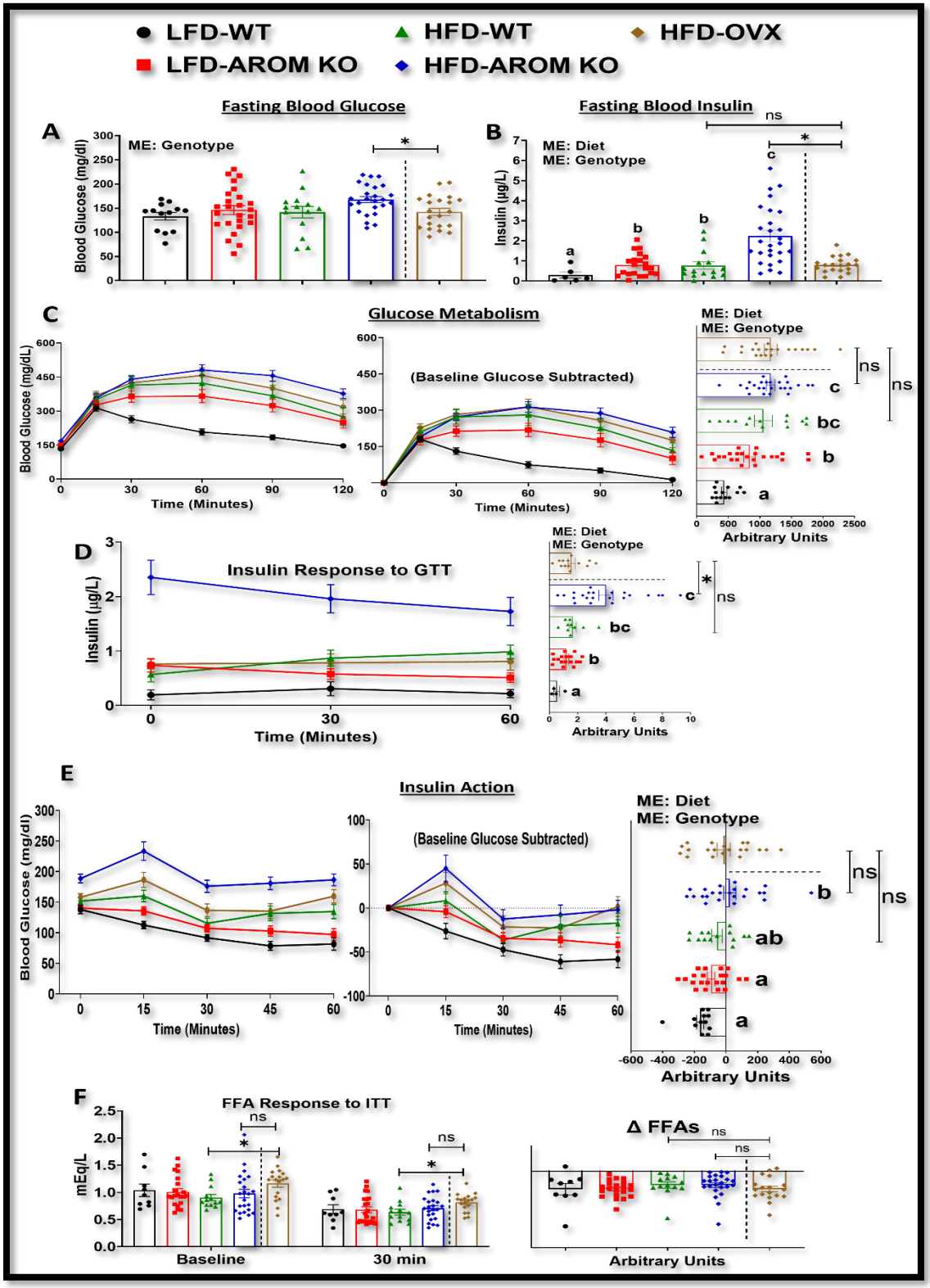
LFD-WT
HFD-WT
HFD-OVX
LFD-AROM KO
HFD-AROM KO
Fasting Blood Glucose
A
ME: Genotype
Blood Glucose (mg/dl)
Fasting Blood Insulin
B
ME: Diet
ME: Genotype
Insulin (µg/L)
ns
Glucose Metabolism
C
Blood Glucose (mg/dL)
Time (Minutes)
(Baseline Glucose Subtracted)
Arbitrary Units
D
Insulin Response to GTT
Insulin (µg/L)
Time (Minutes)
E
Insulin Action
Blood Glucose (mg/dl)
F
FFA Response to ITT
mEq/L
Baseline
30 min
Δ FFAs

Figure 2.4 Estrogen deficiency negatively impacts glucose metabolism and insulin action in Arom KO females, but not in Ovx mice. Arom KO, Wt, and Ovx females consumed either LFD or HFD. A,B fasting (5hrs) blood glucose and insulin, respectively, after 15 weeks of diet administration. C, Glucose tolerance test (GTT) for 2hrs, 30 min intervals, glucose baseline subtracted, then the Area Under the Curve (AUC/arbitrary units) is calculated. D, Insulin response to GTT at 0,30, and 60 min time points, AUC is calculated accordingly. E, insulin tolerance test (ITT) ,on week 16 of diet, for 1hr (15 min intervals), baseline glucose subtracted, then AUC was calculated accordingly. F, FFAs analysis at 0 and 30 min after insulin injection in ITT, ΔFFAs of 0 and 30 min timepoints was calculated. Data are presented as mean ±SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA was used for Arom KO comparisons. ME= Main effect. A two-tailed student's *t* test used for Ovx mice comparisons (* = $P<.05$, ns= not significant)

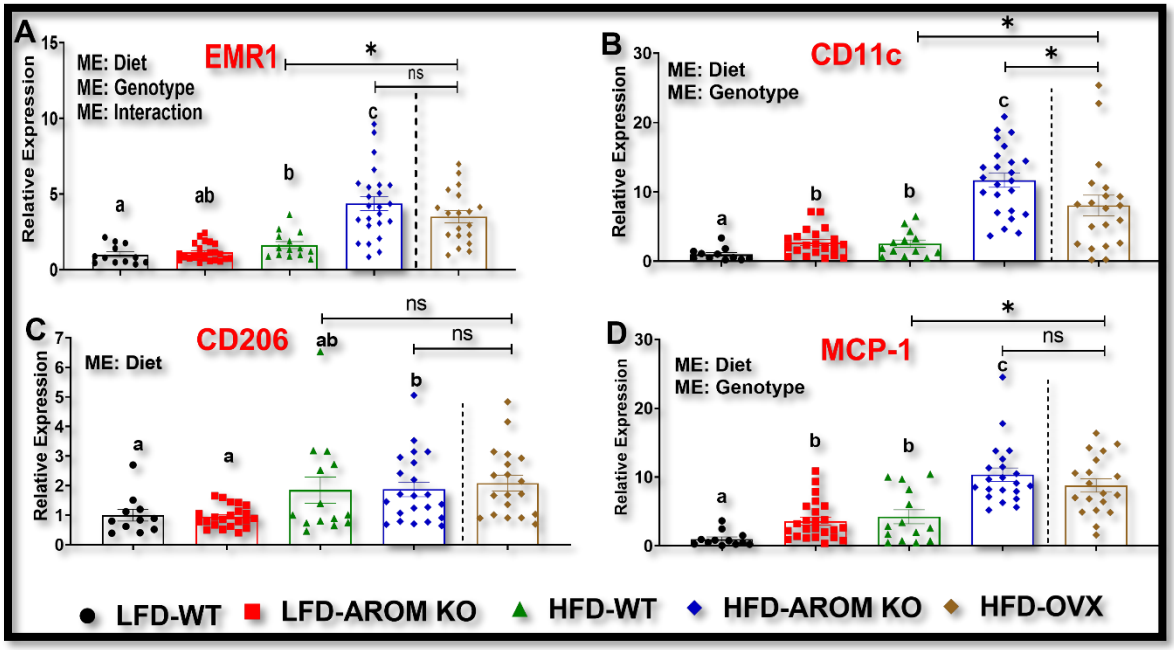

Figure 2.5 Estrogen deficiency promotes adipose tissue inflammation. Gonadal adipose tissue in both of Arom KO, Ovx, and WT were analyzed for gene expression of inflammation. A, EMR-1. B, CD11c. C, CD206. D, MCP-1. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect. A two-tailed student's *t* test used for Ovx mice comparisons (* = $P<.05$, ns= not significant)

CHAPTER THREE

Skeletal muscle aromatase overexpression limits adipose tissue inflammation but does not ameliorate diet-induced obesity in females

3.1 Introduction

Extragonadal production of estrogen is well known and established process, specifically in men and postmenopausal women. The process is catalyzed by aromatase enzyme that converts circulating androgen to estrogen in situ and has a paracrine mode of action[139, 140]. Aromatase expression in several tissues and organs have been assessed and analyzed such as, but not limited to, the brain, skeletal muscle, and AT[56]. After menopause, peripheral aromatization in AT is largely responsible for estrogen biosynthesis, therefore, many human studies have shown a strong correlation between high BMI and expression of aromatase in subcutaneous AT[141]. However, as already established in many clinical studies, high expression level of aromatase and E2, observed in postmenopausal women suffering from breast cancer, were crucially produced by fibroblast of breast AT[142]. Also, it has been established that obesity is highly associated with high aromatase and E2 levels in breast which highly promote breast hyperplasia and cancer[143]. Furthermore, deep analysis on hormone therapy replacement (HTR) among

postmenopausal women in the Women's Health Initiative (WHI) trials have shown that even though HTR showed essential benefits in energy homeostasis improvement and decrease insulin resistance. However, several challenges exist, one of them is that high risk of breast cancer with chronic HT administration have been registered among participating postmenopausal women. Therefore, balance of potential benefits and risks should be re-evaluated for each individual woman[144]. Lastly, aromatase physiological activity is highly tissue-specific action, as aromatase inhibitor (AI) adjuvant treatment is used to reduce local breast E2 to combat breast cancer. However, half of the women who treated with AI suffer from AI-associated musculoskeletal syndrome (AIMSS)[145]. AIMSS is mainly characterized of AI-associated bone loss and arthralgia due to reduction of BMD and weakening muscle strength that detrimentally impact patient life quality and treatment adherence[146]. Interestingly, exercise increases local skeletal muscle aromatase expression and sex hormone levels in ovariectomized rat which in turn improve their BMD[58]. For that we hypothesized that skeletal muscle aromatase overexpression might improve metabolic homeostasis and energy expenditure in females preventing them against diet-induced obesity and this tissue-specific regulation of aromatase level would not potentially affect breast cell proliferation and growth. Interestingly, skeletal muscle encompasses 30-40% of human tissue size and 75-85% of glucose disposal occurs in such a metabolic active organ on the other hand, our tissue-specific inducible model would not affect the AT aromatase expression. To test our hypothesis, we generated a novel inducible double transgenic murine model using Tet-on system combined with reverse tetracycline-controlled transactivator (rtTA) linked to human skeletal actine promoter (HSA). Upon

subclinical doxycycline dose administration, aromatase expression will be augmented specifically in skeletal muscle of adult female mice. This novel double transgenic model will be combined with surgical-induced E2-dficient approach to test the effect of muscle aromatase overexpression in E-deficient condition in addition to sufficient status.

3.2 Method and materials

Mice: To generate our novel double transgenic mouse model, two transgenic mice (C57Bl6 background) were bred. First transgenic mouse (rtTA-HSA), which harbors rtTA promoter that drives the expression of (rtTA). Animals were screened by PCR for the presence of the rtTA sequence using genomic DNA isolated from tail snips with the following primers:F, 5′ATGTCTAGACTGGACAAGAGCAAAG-3′; R, 5′-TTACCCGGGGAGCATGTC-3′ [147]. Second mouse has tetracycline response element Tet-on promoter that drive aromatase expression (Tet-Arom). For genotyping, following primers were used: F, 5'- CGAGCTCGGTACCCGGGTCG -3' and R, 5'-CAGGCATGGCTTCAGGCACGA -3' [148]. When rtTA-HSA$^{+/+}$ and Tet-Arom$^{+/-}$ mice were bred, both rtTA-HSA$^{+/-}$, Tet-arom$^{+/-}$ and rtTA-HSA$^{+/-}$, Tet-arom$^{-/-}$ offspring were used as experimental and WT littermate control groups, respectively. Doxycycline administration (0.1 mg/ml) with drinking water was provided to both groups. In this reversable process, only rtTA-HSA$^{+/-}$, Tet-arom$^{+/-}$ mice will overexpress skeletal muscle aromatase upon doxycycline administration.

Diet: At 10 weeks of age, animals were randomly assigned to 1 of 4 groups: LFD-WT, LFD-SkM-*Arom*$^{\uparrow}$, HFD-WT, and HFD-SkM-*Arom*$^{\uparrow}$ in intact (E2-efficient mice) and same groups in Ovx (E2-deficient mice) as well (n= 15-25/group). The LFD utilized in this study was open-source, purified AIN-76A diet (3.77 kcal/g). The HFD was a custom-made purified HFD (4.57 kcal/g) comprised of 47%, 40%, and 13% of total calories from carbohydrates, fat, and protein, respectively, with saturated sat make up 12% of total calories in order to mimic the standard American diet (Bio-Serv, Frenchtown, NJ).

Ovariectomy: For E2-deficient groups, bilateral ovariectomy was done to animals at 8th week of age one week recovery time before the study was started. Mice were housed, 3-5/cage, maintained on 12:12-h light-dark cycle in a low stress environment (22 C, 50% humidity, low noise), and given food and water as libitum. All methods were in accordance with the American Association for Laboratory Animal Science, and the Institutional Animal Care and Usage Committee of the University of South Carolina approved all experiments.

Body weight and body composition

Body weight was monitored on a weekly basis throughout the study. Body composition was assessed after 11 weeks of diet to use lean mass as the basis for the dose of glucose and insulin administration for glucose and insulin tolerance tests, respectively. For this procedure, mice were briefly anesthetized via isoflurane inhalation, and lean mass,

fat mass, and percent body fat were assessed by dual-energy X-ray absorptiometry (Lunar PIXImus).

Metabolic assessments

Fasting blood glucose and insulin levels were assessed after 12 weeks of dietary treatment. After a 5-hour fast, blood samples were collected from the tip of the tail. A glucometer (Bayer Contour, Mishawaka, IN) was used to determine blood glucose concentrations in whole blood. Collected blood was centrifuged at 4,000 rpm for 10 minutes at 4°C. Plasma insulin concentrations were analyzed according to the manufacturer's instructions using a mouse insulin ELISA kit (Mercodia, Winston Salem, NC). Glucose and insulin tolerances tests (GTTs and ITTs, respectively) were performed after 12 and 13 weeks of dietary treatment, respectively. For these procedures, mice were fasted for 5 hours, and glucose or insulin was administered intraperitoneally at 2 g/kg or 0.75 U/kg lean mass, respectively. A glucometer (Bayer Contour) was used to measure blood glucose concentrations (tail sampling) intermittently over a 2-h period (0, 15, 30, 60, 90, and 120 minutes) for GTTs and intermittently over a 1-h period (0, 15, 30, 45, and 60 minutes) for ITTs. Baseline glucose levels were subtracted accordingly, and Area of the curve (AOC) was generated as previously described[149]. Area under the curve (AUC) of AOC was calculated using the trapezoidal rule. Blood was collected from the tip of the tail during the GTT (0, 15, 30, and 60 minutes) to assess the insulin response to the GTT for a subset of mice from each group. Fasting serum was collected (using non-heparinized capillary tubes) for free fatty acid (FFA) analysis at the 0- and 30-min time points of the

ITT to assess insulin's ability to inhibit lipolysis. FFAs were analyzed using a commercially available kit according to the manufacturer's instructions (Wako Diagnostics, Richmond, VA).

Tissue collection

After 13 weeks of dietary treatment, mice were euthanized via isoflurane inhalation for tissue collection. Gonadal, mesenteric, and perirenal fat pads, as well as the liver, and skeletal muscle (gastrocnemius and quadriceps) were removed, weighed, and immediately snap-frozen in liquid nitrogen and stored at −80°C or fixed in 4% formalin until analysis.

Quantitative real-time PCR

An EZNA Total RNA Kit (Omega Bio-Tek, Norcross, GA) was used to isolate RNA from gonadal adipose tissue. To confirm changes in a subset of genes identified by the microarray, we performed qRT-PCR. Bio-Rad reverse transcription reagents and probe assays (Bio-Rad, Hercules, CA) were used to reverse transcribe and analyze the expression of the following genes in adipose tissue: EMR1, CD11c, CD206, MCP-1, TNFα, and IL-10, Potential reference genes (HPRT, 18s, GAPDH, β-Actin, HMBS, TBP, H2AFV, and B2M) were analyzed for stability using Qbase+software (Biogazelle, Ghent, Belgium) for each tissue analyzed. The optimal number of reference genes was determined by Qbase+, and the geometric mean of these genes was used as the normalization factor for each analysis: gonadal adipose tissue (GAPDH, B2 M, and H2AFV). Gene expression was quantified using the ΔΔCT method and Qbase+software[134].

Statistical analysis

Data were analyzed using commercially available statistical software: Prism 9 (GraphPad Software, La Jolla, CA). A two-way ANOVA (diet x genotype) followed by a Tukey post hoc analysis was used for the outcomes analyzed in both E2-efficient and E2-deficient status, separately. Any statistical test that did not pass the equal-variance test (Bartlett's test for equal variances) values were transformed accordingly and then reanalyzed.

3.3 Results

3.3.1 Novel inducible model successively augments aromatase gene expression in skeletal muscle

To test skeletal muscle aromatase expression, gene expression of aromatase was quantified in gastrocnemius muscle. Interestingly, gene analysis revealed that aromatase expression significantly increased after 13 weeks of gene activation in SkM-Arom$^{\uparrow}$ animals compared to WT littermates (figure 3.1 A)

3.3.2 Skeletal muscle aromatase overexpression does not mitigate diet-induced obesity in females

Independent to diet formula and E2 status, data collected from different studied animal groups fed on corresponding diet revealed that induced overexpression of muscle aromatase did not mitigate obesity phenotype in females. Body weight gain (figure3.1 B-

C), lean mass, total fat mass, body fat percent (figure 3.2 A-B), total visceral fat, and liver weight (figure 3.2 C-D) of SkM-Arom$^{\uparrow}$ females reported same levels as of their corresponding WT littermate controls. HFD was the main effect to increase all previous outcomes when compared to LFD groups and independent to the estrogen status ($P < .05$) (figure 3.1 and 3.2).

3.3.3 Skeletal muscle aromatase overexpression increases bone mineralization density and promotes uterus mass in females

Augmentation of muscle Aromatase, independent to diet and E2 status, improved BMD in SkM Arom$^{\uparrow}$ females in whom their genotype was the main effect to increase BMD (figure 3.3 A,B). Post-hoc analysis showed that ovx SkM Arom$^{\uparrow}$ mice registered highest BMD levels, independent to diet, when compared to their littermate controls ($P < .05$) (figure 3.3 B). Regarding of uterus size, SkM Arom$^{\uparrow}$ was the main effect to increase the uterus weight in females (figure 3.3 C,D). Post-hoc analysis revealed that intact SkM Arom$^{\uparrow}$ reported heaviest uterus, independent to the diet, ($P < .05$) (figure 3.3 C). However, in ovx mice, both SkM Arom$^{\uparrow}$ and HFD were the main effect to increase uterus weight where SkM Arom$^{\uparrow}$ ovx on HFD registered heaviest uterus among all other groups ($P < .05$) (figure 3.3 D)

3.3.4 Muscle aromatase overexpression does not impact glucose metabolism and insulin action in female mice

Collected data from GTT in intact females revealed that diet is the main effect of glucose metabolism as HFD-fed animals showed higher fasting glucose ($P < .05$) (figure 3.4 A) and insulin levels (figure 4.1 B) than LFD groups. However, HFD and SkM Arom$^{\uparrow}$ were the main effect to increase the AUC of glucose metabolism of intact females in response to glucose administration (figure 3.4 C). Furthermore, intact females on HFD showed highest levels of insulin response (AUC) to GTT, independent to the genotype ($P < .05$) (figure 3.4D). Also, systemic glucose response to insulin in ITT showed no effect of genotype neither diet among different intact female groups, as no difference in the AUC of insulin was observed (figure 3.4E). Similar effect was observed on FFAs levels in both baseline and after 30 min of insulin administration (figure 3.4F). However, when ΔFFAs between 0- and 30-min time points was assessed, HFD was the main effect to decrease insulin inhibitory effect on adipose tissues lipolysis (figure 3.4F). On the Ovx mice, HFD was the main effect to increase fasting blood glucose and insulin levels, independent to the genotype (figure 3.5 A,B). In addition to the HFD, SkM Arom$^{\uparrow}$ showed main effect to increase fasting blood insulin in Ovx mice ($P < .05$) (figure 3.5D). Regarding of GTT, data analysis revealed that there was a main effect of SkM-Arom$^{\uparrow}$ to decrease and HFD to increase the AUC of glucose metabolism in Ovx mice ($P < .05$) (figure 3.5C). Post-hoc analysis showed that SkM-Arom$^{\uparrow}$ mice on LFD had significantly the lowest AUC among animal groups ($P < .05$) as indicative of glucose metabolism improvement. Furthermore,

HFD was the main effect to increase the AUC of glucose stimulated-insulin response in Ovx mice regardless to the genotype (figure 3.5D). Surprisingly, insulin action on circulating glucose was significantly decreased ($P < .05$) in HFD fed- SkM-Arom$^{\uparrow}$ animals compared to WT control (figure 5E). Regarding FFAs level, there was no significant differences between the animals in the baseline level, however, 30min time point and ΔFFAs levels showed main effect of HFD among experimental groups (figure 5F).

3.3.5 Skeletal muscle aromatase overexpression decreases M1 macrophage gene marker adipose tissue

To assess macrophage infiltration in adipose tissue, gene expression profile was conducted on all experimental groups using qPCR technique. Gene expression analysis of both intact and Ovx groups revealed that HFD was the main effect to increase EMR1 (Macrophage marker) in adipose tissue, independent to the genotype ($P < .05$) (figure 3.6 A). However, in intact females, SkM-Arom$^{\uparrow}$ was the main effect to decrease adipose CD11c gene expression (M1 macrophage marker). Post-hoc analysis revealed that muscle aromatase overexpression significantly decreased CD11c expression only on HFD fed females ($P < .05$) (figure 3.6 B). On the other hand, in the Ovx mice, HFD and SkM-Arom$^{\uparrow}$ were main effects to significantly decrease CDc11 gene expression in adipose tissue ($P < .05$) (figure 3.6 B). Regarding CD206 (M2 macrophage), there was no significant difference in gene expression among different animal groups (figure 3.6 C).

3.3.6 Skeletal muscle aromatase overexpression inhibits pro-inflammatory markers in adipose tissue

Gene expression of inflammation markers and cytokines was assessed in adipose tissue of the experimental groups. Inflammatory markers gene profile analysis revealed that SkM-Arom$^{\uparrow}$ was the main effect to decrease gene expression of MCP1 and TLR2 in intact females ($P < .05$) (figure 3.7 A,B). in the Ovx mice, HFD and SkM-Arom$^{\uparrow}$ were the main effect to significantly decrease MCP1 and TLR2 gene expression ($P < .05$) (figure 3.7 A,B).

3.4 Discussion

Lacking ovarian estrogen production, extragonadal aromatase expression plays a pivotal role in process of estrogen biosynthesis in men and postmenopausal women. Aromatase gene expression is regulated by tissue-specific promotor activation via different splicing process, however, the expressed protein is the same regardless of the splicing pattern[150]. At low level in circulation compared to premenopausal time, this extragonadal estrogen production doesn't protect postmenopausal women against obesity, metabolic dysfunction, and other health complications such as breast cancer. On the other hand, clinical data already confirmed high level of aromatase activity and its product, E2, in malignant breast epithelial cells as well as adipose fibroblasts proximal to tumor. Also, intra-tumoral E2 level is even higher than plasma concentrations in postmenopausal women and higher than normal breast areas[151-153]. Therefore, we proposed that

promotion of aromatase activity in peripheral tissue, other than adipose tissue, might mitigate diet-induced obesity and metabolic impairment in females. With no noticeable any breast tissue changes, our data showed no effect of overexpression of SkM-aromatase on total body weight gain, and fat deposition regardless to diet and E2 status among different experimental groups compared to their corresponding WT controls. However, we have observed systemic effects of E2 increasing as SkM-Arom animals present increasing uterus weight and improvement of BMD compared to their WT littermate controls. Previous study conducted by Peng et al, in which global aromatase overexpression significantly increased circulating E2, decreased total body weight, and elevated BMD in (AROM+) mouse model [154]. It's worth to note that AROM+ mouse is from birth, hence the effect of high level of estrogen on developmental process cannot be rule out. Also, crucial structural and functional alterations have been observed in the reproductive organs of AROM+ males. Furthermore, Ohlsson et al already studied the effect of adipose tissue aromatase overexpression, from birth. They concluded that the females in their model were unsuitable for studying the effects of increasing adipose E2 production due to high normal variations of circulating E2 and limited amount of the substrate, testosterone. Therefore, Ap2-arom females cannot increase their local E2 above the normal levels. Furthermore, increase aromatase activity locally in adipose tissue is a perilous approach which might induce hyperplastic and dysplastic lesions in female mammary glands[155], hence they focused on male only in their study. Even though our transgenic model showed evidence of circulating E2 elevation, yet glucose metabolism and insulin sensitivity not improved, however, adipose tissue inflammatory markers were significantly decreased. To our

knowledge, our findings are first evidence of the regulatory effect of muscle aromatase overexpression on adipose tissue inflammation in females by decreasing gene expression of pro-inflammatory markers; CD11c, MCP-1, and TLR2. In previous study, Ap2-arom males did not present circulating E2 improvement, however, promotion of adipose aromatase protected the animals against inflammation and improved their insulin sensitivity. Contradictory results regarding E2 circulation between SkM-Arom and Ap2-arom animals may be due to lipophilic property of E2 which is sequestered inside adipose tissue thus, non-detectable levels of circulating E2 have been detected in Ap2-arom animals. This might raise a concern regarding potential mammary glands transformation might have been observed in the females, thus they were eliminated from the study. The necessity of sensitive steroids quantification methodology should be considered for more accurate assessment and better evaluate the levels of different estrogen types in SkM-Arom females. Furthermore, one of the limitations of our study is that local aromatase level in skeletal muscle naturally is very low therefore the splicing promotor action is not enough to combat obesity and metabolic dysfunction in females. In conclusion, promoting of aromatase activity in skeletal muscle might be considered in future studies to combat AI-associated complications in muscular and skeletal systems as well as decrease adipose tissue inflammation in obese postmenopausal women. Also, inducible tissue specific model might be a potential approach to genetically or pharmacologically augmentation of aromatase activity in different tissues, such as brain, where natural expression originally higher than skeletal muscle.

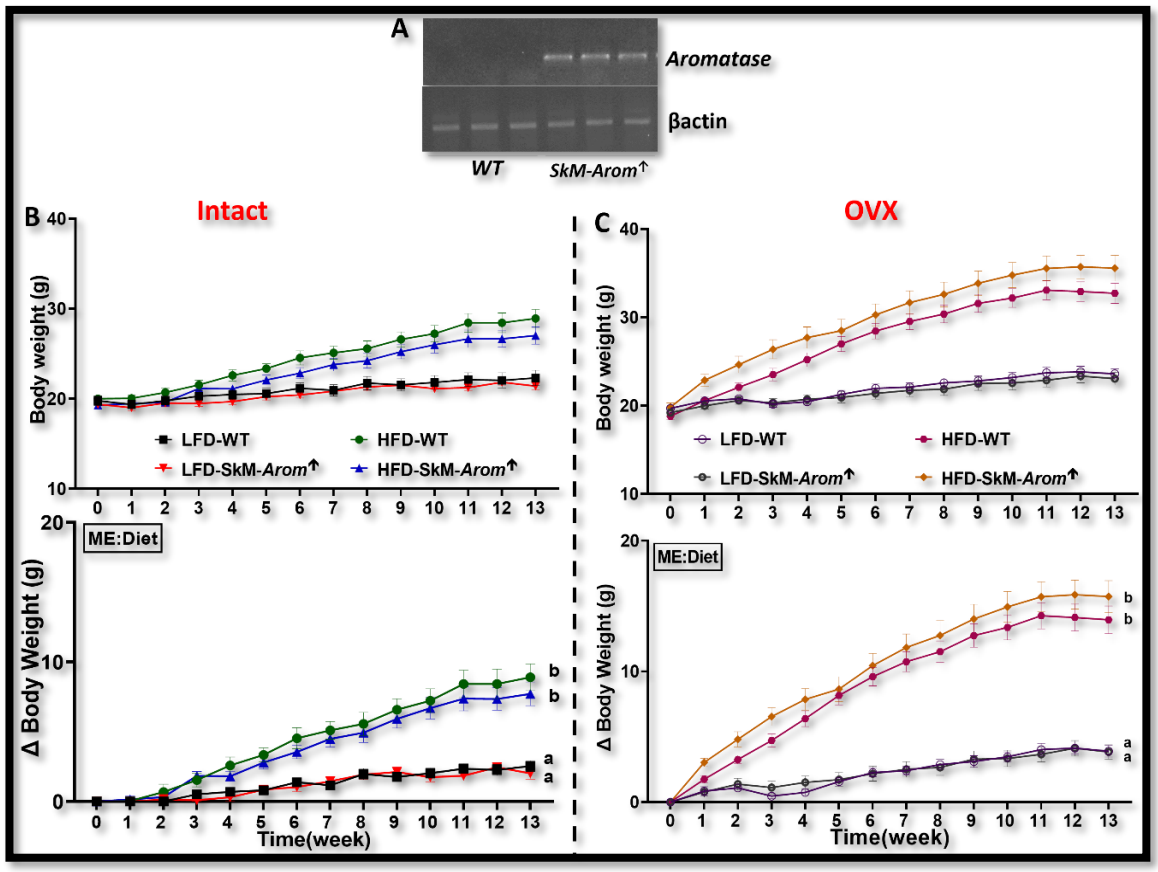

Figure 3.1 Skeletal muscle Arom overexpression does not protect females against diet-induced weight gain independent to the E2 level. SkM-Arom$^{\uparrow}$ and WT littermate females were either intact or ovariectomized (Ovx), on LFD or HFD, and doxycycline-treated (DOX) drinking water for 13 weeks (n=15-25/group). A, northern blot of Aromatase gene PCR product. B, body weight. C, Δ body weight. Data are presented as mean ±SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect.

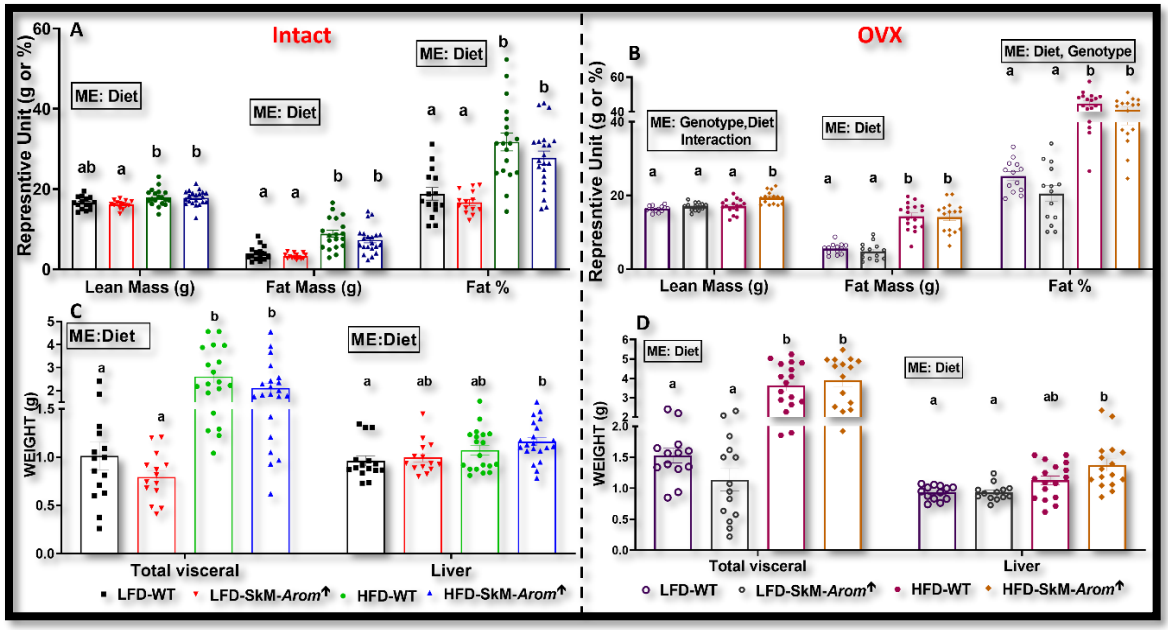

Figure 3.2 Skeletal muscle Arom overexpression does not influence fat deposition in females. SkM-Arom$^{\uparrow}$ and WT littermate females were either intact or Ovx, on LFD or HFD, and on Dox with drinking water (n=15-25/group). A and B lean mass, total body fat mass and Fat% were (analyzed by DEXA at week 11 of diet and Dox treatment). C and D, wet weight of Visceral and liver were measured at the time of euthanization. Data are presented as mean $\pm$SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect.

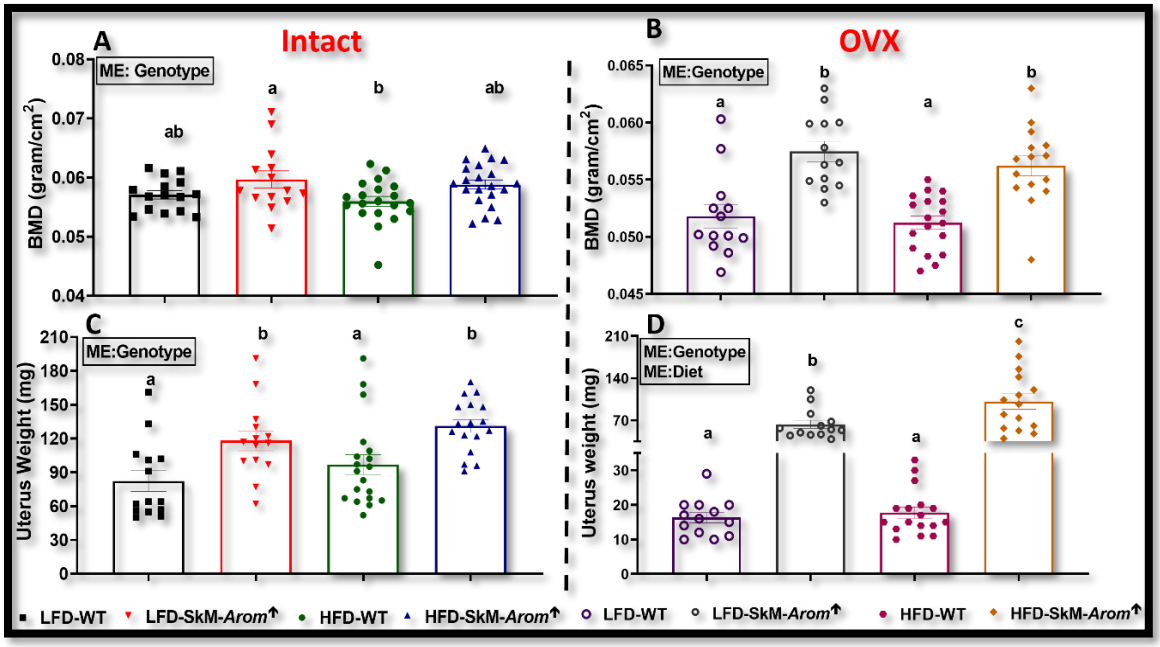

Figure 3.3 Skeletal muscle Arom overexpression enhances BMD and increases uterus size independent to diet and estrogen level, evidence of increasing circulating estrogen level SkM-Arom↑ and WT littermate females were either intact or Ovx, on LFD or HFD, and on Dox with drinking water (n=15-25/group). A and B Bone Density mineralization (BMD) was analyzed by DEXA at week 11 of diet and Dox treatment. C and D, wet weight of Uterus measured at the time of euthanization. Data are presented as mean ±SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect.

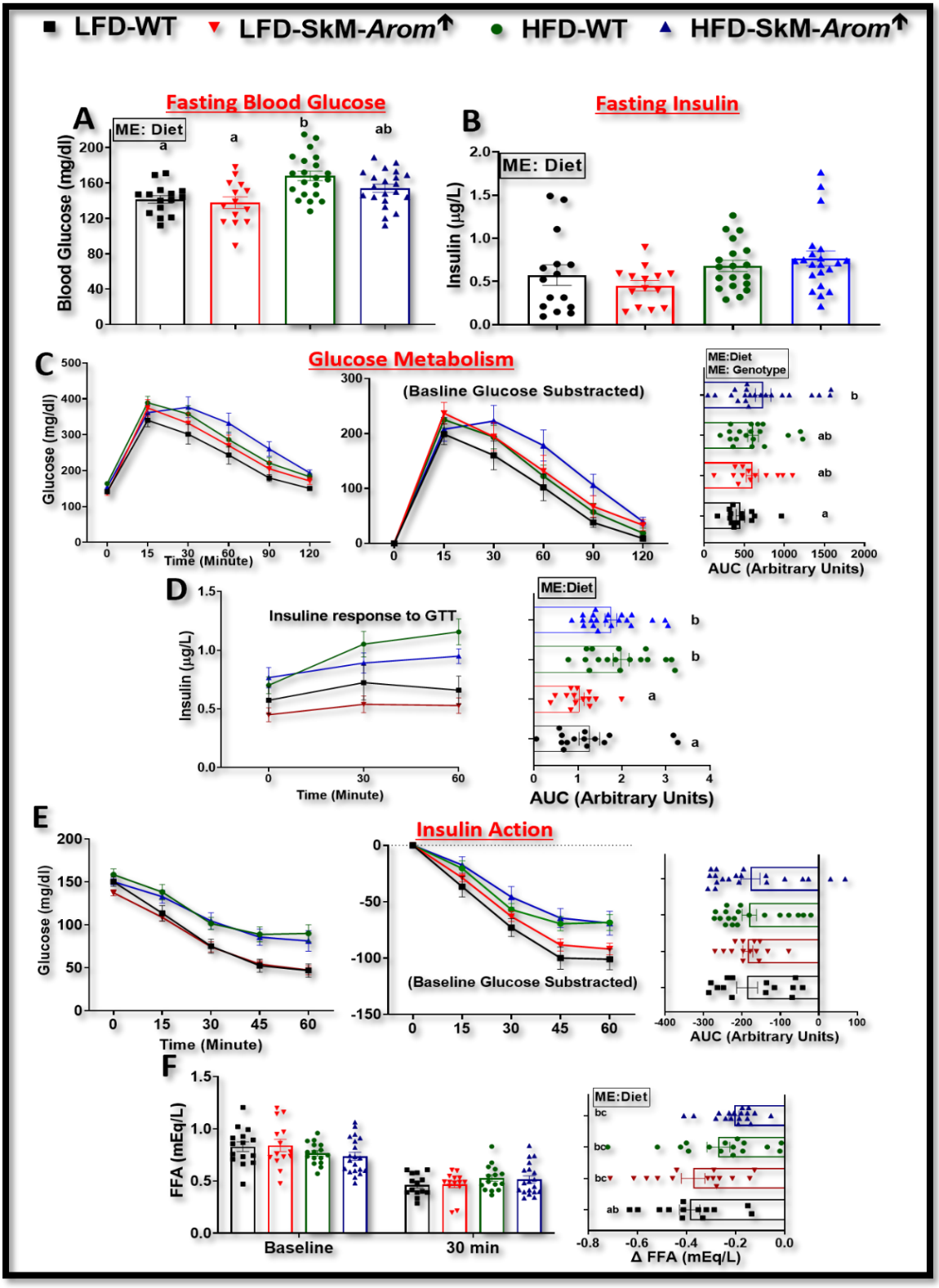
LFD-WT
LFD-SkM-Arom↑
HFD-WT
HFD-SkM-Arom↑
A
Fasting Blood Glucose
ME: Diet
Blood Glucose (mg/dl)
B
Fasting Insulin
ME: Diet
Insulin (µg/L)
C
Glucose Metabolism
Glucose (mg/dl)
Time (Minute)
(Basline Glucose Substracted)
ME:Diet
ME: Genotype
AUC (Arbitrary Units)
D
Insuline response to GTT
Insulin (µg/L)
Time (Minute)
ME:Diet
AUC (Arbitrary Units)
E
Glucose (mg/dl)
Time (Minute)
Insulin Action
(Baseline Glucose Substracted)
AUC (Arbitrary Units)
F
FFA (mEq/L)
Baseline
30 min
ME:Diet
Δ FFA (mEq/L)

Figure 3.4 Skeletal muscle aromatase overexpression does not regulate glucose metabolism and insulin action in intact females. Intact SkM-Arom$^{\uparrow}$ and WT littermate females were on LFD or HFD, and on Dox with drinking water (n=15-25/group). A,B fasting (5hrs) blood glucose and insulin, respectively, after 12 weeks of diet and Dox administration. C, Glucose tolerance test (GTT) for 2hrs, 30 min intervals, glucose baseline subtracted, then the Area Under the Curve (AUC/arbitrary units) is calculated. D, Insulin response to GTT at 0,30, and 60 min time points, AUC is calculated accordingly. E, insulin tolerance test (ITT), on week 13 of diet, for 1hr (15 min intervals), baseline glucose subtracted, then AUC was calculated accordingly. F, FFAs analysis at 0 and 30 min after insulin injection in ITT, ΔFFAs of 0 and 30 min timepoints was calculated. Data are presented as mean $\pm$SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect.

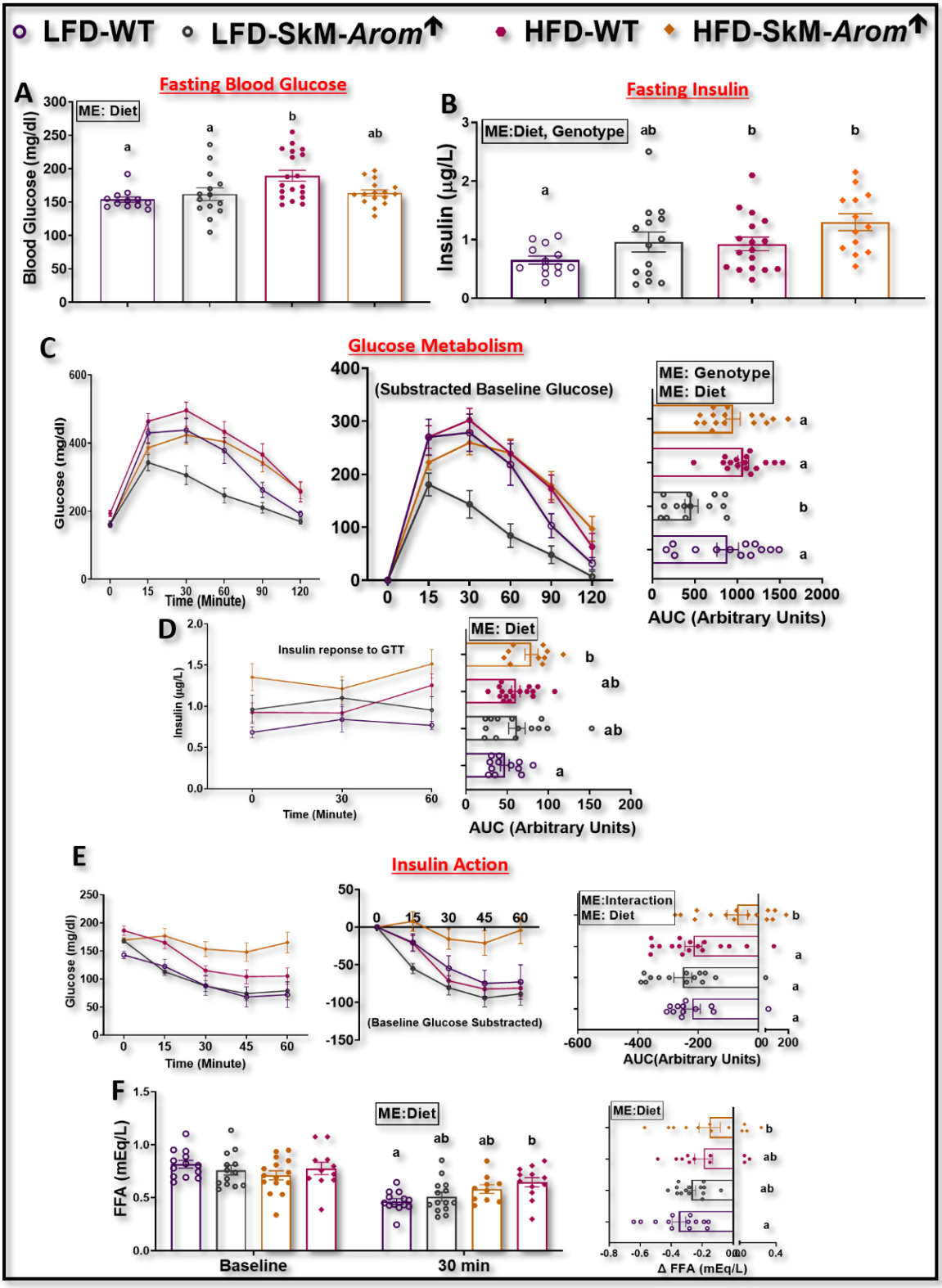
LFD-WT
LFD-SkM-Arom↑
HFD-WT
HFD-SkM-Arom↑
A
Fasting Blood Glucose
ME: Diet
Blood Glucose (mg/dl)
B
Fasting Insulin
ME:Diet, Genotype
Insulin (µg/L)
C
Glucose Metabolism
Glucose (mg/dl)
Time (Minute)
(Substracted Baseline Glucose)
ME: Genotype
ME: Diet
AUC (Arbitrary Units)
D
Insulin reponse to GTT
Insulin (µg/L)
Time (Minute)
ME: Diet
AUC (Arbitrary Units)
E
Insulin Action
Glucose (mg/dl)
Time (Minute)
(Baseline Glucose Substracted)
ME:Interaction
ME: Diet
AUC(Arbitrary Units)
F
FFA (mEq/L)
ME:Diet
Baseline
30 min
ME:Diet
Δ FFA (mEq/L)

Figure 3.5 Skeletal muscle aromatase overexpression does not regulate glucose metabolism and insulin action in Ovx females. Ovx SkM-Arom$^{\uparrow}$ and WT littermate females were on LFD or HFD, and Doxycyclin-treated drinking water (n=15-25/group). A,B fasting (5hrs) blood glucose and insulin, respectively, after 12 weeks of diet and Dox administration. C, Glucose tolerance test (GTT) for 2hrs, 30 min intervals, glucose baseline subtracted, then the Area Under the Curve (AUC/arbitrary units) is calculated. D, Insulin response to GTT at 0,30, and 60 min time points, AUC is calculated accordingly. E, insulin tolerance test (ITT) ,on week 13 of diet, for 1hr (15 min intervals), baseline glucose subtracted, then AUC was calculated accordingly. F, FFAs analysis at 0 and 30 min after insulin injection in ITT, ΔFFAs of 0 and 30 min timepoints was calculated. Data are presented as mean $\pm$SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect.

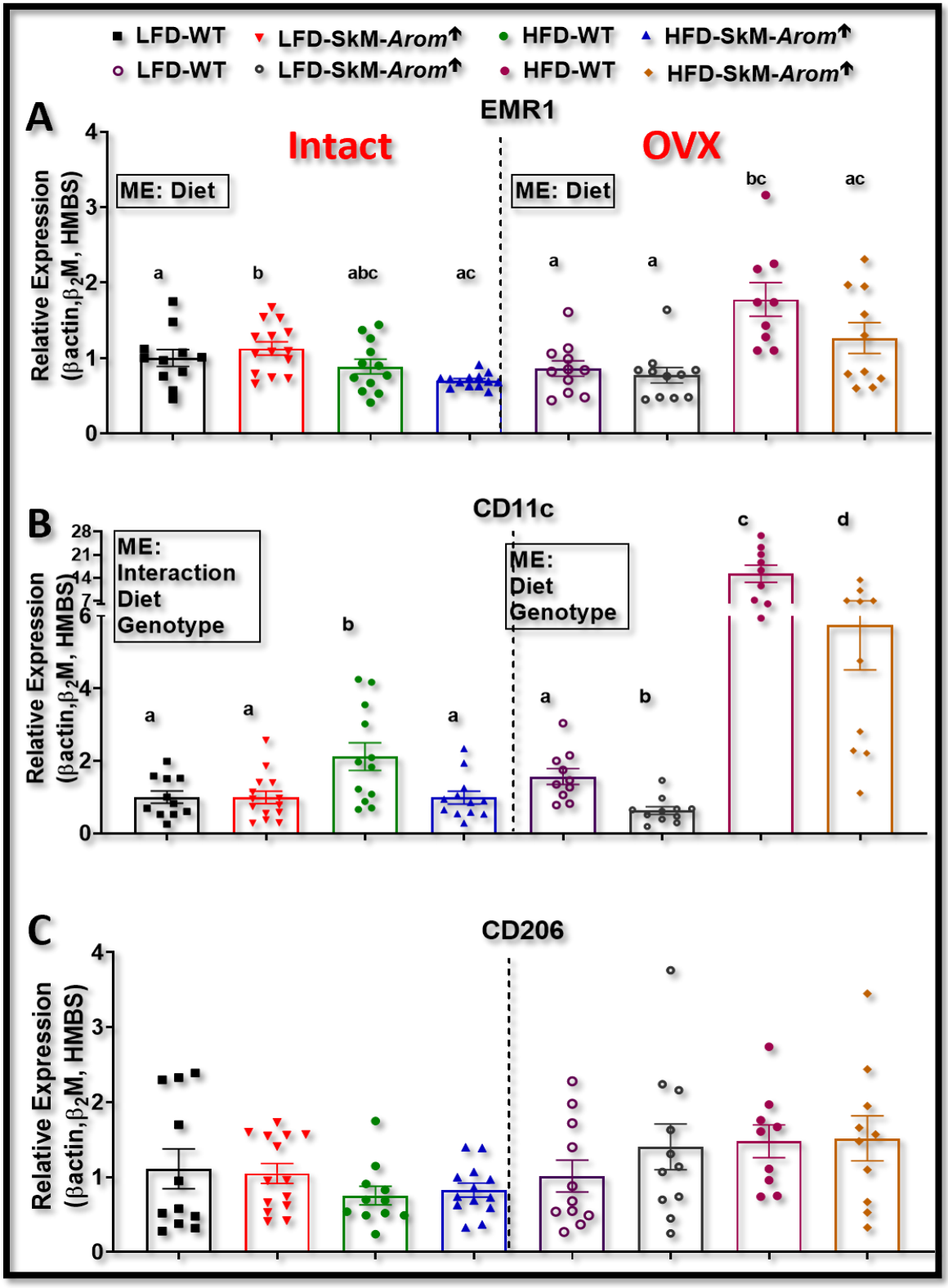
LFD-WT
LFD-SkM-Arom↑
HFD-WT
HFD-SkM-Arom↑
LFD-WT
LFD-SkM-Arom↑
HFD-WT
HFD-SkM-Arom↑
A
EMR1
Intact
OVX
ME: Diet
ME: Diet
Relative Expression (βactin,β2M, HMBS)
a
b
abc
ac
a
a
bc
ac
B
CD11c
ME: Interaction Diet Genotype
ME: Diet Genotype
Relative Expression (βactin,β2M, HMBS)
a
a
b
a
a
b
c
d
C
CD206
Relative Expression (βactin,β2M, HMBS)

Figure 3.6 Skeletal muscle aromatase overexpression decreases M1 macrophage gene marker in adipose tissue. Macrophage gene expression in gonadal adipose tissue in both intact and Ovx females. A, EMR1. B, CD11c. C, CD206. Data are presented as mean ±SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect.

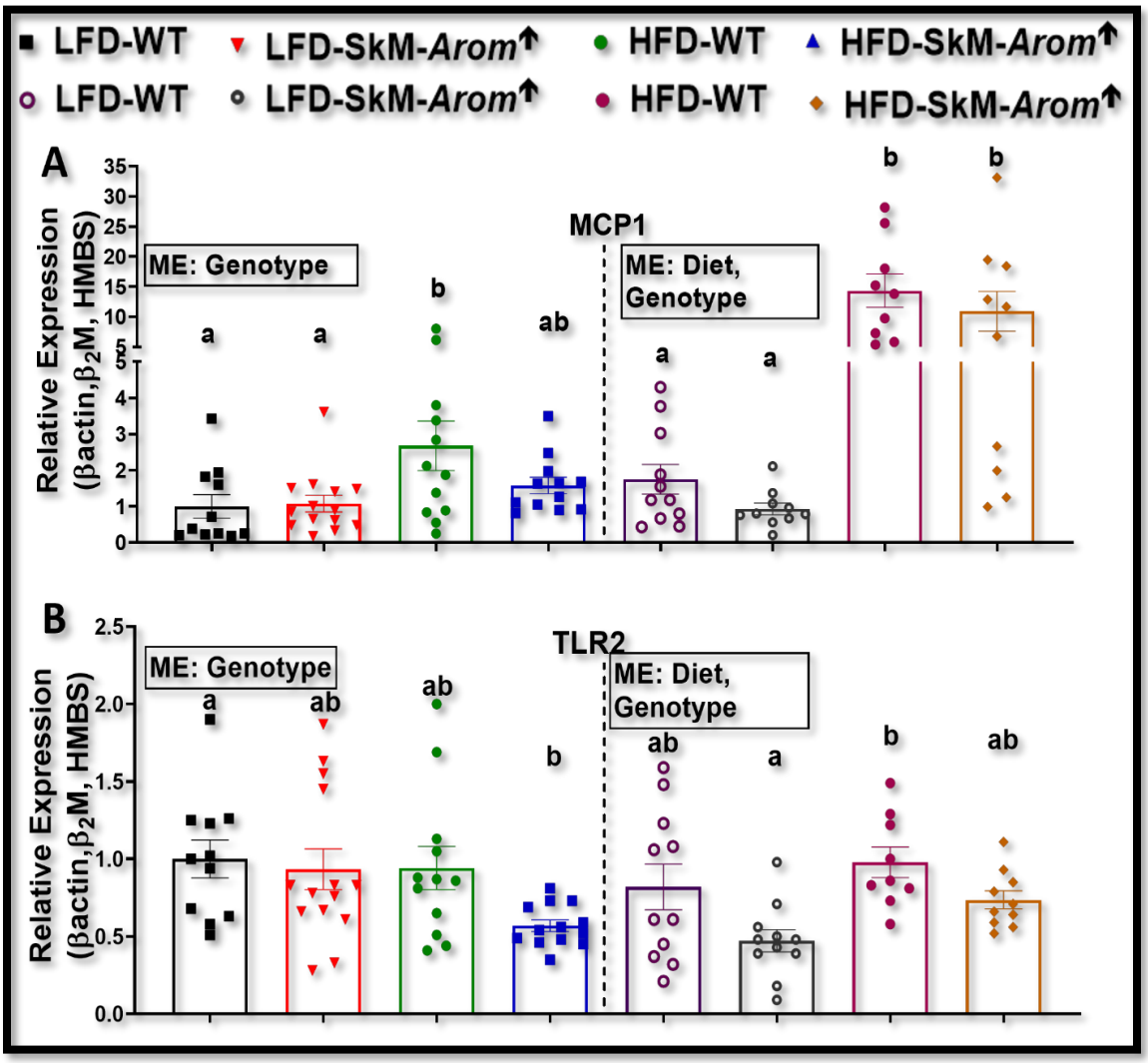

Figure 3.7 Skeletal muscle aromatase overexpression decreases pro-inflammatory gene expression in adipose tissue. Gonadal adipose tissue in both intact and Ovx females. A, MCP-1. B, TLR2. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect.

CHAPTER FOUR

Skeletal muscle ERα overexpression ameliorates HFD-induced obesity and metabolic dysregulation in females independent to the estrogen status

4.1 Introduction

Broadly expressed in many organs and tissues such as central nervous system, skeletal muscle, adipose tissues, liver, and immune cells, role of ERα in metabolism and energy homeostasis have been investigated extensively. Deletion of ERα, globally, have shown that estrogen, acting via ERα, regulates glucose homeostasis and insulin sensitivity. Also, females are lacking ERα action develop central obesity with increase white AT and weight gain due to reductions in energy expenditure as well[105, 112]. However, in context of whether ERα mechanism action is a central and/or peripheral mechanism, tissue dissection approach to studying ERα using mice with conditional deletion allele had opened new horizons to researchers to delineate unique aspects and novel findings of ERα biology in a tissue-specific context[96, 114]. Clinical observational findings indicated that ERα expression levels were reduced in skeletal muscle of women with metabolic syndrome and the ERα level were inversely correlated with adiposity and fasting insulin. Remarkably, similar findings were observed across several strains of inbred female mice as well as

genetically induced-obese mice[106]. Hevener and her colleagues successively induced skeletal muscle (SkM)- specific deletion of ERα in MERK murine model. Major findings confirmed that MERK females showed insulin signaling impairment and high fat accumulation due to mitochondrial malfunction. Similar metabolic phenotype had been reported when global ERα KO mouse model was utilized. However, ERα deletion in both animal models, global ERα KO and MERK, were from birth and were confounded by altered developmental programing. Also, secondary side effects of ERα deletion from birth might significantly affect the interpretation of the results. Therefore, inducible deletion of SkM ERα approach was proposed by Spangenburg *et al* using tamoxifen to induce SkM ERα deletion in ERαKO^{ism} females[122]. Their results were contradicting Hevener findings as they found that muscle ERα was not required for regulation of muscle insulin sensitivity and mitochondrial function. However, further, and careful analysis of ERαKOism model uncovered to us two major problematic issues. First, tamoxifen is one of selective estrogen receptor modulator (SERM) that is used to treat breast cancer in premenopausal women[156, 157]. Therefore, using a SERM molecule as an inducer of a deletion transgenic system to study role or ERα is considered as an uncertain approach. Second, according to their model design, Spangenberg and his colleagues were obligated to administer Tamoxifen only to experimental group, not to their littermate control. Thus direct and/or indirect effect of tamoxifen on muscle physiology cannot be ruled out which put their approach as a questionable issue[123]. Furthermore, both SkM- ERα KO approaches, from birth and inducible, have tested the mechanistic pathways of loss of muscle ERα function involved in global metabolism and energy homeostasis. To our knowledge, gain of function approach have not been done yet by any other group. Thus,

using a non-tamoxifen inducible model to specifically overexpress SkM-ERα is a novel and more therapeutically relevant model. Also, novel therapeutic molecular targets may be uncovered to combat diet-induced obesity in women. Therefore, we developed an inducible double transgenic model which has rtTA transgene linked to HSA promoter and Tet operon which control ERα expression. In HSA- rtTA $^{+/-}$, Tet-ERα$^{+/-}$ mice, administration of doxycycline with drinking water will activate rtTA molecule, in skeletal muscle only, to augment SkM ERα expression. Wild type littermate control had been equally treated with doxycycline as well. Our central hypothesis focuses on overexpression of SkM ERα will beneficially improve global glucose metabolism and enhance insulin sensitivity which in turn will combat diet-induced obesity in females.

4.2 Method and materials

Mice: To generate our novel double transgenic mouse model, two transgenic mice (C57Bl6 background) were bred. First transgenic mouse harbors human skeletal actin (HSA) promoter that drive the expression of reverse tetracyclin-controled transactivator (rtTA) (rtTA-HSA)[147]. Second mouse has tetracycline response element Tet-on promoter that drive ERα (Tet-ER)[148]. Animals were screened by PCR for the presence of the target sequence using genomic DNA isolated from tail snips with the following primers: for rtTA-HSA; F, 5′-ATGTCTAGACTGGACAAGAGCAAAG-3′; R, 5′-TTACCCGGGGAGCATGTC-3′. For Tet-ER; For Tet-ER; F, 5′-CGAGCTCGGTACCCGGGTCG-3’, R 5’-GAACACAGTGGGCTTGCTGTTG-3’. When rtTA-HSA$^{+/+}$ and Tet- ERα $^{+/-}$ mice were bred, both rtTA-HSA$^{+/-}$, Tet- ERα $^{+/-}$ and rtTA-HSA$^{+/-}$,Tet- ERα $^{-/-}$ offspring were used as experimental and WT littermate control

groups, respectively. Doxycycline administration (0.1 mg/ml) with drinking water was provided to both groups. In this reversable process, only rtTA-HSA$^{+/-}$, Tet- ERα $^{+/-}$ mice will overexpress skeletal muscle ERα upon doxycycline administration.

Diet: At 10 weeks of age, animals were randomly assigned to 1 of 4 groups: LFD-WT, LFD-SkM- ERα$^{\uparrow}$, HFD-WT, and HFD-SkM- ERα$^{\uparrow}$ in intact (E2-efficient mice) and same groups in Ovx (E2-deficient mice) as well (n= 15-25/group). The LFD utilized in this study was open-source, purified AIN-76A diet (3.77 kcal/g). The HFD was a custom-made purified HFD (4.57 kcal/g) comprised of 47%, 40%, and 13% of total calories from carbohydrates, fat, and protein, respectively, with saturated sat make up 12% of total calories in order to mimic the standard American diet (Bio-Serv, Frenchtown, NJ).

Ovariectomy: For E2-deficient groups, bilateral ovariectomy was done to animals at 8th week of age one week recovery time before the study was started. Mice were housed, 3-5/cage, maintained on 12:12-h light-dark cycle in a low stress environment (22 C, 50% humidity, low noise), and given food and water as libitum. All methods were in accordance with the American Association for Laboratory Animal Science, and the Institutional Animal Care and Usage Committee of the University of South Carolina approved all experiments.

Body weight and body composition

Body weight was monitored on a weekly basis throughout the study. Body composition was assessed after 11 weeks of diet to use lean mass as the basis for the dose of glucose and insulin administration for glucose and insulin tolerance tests, respectively.

For this procedure, mice were briefly anesthetized via isoflurane inhalation, and lean mass, fat mass, and percent body fat were assessed by dual-energy X-ray absorptiometry (Lunar PIXImus).

Food- intake assessment:

To assess food-intake in this model, the animals were single-housed and ad libitum HFD with free access to water and body weight and food intake were weekly recorded, then body weight difference of beginning day vs end day was calculated as well as total Kcal consumption was calculated accordingly.

Metabolic assessment

Fasting blood glucose and insulin levels were assessed after 12 weeks of dietary treatment. After a 5-hour fast, blood samples were collected from the tip of the tail. A glucometer (Bayer Contour, Mishawaka, IN) was used to determine blood glucose concentrations in whole blood. Collected blood was centrifuged at 4,000 rpm for 10 minutes at 4°C. Plasma insulin concentrations were analyzed according to the manufacturer's instructions using a mouse insulin ELISA kit (Mercodia, Winston Salem, NC). Glucose and insulin tolerances tests (GTTs and ITTs, respectively) were performed after 12 and 13 weeks of dietary treatment, respectively. For these procedures, mice were fasted for 5 hours, and glucose or insulin was administered intraperitoneally at 2 g/kg or 0.75 U/kg lean mass, respectively. A glucometer (Bayer Contour) was used to measure blood glucose concentrations (tail sampling) intermittently over a 2-h period (0, 15, 30, 60, 90, and 120 minutes) for GTTs and intermittently over a 1-h period (0, 15, 30, 45, and

60 minutes) for ITTs. Baseline glucose levels were subtracted accordingly, and Area of the curve (AOC) was generated as previously described[149]. Area under the curve (AUC) of AOC was calculated using the trapezoidal rule. Blood was collected from the tip of the tail during the GTT (0, 15, 30, and 60 minutes) to assess the insulin response to the GTT for a subset of mice from each group. Fasting serum was collected (using non-heparinized capillary tubes) for free fatty acid (FFA) analysis at the 0- and 30-min time points of the ITT to assess insulin's ability to inhibit lipolysis. FFAs were analyzed using a commercially available kit according to the manufacturer's instructions (Wako Diagnostics, Richmond, VA).

Tissue collection

After 13 weeks of dietary treatment, mice were euthanized via isoflurane inhalation for tissue collection. Gonadal, mesenteric, and perirenal fat pads, as well as the liver, and skeletal muscle (gastrocnemius and quadriceps) were removed, weighed, and immediately snap-frozen in liquid nitrogen and stored at −80°C or fixed in 4% formalin until analysis.

Statistical analysis

Data were analyzed using commercially available statistical software, GraphPad Prism 9 (GraphPad Software, La Jolla, CA). A two-way ANOVA (diet x genotype) followed by a Tukey post hoc analysis was used for the outcomes analyzed in both E2-efficient and E2-deficient status, separately. Any statistical test that did not pass the equal-variance test (Bartlett's test for equal variances) values were transformed accordingly and then reanalyzed.

4.3 Results:

4.3.1 Skeletal muscle ERα gene and protein expression were elevated in novel inducible SkM- ERα$^{\uparrow}$ model after 7 days of doxycycline administration

To assess the efficiency of our novel model promoting SkM-ERα, gene, and protein expression of gastrocnemius SkM-ERα were significantly increased after seven days of doxycycline administration to SkM- ERα$^{\uparrow}$ mice compared to non-treated animals ($P < .05$) (figure 4.1A).

4.3.2 SkM-ERα overexpression ameliorates diet-induced obesity in females independent to E2 status

Induced SkM-ERα overexpression protects HFD-fed females against diet-induced body weight gain (figure 4,1B). Post-hoc analysis revealed that intact SkM- ERα$^{\uparrow}$ females gained significantly less weight than littermate WT controls ($P < .05$) (figure 4.1B). This outcome was more obvious in the Ovx mice as SkM- ERα$^{\uparrow}$ and HFD were the main effects to reduce body weight. Post-hoc analysis showed that Ovx SkM- ERα$^{\uparrow}$mice gained as much as LFD-animals and significantly less than their counterpart controls ($P < .05$) (figure 4.1C). Furthermore, body composition analysis showed main effect of genotype and HFD to decrease all body composition parameters ($P < .05$) (figure 4.2). Post-hoc analysis confirmed that SkM- ERα$^{\uparrow}$ significantly decreased lean mass, fat mass, as well as fat mass % compared to their corresponding WT counterparts and independent of E2 status ($P < .05$) (figure 4.2 A,B). Regarding of visceral fat and liver weights, post-hoc analysis revealed that intact SkM-ERα$^{\uparrow}$ females on HFD significantly had lower levels than their

counterpart WT control ($P < .05$) (figure 4.3 A,B). For the Ovx mice, HFD and SkM- ERα$^{\uparrow}$ were the main effects to decrease visceral fat and liver weights ($P < .05$) (figure 4.3 C,D). Post-hoc analysis revealed that liver weight levels were significantly decreased in Ovx SkM- ERα$^{\uparrow}$ animals, independent of the fed diet ($P < .05$) (figure 4.3 C).

4.3.3 Muscle ERα overexpression does not affect food intake and total consumed calories

In order to assess the animal feeding behavior, food intake study was conducted on single housed ad libitum HFD-fed animals. Collected data revealed that SkM-ERα$^{\uparrow}$ animals gained significantly less weight compared to WT littermate control mice after 8 weeks of HFD feeding ($P < .05$) (figure 4.4 A). Also, data analysis revealed that weekly food intake as well as total kcal consumption were not changed in SkM- ERα↑ animals compared to their littermate controls.

4.3.4 SkM-ERα overexpression improves glucose metabolism and decreases fasting blood insulin levels in females independent to E2 status

Metabolic test GTT was done to asses global glucose metabolism after 12 wks of diet treatments among different female groups (figure 4.5 A,F). Data analysis revealed that there was main effect of SkM-ERα$^{\uparrow}$ to decrease, and inversely HFD to increase, the AUC of glucose metabolism in intact females ($P < .05$). Post-hoc analysis showed that SkM-ERα$^{\uparrow}$ females exhibited significantly lowest AUC levels of glucose metabolism independent to the fed-diet (figure 4.5 B). Same relationship was found regarding of fasting blood glucose and insulin levels (figure 4.5 C,E). Post-hoc analysis revealed that SkM-

ERα↑ significantly decreased fasting blood glucose only in HFD animals when compared to their WT littermate controls ($P < .05$). Furthermore, SkM-ERα↑ significantly decreased fasting insulin levels independent to the fed-diet compared to the counterpart WT controls ($P < .05$) (figure 4.5 E). In the Ovx mice, GTT data analysis revealed that SkM-ERα↑ was the main effect to decrease the AUC of glucose metabolism (figure 4.5 G). Post-hoc analysis showed that Ovx SkM-ERα↑ mice exhibited the lowest AUC levels when compared to their WT controls and independent to the diet ($P < .05$). Also, main effect of HFD and SkM-ERα↑ were observed to decrease fasting blood glucose and insulin levels among different experimental groups ($P < .05$) (figure4.5 H,I) . Post-hoc analysis found that SkM-ERα↑ in HFD significantly decreased fasting blood insulin when compared to their corresponding WT controls ($P < .05$) (figure 4.5 I).

4.3.5 Insulin action was improved after promotion of SkM-ERα in intact but not Ovx animals, however, Adipose tissue lipolysis was inhibited regardless of E2 and diet

Insulin sensitivity was assessed after 13 weeks of diet treatment among different animal groups (figure 4.6 A,C). Data analysis of ITT revealed that SkM-ERα↑ was the main effect to decrease the AUC of insulin action in the intact females ($P < .05$) (figure4.6 A). However, in the Ovx mice no changes on insulin action were detected among the studied groups (figure4.6 B). Regarding blood concentration of FFAs levels, in intact females, there were main effects of SkM-ERα↑ to decrease, and HFD to increase, FFAs among animal groups. Post-hoc analysis revealed that HFD-fed SkM-ERα↑ registered significant decreasing of FFA levels in both baseline and 30 min time points when compared to their

littermate controls ($P < .05$) (figure4.6 B). However, when ΔFFAs was calculated, there was no change between different intact female groups. On the other hand, Ovx mice data analysis did not show change in the AUC of insulin action among different test groups (figure 4.6 C). Regarding blood concentration of FFAs levels, Ovx mice data analysis showed main effects of SkM-ERα$^{\uparrow}$ and LFD to increase baseline FFAs levels among different groups ($P < .05$) (figure4.6 D). Interestingly, when ΔFFAs was calculated, data analysis showed that SkM-ERα$^{\uparrow}$ and LFD were the main effects to increase the inhibitory effect of insulin on adipose tissue lipolysis ($P < .05$) (figure4.6 D).

4.4 Discussion

Role of ERα in metabolism and energy balance was discovered and studied for long time. It has been established that ERα by several potential mechanism controls glucose metabolism, insulin action, and mitochondrial activity[93, 96, 105]. Furthermore, SkM-ERα confirmed same action as global ERα which have been reported using global and tissue specific Era deletion models. Also, clinical data suggested inverse correlation between SkM-ERα level and obesity in women regardless to their E2 level[106]. However, major discrepancies and contradicting data were collected from different SkM-ERα transgenic models which lead to question the exact role of SkM-ERα on regulating metabolism and obesity in females. As MERKO mice by Hevener *et al* have confirmed the regulatory function of SkM-ERα in glucose metabolism and mitochondrial function combating diet-induced obesity. On the other hand, ERαKO^{ism} mouse by Spangenburg *et al* showed no beneficial effect of SkM-ERα on insulin sensitivity and glucose metabolism[105, 106, 122]. Two major limitations were discovered in previous works

which might be the reason for these contradicting findings and uncertain conclusions. In MERKO model, the SkM-ERα is deleted from birth which is considered a profound factor on developmental program of skeletal muscle. On the other hand, ERαKO^{ism} used tamoxifen (a SERM molecule that prevents ERα-mediated action) to induce SkM-ERα deletion. Furthermore, according to our knowledge, none of previous work have used gain of ERα function approach. Therefore, using an inducible non-tamoxifen model might be a solution to such generated contradicting observations. Therefore, we generated an inducible novel transgenic mouse model that for first time employed gain of function of SkM-ERα to combat diet-induced obesity in female mice. Furthermore, testing our approach in different E2 status and on animals fed on different purified diet were part of our main goals for better understanding of SkM-ERα role in metabolism and energy balance. In order to concrete our findings, we utilized littermate controls and large sample size (n=15-25). Our results regarding inducible overexpression of SkM-ERα agree with Hevner *et al* study who found that deletion of SkM-ERα from birth promotes obesity and metabolic dysfunction in female mice. Importantly, our data concluded the power of SkM-ERα regulating whole body weight and metabolism regardless to the global estrogen level as well as fed diet. Also, among the study, general health status was monitored either by doing several function tests, such as complete blood count CBC, or global navigation of internal organs at euthanizing time. All SkM-ERα animals look healthy with 100% survival rate over time of the study. However, doxycycline dose response study is necessary to be done, ongoing study, to optimize best physiological SkM-ERα expression to accomplish proposed phenotype with no potential side effects. Our collected data in the Ovx mice confirmed that independent to the ligand (E2), SkM-ERα overexpression combats diet-

induced obesity and metabolic dysregulation. These data led us to reconsider the importance of ERs vs E2 in regulation of metabolism in females and further investigation is required to uncover potential mechanistic pathways downstream of SkM-ERα. To our knowledge, loss of ligand-dependency of ERα activation has been observed only in human breast cancer cell lines and in some murine breast cancer models [158-161]. However, our data provided first evidence of role of SkM-ERα in regulating of metabolism independent to E2 status. To exclude extragonadal estrogen that Ovx mice have, it's interesting to assess the effect of our inducible SkM-ERα$^{\uparrow}$ model on another E2-dedeicent background such as Arom KO model. Also, mechanistic *In vitro* studies using various ERα mutants are required to uncover a potential non-estrogen ligand might activate SkM-ERα. A limitation of our study is that we did not measure systemic ERα in serum after 13 weeks of SkM-ERα induction to find any potential leaking of the receptor into another organ which might be the direct or indirect cause of the observed phenotype. Also, for better understanding and reveal a new mechanistic pathways, muscle, and adipose tissues gene microarray analysis should be considered and the outcomes could be utilized in additional *In vitro* works on primary myotubes cell cultures. Also, for better understanding of behavioral, feeding, and specific fuel oxidation, indirect calorimetric metabolic cage system needs to be used for continuous real-time thorough analyses and assessments. Based on our findings, our novel transgenic inducible mouse model showed for the first time the power of SkM-ERα in regulating whole body weight, fat deposition and distribution, without effecting food intake and food consumption suggesting a peripheral mechanism of ERα action on metabolism. Also, SkM- ERα overexpression has the power to enhance global glucose metabolism as well as insulin action, even in lower level of insulin than WT controls by preventing

lipolysis as FFAs analysis showed. Finally, this novel inducible transgenic mouse model might be utilized to control tissue specific ERα expression either in loss or gain function protocols and in other different tissues such as adipose tissues or brain using appropriate specific promoter.

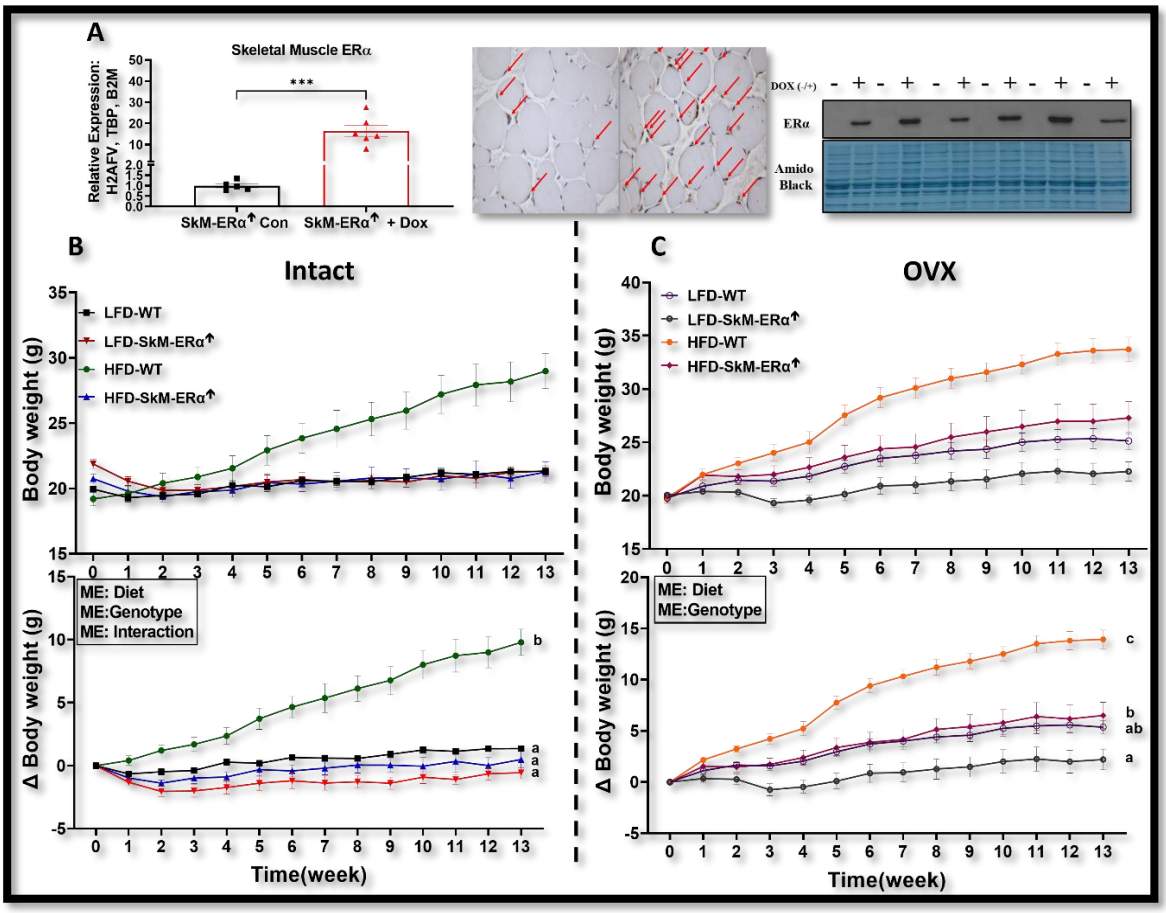

Figure 4.1 Skeletal muscle ERα overexpression protect females against diet-induced weight gain independent to the estrogen level. SkM-ERα$^{\uparrow}$ and WT littermate females were either intact or ovariectomized (Ovx), on LFD or HFD, and doxycycline-treated (DOX) drinking water for 13 weeks (n=15-25/group). A, ERα gene (qPCR) and protein expression (IHC and WB) in skeletal muscle after 7-days of Dox treatment. B, body weight and Δ body weight after 13 weeks of diet treatment for intact females. C, body weight and Δ body weight after 13 weeks of diet treatment for Ovx females. Data are presented as mean ±SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect.

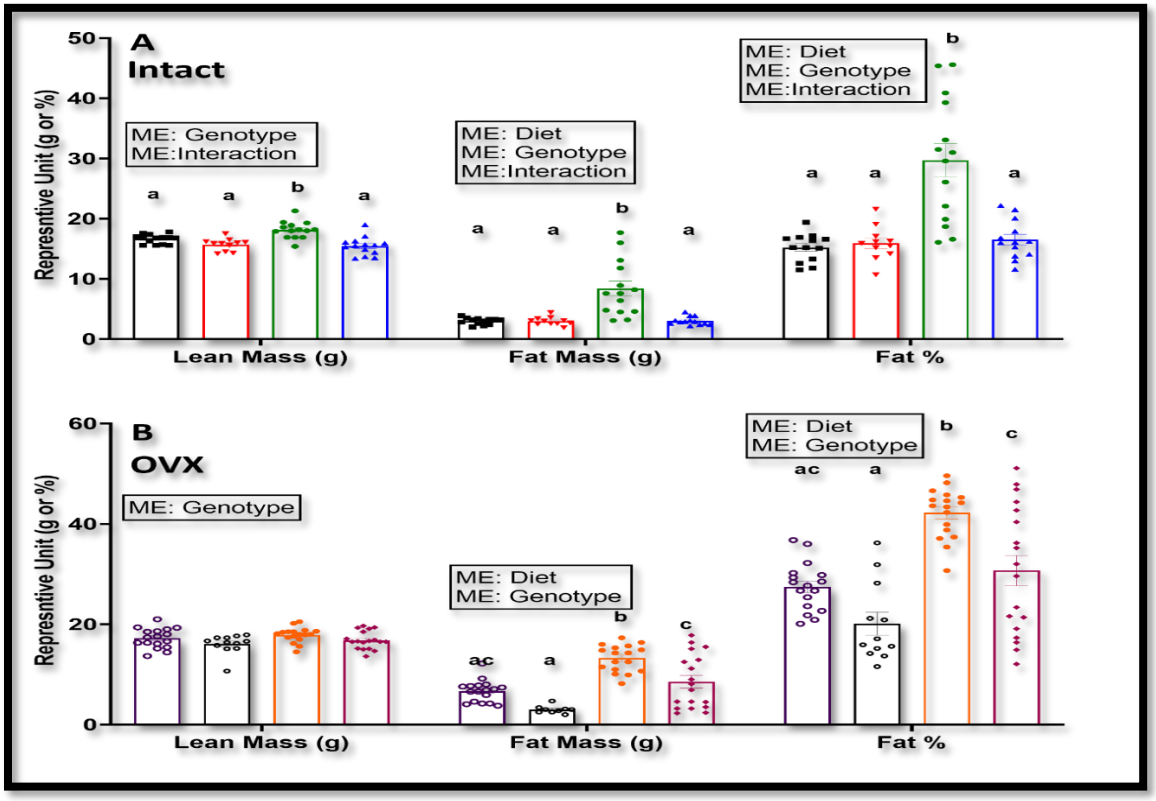

Figure 4.2 Skeletal muscle ERα overexpression decreases total fat mass and fat percentage regardless to the estrogen status. SkM-ERα$^{\uparrow}$ and WT littermate females were either intact or ovariectomized (Ovx), on LFD or HFD, and doxycycline-treated (DOX) drinking water for 13 weeks (n=15-25/group). Lean mass, total body fat mass, and Fat% were analyzed by DEXA at week 11 of diet and Dox treatment for intact A, and Ovx B. Data are presented as mean ±SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect.

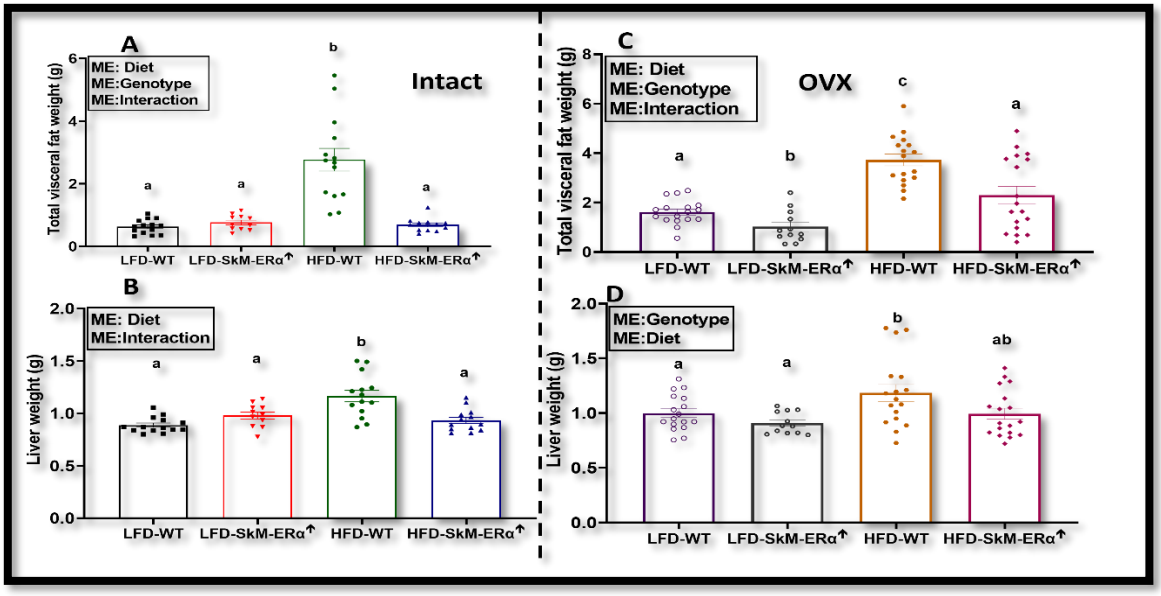

Figure 4.3 Skeletal muscle ERα overexpression significantly decreases total visceral weight and liver weight regardless to estrogen level. Wet weight of Visceral and liver were measured at the time of euthanization for intact females A and B, and Ovx females C and D. Data are presented as mean ±SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect.

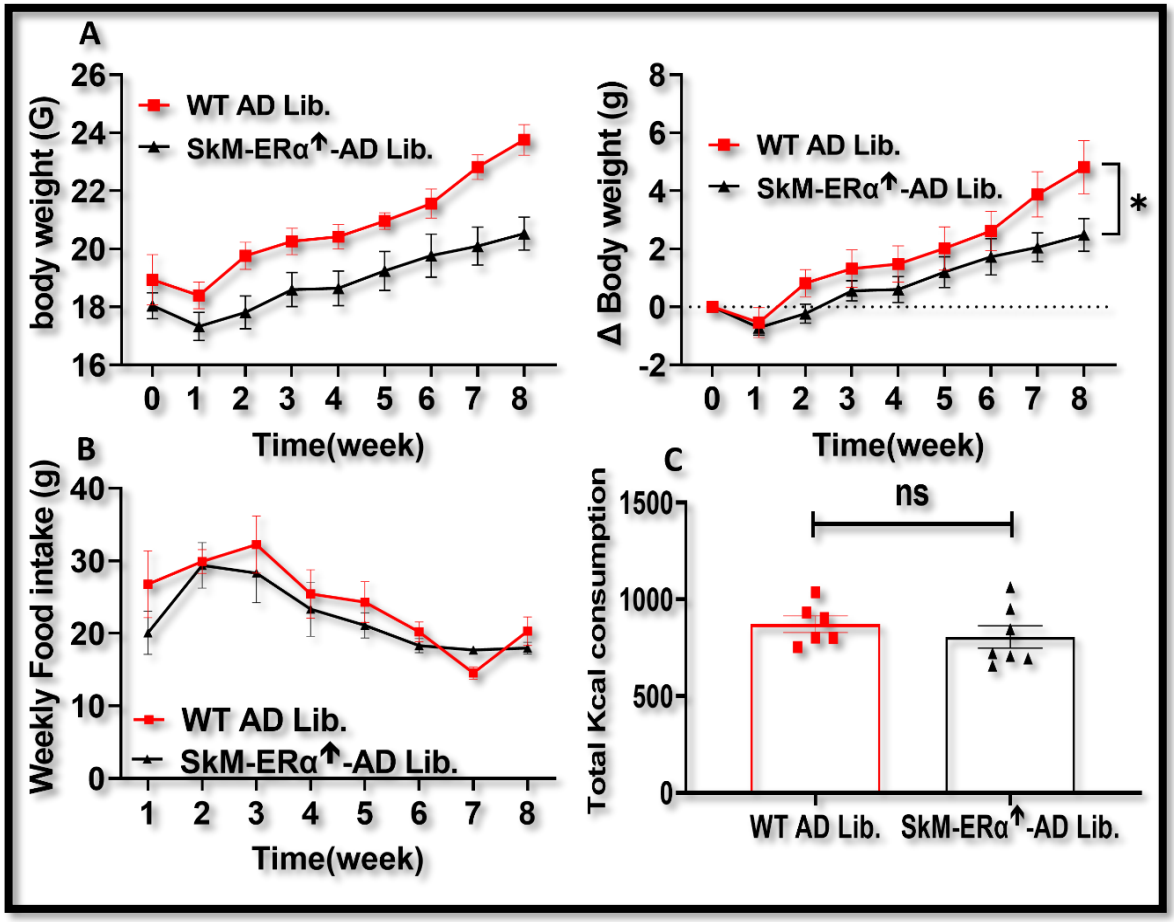

Figure 4.4 Skeletal muscle ERα overexpression does not affect food intake and total calories consumption even though SkM ERα positive gains less weight than WT. SkM-ERα$^{\uparrow}$ and WT littermate females were single-housed and fed on HFD Ad Libitum and doxycycline-treated water for 8 weeks (n= 5-7 mice/group). A, body weight registration. B, Δ body weight calculation. C, weekly food intake in gram. D, Total Kcal consumption. A two-tailed student's *t* test used for statistical comparisons (* = *P*<.*05*).

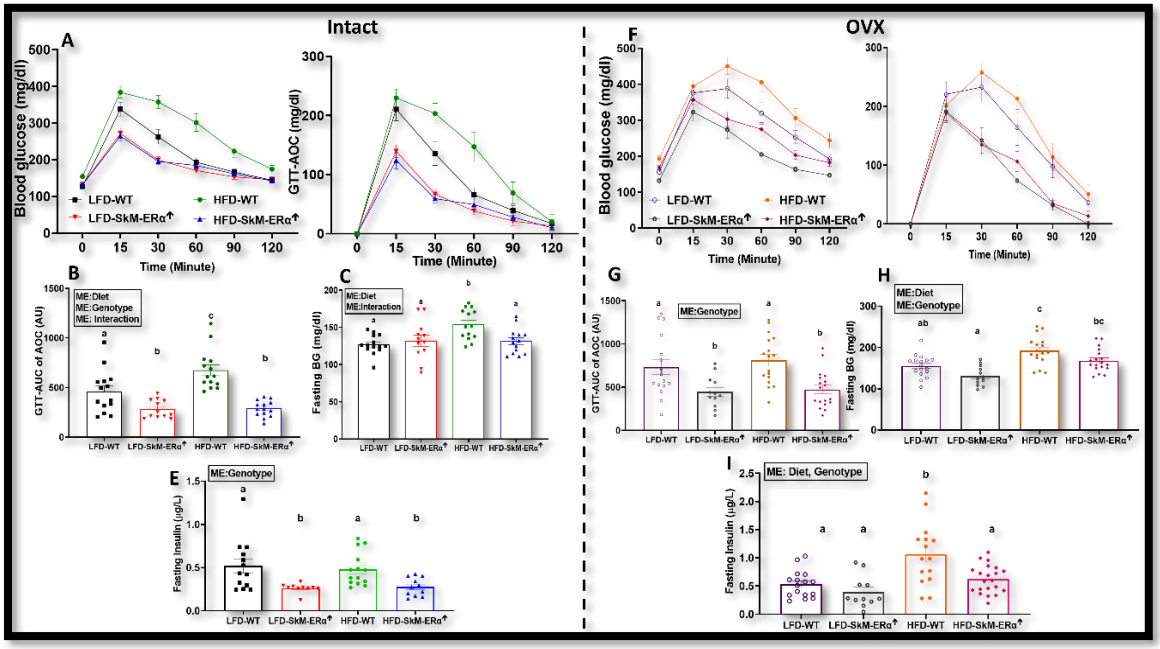

Figure 4.5 Skeletal muscle ERα overexpression enhances glucose metabolism independent to estrogen status and diet and decreases circulating fasting insulin level. Both intact and Ovx SkM- ERα$^{\uparrow}$ and WT littermate females were on LFD or HFD, and Doxycyclin-treated drinking water (n=15-25/group). Glucose tolerance test (GTT) for 2hrs, 30 min intervals, after 12 weeks of diet and Dox administration, glucose baseline subtracted (for intact, A) and (for Ovx, F). The Area Under the Curve (AUC/arbitrary units) is calculated (For intact, B) and (for Ovx, G). For intact females, C and E, fasting (5hrs) blood glucose and insulin, respectively, and H and I for Ovx females. Data are presented as mean ±SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple

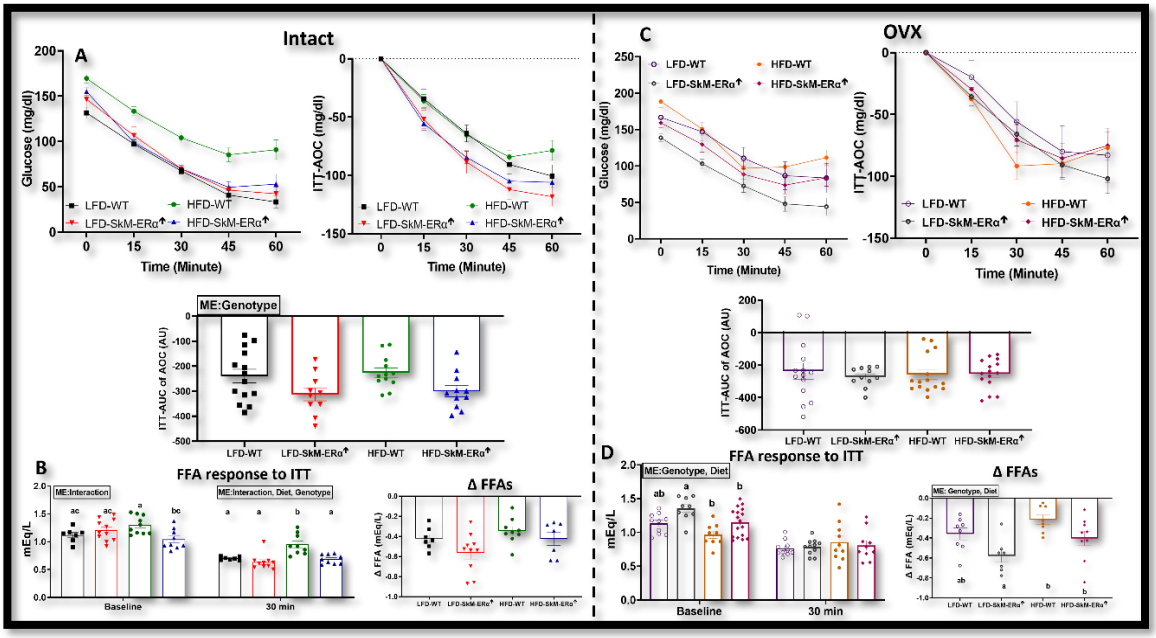

Figure 4.6 Skeletal muscle ERα overexpression increases global insulin sensitivity in intact females independent to the diet and significantly enhances insulin's inhibitory effect on lipolysis in Ovx mice. Insulin tolerance test (ITT), on week 13 of diet, was conducted for 1hr after IP insulin injection (15 min intervals), baseline glucose subtracted, then AUC was calculated accordingly for intact females A, and Ovx females C. B and D, FFAs analysis at 0 and 30 min after insulin injection in ITT, ΔFFAs of 0 and 30 min timepoints was calculated. Data are presented as mean +SEM. Graphs are not sharing same letter are significantly different from one another, Two-way ANOVA (Diet X Genotype) was used for multiple comparisons. ME= Main effect.

CHAPTER FIVE

CONCLUSIONS

Utilizing various transgenic and surgical animal models, we show that estrogen deficiency as well as skeletal muscle ERa and aromatase overexpression have differing effects on the development of the obese phenotype. In the first aim, we utilized two different estrogen-deficient models to determine the impact that congenital estrogen deficiency (Arom KO) versus adult estrogen deficiency (OVX) has on obesity development. We found that both models led to similar degrees of body weight gain, however, the Arom KO mice presented with greater metabolic dysfunction and adipose tissue inflammation than the OVX model. Furthermore, our data suggests that the estrogen-deficient increase in adiposity is largely driven by decreased physical activity rather than changes in food intake.

In the second and third aims, we created novel inducible models to overexpress aromatase and ERα, separately, in the skeletal muscle. These models were designed to address critical limitations in previous work. We found that overexpression of skeletal muscle aromatase improves bone mineralization and limits adipose tissue inflammation in female mice. However, diet-induced obesity and metabolic dysregulation were not

ameliorated in our novel transgenic model. On the other hand, our data showed the power of skeletal muscle ERα overexpression to combat adiposity and significantly improve glucose metabolism and insulin sensitivity independent of estrogen status. Our findings suggest that either another ligand, other than estrogen, is responsible for activating the ERα pathway or a non-ligand activation mechanism was promoted to induce the observed phenotype. Therefore, further investigations are required to explore potential mechanistic pathways downstream of ERα activation. Furthermore, the power of inducible transgenic models can be further employed and developed in different tissues to promote/delete target molecules of interest in preclinical murine models.

REFERENCES

[1] K. B. Smith and M. S. Smith, "Obesity Statistics," (in eng), *Prim Care,* vol. 43, no. 1, pp. 121-35, ix, Mar 2016, doi: 10.1016/j.pop.2015.10.001.

[2] D. Albuquerque, E. Stice, R. Rodríguez-López, L. Manco, and C. Nóbrega, "Current review of genetics of human obesity: from molecular mechanisms to an evolutionary perspective," (in eng), *Mol Genet Genomics,* vol. 290, no. 4, pp. 1191-221, Aug 2015, doi: 10.1007/s00438-015-1015-9.

[3] M. Hopkins and J. E. Blundell, "Energy balance, body composition, sedentariness and appetite regulation: pathways to obesity," (in eng), *Clin Sci (Lond),* vol. 130, no. 18, pp. 1615-28, 09 01 2016, doi: 10.1042/CS20160006.

[4] M. W. Schwartz *et al.*, "Obesity Pathogenesis: An Endocrine Society Scientific Statement," (in eng), *Endocr Rev,* vol. 38, no. 4, pp. 267-296, 08 01 2017, doi: 10.1210/er.2017-00111.

[5] F. Zatterale *et al.*, "Chronic Adipose Tissue Inflammation Linking Obesity to Insulin Resistance and Type 2 Diabetes," (in eng), *Front Physiol,* vol. 10, p. 1607, 2019, doi: 10.3389/fphys.2019.01607.

[6] W. E. Consultation, "Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies," (in eng), *Lancet,* vol. 363, no. 9403, pp. 157-63, Jan 10 2004, doi: 10.1016/S0140-6736(03)15268-3.

[7] C. L. Ogden *et al.*, "Trends in Obesity Prevalence by Race and Hispanic Origin—1999-2000 to 2017-2018," *JAMA,* vol. 324, no. 12, p. 1208, 2020-09-22 2020, doi: 10.1001/jama.2020.14590.

[8] Z. J. Ward *et al.*, "Projected U.S. State-Level Prevalence of Adult Obesity and Severe Obesity," *New England Journal of Medicine,* vol. 381, no. 25, pp. 2440-2450, 2019-12-19 2019, doi: 10.1056/nejmsa1909301.

[9] A. C. Skinner, S. N. Ravanbakht, J. A. Skelton, E. M. Perrin, and S. C. Armstrong, "Prevalence of Obesity and Severe Obesity in US Children, 1999–2016," *Pediatrics,* vol. 141, no. 3, p. e20173459, 2018-03-01 2018, doi: 10.1542/peds.2017-3459.

[10] G. A. Bray, "Medical Consequences of Obesity," *The Journal of Clinical Endocrinology & Metabolism,* vol. 89, no. 6, pp. 2583-2589, 2004-06-01 2004, doi: 10.1210/jc.2004-0535.

[11] M. L. Butryn, V. Webb, and T. A. Wadden, "Behavioral Treatment of Obesity," *Psychiatric Clinics of North America,* vol. 34, no. 4, pp. 841-859, 2011-12-01 2011, doi: 10.1016/j.psc.2011.08.006.

[12] R. J. F. Loos, "Genetic determinants of common obesity and their value in prediction," *Best Practice & Research Clinical Endocrinology & Metabolism,* vol. 26, no. 2, pp. 211-226, 2012-04-01 2012, doi: 10.1016/j.beem.2011.11.003.

[13] S. Z. Gebreab *et al.*, "Psychosocial Stress Over the Lifespan, Psychological Factors, and Cardiometabolic Risk in the Community," *Psychosomatic Medicine*, vol. 80, no. 7, pp. 628-639, 2018-09-01 2018, doi: 10.1097/psy.0000000000000621.
[14] I. Sommer *et al.*, "Socioeconomic inequalities in non-communicable diseases and their risk factors: an overview of systematic reviews," *BMC Public Health*, vol. 15, no. 1, 2015-12-01 2015, doi: 10.1186/s12889-015-2227-y.
[15] J. F. Sallis and K. Glanz, "Physical Activity and Food Environments: Solutions to the Obesity Epidemic," *Milbank Quarterly*, vol. 87, no. 1, pp. 123-154, 2009-03-01 2009, doi: 10.1111/j.1468-0009.2009.00550.x.
[16] F. L. Greenway, "Physiological adaptations to weight loss and factors favouring weight regain," *International Journal of Obesity*, vol. 39, no. 8, pp. 1188-1196, 2015-08-01 2015, doi: 10.1038/ijo.2015.59.
[17] M. Ng *et al.*, "Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013," *The Lancet*, vol. 384, no. 9945, pp. 766-781, 2014-08-01 2014, doi: 10.1016/s0140-6736(14)60460-8.
[18] X. Zhang, A. M. Lewis, J. R. Moley, and J. R. Brestoff, "A systematic review and meta-analysis of obesity and COVID-19 outcomes," *Scientific Reports*, vol. 11, no. 1, 2021, doi: 10.1038/s41598-021-86694-1.
[19] M. Thevis, A. Thomas, and W. Schänzer, "Insulin," in *Handbook of Experimental Pharmacology*: Springer Berlin Heidelberg, 2009, pp. 209-226.
[20] M. C. Petersen and G. I. Shulman, "Mechanisms of Insulin Action and Insulin Resistance," *Physiological Reviews*, vol. 98, no. 4, pp. 2133-2223, 2018-10-01 2018, doi: 10.1152/physrev.00063.2017.
[21] G. I. Shulman, "Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease," (in eng), *N Engl J Med*, vol. 371, no. 12, pp. 1131-41, Sep 18 2014, doi: 10.1056/NEJMra1011035.
[22] R. A. DeFronzo *et al.*, "Type 2 diabetes mellitus," (in eng), *Nat Rev Dis Primers*, vol. 1, p. 15019, 07 23 2015, doi: 10.1038/nrdp.2015.19.
[23] M. C. Petersen, D. F. Vatner, and G. I. Shulman, "Regulation of hepatic glucose metabolism in health and disease," *Nature Reviews Endocrinology*, vol. 13, no. 10, pp. 572-587, 2017-10-01 2017, doi: 10.1038/nrendo.2017.80.
[24] V. L. Tokarz, P. E. Macdonald, and A. Klip, "The cell biology of systemic insulin function," *Journal of Cell Biology*, vol. 217, no. 7, pp. 2273-2289, 2018-07-02 2018, doi: 10.1083/jcb.201802095.
[25] H. V. Lin and D. Accili, "Hormonal regulation of hepatic glucose production in health and disease," (in eng), *Cell Metab*, vol. 14, no. 1, pp. 9-19, Jul 06 2011, doi: 10.1016/j.cmet.2011.06.003.
[26] S. Lillioja *et al.*, "Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus. Prospective studies of Pima Indians," (in eng), *N Engl J Med*, vol. 329, no. 27, pp. 1988-92, Dec 30 1993, doi: 10.1056/NEJM199312303292703.
[27] F. Magkos *et al.*, "Effects of Moderate and Subsequent Progressive Weight Loss on Metabolic Function and Adipose Tissue Biology in Humans with Obesity,"

Cell Metabolism, vol. 23, no. 4, pp. 591-601, 2016-04-01 2016, doi: 10.1016/j.cmet.2016.02.005.

[28] B. Vandanmagsar *et al.*, "The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance," *Nature Medicine,* vol. 17, no. 2, pp. 179-188, 2011-02-01 2011, doi: 10.1038/nm.2279.

[29] K. M. V. Narayan, J. P. Boyle, T. J. Thompson, E. W. Gregg, and D. F. Williamson, "Effect of BMI on Lifetime Risk for Diabetes in the U.S.," *Diabetes Care,* vol. 30, no. 6, pp. 1562-1566, 2007-06-01 2007, doi: 10.2337/dc06-2544.

[30] "Reduction in the Incidence of Type 2 Diabetes with Lifestyle Intervention or Metformin," *New England Journal of Medicine,* vol. 346, no. 6, pp. 393-403, 2002-02-07 2002, doi: 10.1056/nejmoa012512.

[31] L. F. Van Gaal, I. L. Mertens, and C. E. De Block, "Mechanisms linking obesity with cardiovascular disease," *Nature,* vol. 444, no. 7121, pp. 875-880, 2006-12-01 2006, doi: 10.1038/nature05487.

[32] T. Y. Li *et al.*, "Obesity as Compared With Physical Activity in Predicting Risk of Coronary Heart Disease in Women," *Circulation,* vol. 113, no. 4, pp. 499-506, 2006-01-31 2006, doi: 10.1161/circulationaha.105.574087.

[33] L. Akil and H. A. Ahmad, "Relationships between Obesity and Cardiovascular Diseases in Four Southern States and Colorado," *Journal of Health Care for the Poor and Underserved,* vol. 22, no. 4A, pp. 61-72, 2011-01-01 2011, doi: 10.1353/hpu.2011.0166.

[34] J. E. Manson *et al.*, "Body Weight and Mortality among Women," *New England Journal of Medicine,* vol. 333, no. 11, pp. 677-685, 1995-09-14 1995, doi: 10.1056/nejm199509143331101.

[35] J. Stevens, "Fitness and Fatness as Predictors of Mortality from All Causes and from Cardiovascular Disease in Men and Women in the Lipid Research Clinics Study," *American Journal of Epidemiology,* vol. 156, no. 9, pp. 832-841, 2002-11-01 2002, doi: 10.1093/aje/kwf114.

[36] F. B. Hu, W. C. Willett, T. Li, M. J. Stampfer, G. A. Colditz, and J. E. Manson, "Adiposity as Compared with Physical Activity in Predicting Mortality among Women," *New England Journal of Medicine,* vol. 351, no. 26, pp. 2694-2703, 2004-12-23 2004, doi: 10.1056/nejmoa042135.

[37] A. Chawla, "Control of Macrophage Activation and Function by PPARs," *Circulation Research,* vol. 106, no. 10, pp. 1559-1569, 2010-05-28 2010, doi: 10.1161/circresaha.110.216523.

[38] I. Hameed, S. R. Masoodi, S. A. Mir, M. Nabi, K. Ghazanfar, and B. A. Ganai, "Type 2 diabetes mellitus: From a metabolic disorder to an inflammatory condition," *World Journal of Diabetes,* vol. 6, no. 4, p. 598, 2015-01-01 2015, doi: 10.4239/wjd.v6.i4.598.

[39] K. T. Uysal, S. M. Wiesbrock, and G. K. S. Hotamisligil, "Functional Analysis of Tumor Necrosis Factor (TNF) Receptors in TNF-α-Mediated Insulin Resistance in Genetic Obesity**This work is supported in part by a grant from the NIH (DK-52539)." *Endocrinology,* vol. 139, no. 12, pp. 4832-4838, 1998-12-01 1998, doi: 10.1210/endo.139.12.6337.

[40] C. N. Lumeng, S. M. Deyoung, J. L. Bodzin, and A. R. Saltiel, "Increased Inflammatory Properties of Adipose Tissue Macrophages Recruited During Diet-Induced Obesity," *Diabetes,* vol. 56, no. 1, pp. 16-23, 2007-01-01 2007, doi: 10.2337/db06-1076.

[41] S. Boulenouar *et al.*, "Adipose Type One Innate Lymphoid Cells Regulate Macrophage Homeostasis through Targeted Cytotoxicity," *Immunity,* vol. 46, no. 2, pp. 273-286, 2017-02-01 2017, doi: 10.1016/j.immuni.2017.01.008.

[42] H. Uemura *et al.*, "Relationships of serum high-sensitivity C-reactive protein and body size with insulin resistance in a Japanese cohort," *PLOS ONE,* vol. 12, no. 6, p. e0178672, 2017-06-02 2017, doi: 10.1371/journal.pone.0178672.

[43] C. Phosat *et al.*, "Elevated C-reactive protein, interleukin 6, tumor necrosis factor alpha and glycemic load associated with type 2 diabetes mellitus in rural Thais: a cross-sectional study," *BMC Endocrine Disorders,* vol. 17, no. 1, 2017-12-01 2017, doi: 10.1186/s12902-017-0189-z.

[44] M. Blüher, "Adipose tissue inflammation: a cause or consequence of obesity-related insulin resistance?," (in eng), *Clin Sci (Lond),* vol. 130, no. 18, pp. 1603-14, 09 01 2016, doi: 10.1042/CS20160005.

[45] S. Daemen and J. D. Schilling, "The Interplay Between Tissue Niche and Macrophage Cellular Metabolism in Obesity," (in eng), *Front Immunol,* vol. 10, p. 3133, 2019, doi: 10.3389/fimmu.2019.03133.

[46] B. Lauby-Secretan, C. Scoccianti, D. Loomis, Y. Grosse, F. Bianchini, and K. Straif, "Body Fatness and Cancer — Viewpoint of the IARC Working Group," *New England Journal of Medicine,* vol. 375, no. 8, pp. 794-798, 2016-08-25 2016, doi: 10.1056/nejmsr1606602.

[47] A. G. Renehan, M. Tyson, M. Egger, R. F. Heller, and M. Zwahlen, "Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies," *The Lancet,* vol. 371, no. 9612, pp. 569-578, 2008-02-01 2008, doi: 10.1016/s0140-6736(08)60269-x.

[48] "Handbook of cancer prevention," in *Weight control and physical activity* vol. 6, ed. Lyon, France: International agency for research on cancer, 2002, pp. 1-315.

[49] E. E. Calle, C. Rodriguez, K. Walker-Thurmond, and M. J. Thun, "Overweight, Obesity, and Mortality from Cancer in a Prospectively Studied Cohort of U.S. Adults," *New England Journal of Medicine,* vol. 348, no. 17, pp. 1625-1638, 2003-04-24 2003, doi: 10.1056/nejmoa021423.

[50] L.-L. Hwang *et al.*, "Sex Differences in High-fat Diet-induced Obesity, Metabolic Alterations and Learning, and Synaptic Plasticity Deficits in Mice," *Obesity,* vol. 18, no. 3, pp. 463-469, 2010-03-01 2010, doi: 10.1038/oby.2009.273.

[51] M. E. E. Jones *et al.*, "Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity," *Proceedings of the National Academy of Sciences,* vol. 97, no. 23, pp. 12735-12740, 2000-11-07 2000, doi: 10.1073/pnas.97.23.12735.

[52] M. E. E. Jones *et al.*, "Aromatase-deficient (ArKO) mice accumulate excess adipose tissue," *The Journal of Steroid Biochemistry and Molecular Biology,* vol. 79, no. 1-5, pp. 3-9, 2001-12-01 2001, doi: 10.1016/s0960-0760(01)00136-4.

[53] M. C. Carr, "The Emergence of the Metabolic Syndrome with Menopause," *The Journal of Clinical Endocrinology & Metabolism,* vol. 88, no. 6, pp. 2404-2411, 2003-06-01 2003, doi: 10.1210/jc.2003-030242.
[54] S. V. Koebele and H. A. Bimonte-Nelson, "Modeling menopause: The utility of rodents in translational behavioral endocrinology research," *Maturitas,* vol. 87, pp. 5-17, 2016-05-01 2016, doi: 10.1016/j.maturitas.2016.01.015.
[55] Z. A. Al-Safi and N. Santoro, "Menopausal hormone therapy and menopausal symptoms," *Fertility and Sterility,* vol. 101, no. 4, pp. 905-915, 2014-04-01 2014, doi: 10.1016/j.fertnstert.2014.02.032.
[56] N. Harada, T. Utsumi, and Y. Takagi, "Tissue-specific expression of the human aromatase cytochrome P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis," *Proceedings of the National Academy of Sciences,* vol. 90, no. 23, pp. 11312-11316, 1993-12-01 1993, doi: 10.1073/pnas.90.23.11312.
[57] J. Blakemore and F. Naftolin, "Aromatase: Contributions to Physiology and Disease in Women and Men," *Physiology,* vol. 31, no. 4, pp. 258-269, 2016-07-01 2016, doi: 10.1152/physiol.00054.2015.
[58] R. Shi *et al.*, "Expression of aromatase and synthesis of sex steroid hormones in skeletal muscle following exercise training in ovariectomized rats," *Steroids,* vol. 143, pp. 91-96, 2019-03-01 2019, doi: 10.1016/j.steroids.2019.01.003.
[59] R. Monteiro, D. Teixeira, and C. Calhau, "Estrogen Signaling in Metabolic Inflammation," *Mediators of Inflammation,* vol. 2014, pp. 1-20, 2014-01-01 2014, doi: 10.1155/2014/615917.
[60] R. Barakat, O. Oakley, H. Kim, J. Jin, and C. J. Ko, "Extra-gonadal sites of estrogen biosynthesis and function," (in eng), *BMB Rep,* vol. 49, no. 9, pp. 488-96, Sep 2016, doi: 10.5483/bmbrep.2016.49.9.141.
[61] M. H. Faulds, C. Zhao, K. Dahlman-Wright, and J. Gustafsson, "The diversity of sex steroid action: regulation of metabolism by estrogen signaling," (in eng), *J Endocrinol,* vol. 212, no. 1, pp. 3-12, Jan 2012, doi: 10.1530/JOE-11-0044.
[62] N. Hetemäki *et al.*, "Adipose tissue estrogen production and metabolism in premenopausal women," *The Journal of Steroid Biochemistry and Molecular Biology,* vol. 209, p. 105849, 2021-05-01 2021, doi: 10.1016/j.jsbmb.2021.105849.
[63] E. Hobeika, M. Armouti, H. Sudheer Kala, and C. Stocco, *Hormonal Signaling in Biology and Medicine*. Academic Press, 2020.
[64] F. Mauvais-Jarvis, D. J. Clegg, and A. L. Hevener, "The Role of Estrogens in Control of Energy Balance and Glucose Homeostasis," *Endocrine Reviews,* vol. 34, no. 3, pp. 309-338, 2013-06-01 2013, doi: 10.1210/er.2012-1055.
[65] E. R. Simpson *et al.*, "Estrogen—the Good, the Bad, and the Unexpected," *Endocrine Reviews,* vol. 26, no. 3, pp. 322-330, 2005-05-01 2005, doi: 10.1210/er.2004-0020.
[66] A. L. Hevener, V. Ribas, T. M. Moore, and Z. Zhou, "The Impact of Skeletal Muscle ERα on Mitochondrial Function and Metabolic Health," *Endocrinology,* vol. 161, no. 2, 2020-02-01 2020, doi: 10.1210/endocr/bqz017.

[67] Y. Shang, X. Hu, J. Direnzo, M. A. Lazar, and M. Brown, "Cofactor Dynamics and Sufficiency in Estrogen Receptor–Regulated Transcription," *Cell,* vol. 103, no. 6, pp. 843-852, 2000-12-01 2000, doi: 10.1016/s0092-8674(00)00188-4.
[68] S. R. Hammes and E. R. Levin, "Extranuclear Steroid Receptors: Nature and Actions," *Endocrine Reviews,* vol. 28, no. 7, pp. 726-741, 2007-12-01 2007, doi: 10.1210/er.2007-0022.
[69] E. R. Levin, "Extranuclear Steroid Receptors Are Essential for Steroid Hormone Actions," *Annual Review of Medicine,* vol. 66, no. 1, pp. 271-280, 2015-01-14 2015, doi: 10.1146/annurev-med-050913-021703.
[70] R. P. Barros, U. F. Machado, and J. A. Gustafsson, "Estrogen receptors: new players in diabetes mellitus," (in eng), *Trends Mol Med,* vol. 12, no. 9, pp. 425-31, Sep 2006, doi: 10.1016/j.molmed.2006.07.004.
[71] A. M. Case and R. L. Reid, "Menstrual cycle effects on common medical conditions," (in eng), *Compr Ther,* vol. 27, no. 1, pp. 65-71, 2001, doi: 10.1007/s12019-001-0010-8.
[72] C. G. Solomon, "Long or Highly Irregular Menstrual Cycles as a Marker for Risk of Type 2 Diabetes Mellitus," *JAMA,* vol. 286, no. 19, p. 2421, 2001-11-21 2001, doi: 10.1001/jama.286.19.2421.
[73] A. Dunaif, K. R. Segal, W. Futterweit, and A. Dobrjansky, "Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome," (in eng), *Diabetes,* vol. 38, no. 9, pp. 1165-74, Sep 1989, doi: 10.2337/diab.38.9.1165.
[74] P. Calmels, L. Vico, C. Alexandre, and P. Minaire, "Cross-sectional study of muscle strength and bone mineral density in a population of 106 women between the ages of 44 and 87 years: relationship with age and menopause," (in eng), *Eur J Appl Physiol Occup Physiol,* vol. 70, no. 2, pp. 180-6, 1995, doi: 10.1007/BF00361547.
[75] J. Viña, J. Sastre, F. V. Pallardó, J. Gambini, and C. Borrás, "Role of mitochondrial oxidative stress to explain the different longevity between genders. Protective effect of estrogens," *Free Radical Research,* vol. 40, no. 12, pp. 1359-1365, 2006-01-01 2006, doi: 10.1080/10715760600952851.
[76] W. J. Wallen, M. P. Belanger, and C. Wittnich, "Sex Hormones and the Selective Estrogen Receptor Modulator Tamoxifen Modulate Weekly Body Weights and Food Intakes in Adolescent and Adult Rats," *The Journal of Nutrition,* vol. 131, no. 9, pp. 2351-2357, 2001-09-01 2001, doi: 10.1093/jn/131.9.2351.
[77] Y. Kamei *et al.*, "Ovariectomy in Mice Decreases Lipid Metabolism-Related Gene Expression in Adipose Tissue and Skeletal Muscle with Increased Body Fat," *Journal of Nutritional Science and Vitaminology,* vol. 51, no. 2, pp. 110-117, 2005-01-01 2005, doi: 10.3177/jnsv.51.110.
[78] V. Saengsirisuwan, S. Pongseeda, M. Prasannarong, K. Vichaiwong, and C. Toskulkao, "Modulation of insulin resistance in ovariectomized rats by endurance exercise training and estrogen replacement," (in eng), *Metabolism,* vol. 58, no. 1, pp. 38-47, Jan 2009, doi: 10.1016/j.metabol.2008.08.004.
[79] M. I. Bader, J. Wober, G. Kretzschmar, O. Zierau, and G. Vollmer, "Comparative assessment of estrogenic responses with relevance to the metabolic syndrome and

to menopausal symptoms in wild-type and aromatase-knockout mice," (in eng), *J Steroid Biochem Mol Biol,* vol. 127, no. 3-5, pp. 428-34, Nov 2011, doi: 10.1016/j.jsbmb.2011.05.004.

[80] S. Ben-Shmuel *et al.*, "Ovariectomy is associated with metabolic impairments and enhanced mammary tumor growth in MKR mice," *Journal of Endocrinology,* vol. 227, no. 3, pp. 143-151, 2015-12-01 2015, doi: 10.1530/joe-15-0310.

[81] D. J. Hamilton *et al.*, "Estrogen receptor alpha activation enhances mitochondrial function and systemic metabolism in high-fat-fed ovariectomized mice," (in eng), *Physiol Rep,* vol. 4, no. 17, 09 2016, doi: 10.14814/phy2.12913.

[82] C. R. Fisher, K. H. Graves, A. F. Parlow, and E. R. Simpson, "Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene," (in eng), *Proc Natl Acad Sci U S A,* vol. 95, no. 12, pp. 6965-70, Jun 09 1998, doi: 10.1073/pnas.95.12.6965.

[83] K. N. Hewitt, W. C. Boon, Y. Murata, M. E. E. Jones, and E. R. Simpson, "The Aromatase Knockout Mouse Presents with a Sexually Dimorphic Disruption to Cholesterol Homeostasis," *Endocrinology,* vol. 144, no. 9, pp. 3895-3903, 2003-09-01 2003, doi: 10.1210/en.2003-0244.

[84] V. R. Walker and K. S. Korach, "Estrogen Receptor Knockout Mice as a Model for Endocrine Research," *ILAR Journal,* vol. 45, no. 4, pp. 455-461, 2004-01-01 2004, doi: 10.1093/ilar.45.4.455.

[85] A. B. Ropero, P. Alonso-Magdalena, I. Quesada, and A. Nadal, "The role of estrogen receptors in the control of energy and glucose homeostasis," (in eng), *Steroids,* vol. 73, no. 9-10, pp. 874-9, Oct 2008, doi: 10.1016/j.steroids.2007.12.018.

[86] J. Hong, R. E. Stubbins, R. R. Smith, A. E. Harvey, and N. P. Núñez, "Differential susceptibility to obesity between male, female and ovariectomized female mice," *Nutrition Journal,* vol. 8, no. 1, p. 11, 2009-12-01 2009, doi: 10.1186/1475-2891-8-11.

[87] A. Hevener, D. Reichart, A. Janez, and J. Olefsky, "Female Rats Do Not Exhibit Free Fatty Acid-Induced Insulin Resistance," *Diabetes,* vol. 51, no. 6, pp. 1907-1912, 2002-06-01 2002, doi: 10.2337/diabetes.51.6.1907.

[88] J. P. Frias, G. B. Macaraeg, J. Ofrecio, J. G. Yu, J. M. Olefsky, and Y. T. Kruszynska, "Decreased susceptibility to fatty acid-induced peripheral tissue insulin resistance in women," (in eng), *Diabetes,* vol. 50, no. 6, pp. 1344-50, Jun 2001, doi: 10.2337/diabetes.50.6.1344.

[89] C. K. Sites *et al.*, "Menopause-related differences in inflammation markers and their relationship to body fat distribution and insulin-stimulated glucose disposal," *Fertility and Sterility,* vol. 77, no. 1, pp. 128-135, 2002-01-01 2002, doi: 10.1016/s0015-0282(01)02934-x.

[90] J. Pfeilschifter, R. KöDitz, M. Pfohl, and H. Schatz, "Changes in Proinflammatory Cytokine Activity after Menopause," *Endocrine Reviews,* vol. 23, no. 1, pp. 90-119, 2002-02-01 2002, doi: 10.1210/edrv.23.1.0456.

[91] K. Timper and J. C. Brüning, "Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity," *Disease Models & Mechanisms,* vol. 10, no. 6, pp. 679-689, 2017-06-01 2017, doi: 10.1242/dmm.026609.

[92] S. Farooqi and S. O'Rahilly, "Genetics of obesity in humans," (in eng), *Endocr Rev,* vol. 27, no. 7, pp. 710-18, Dec 2006, doi: 10.1210/er.2006-0040.
[93] F. Lizcano and G. Guzmán, "Estrogen Deficiency and the Origin of Obesity during Menopause," *BioMed Research International,* vol. 2014, pp. 1-11, 2014-01-01 2014, doi: 10.1155/2014/757461.
[94] D. J. Clegg, L. M. Brown, S. C. Woods, and S. C. Benoit, "Gonadal Hormones Determine Sensitivity to Central Leptin and Insulin," *Diabetes,* vol. 55, no. 4, pp. 978-987, 2006-04-01 2006, doi: 10.2337/diabetes.55.04.06.db05-1339.
[95] P. A. Barros, Rodrigo and J.-Å. Gustafsson, "Estrogen Receptors and the Metabolic Network," *Cell Metabolism,* vol. 14, no. 3, pp. 289-299, 2011-09-01 2011, doi: 10.1016/j.cmet.2011.08.005.
[96] P. A. Heine, J. A. Taylor, G. A. Iwamoto, D. B. Lubahn, and P. S. Cooke, "Increased adipose tissue in male and female estrogen receptor-alpha knockout mice," *Proceedings of the National Academy of Sciences,* vol. 97, no. 23, pp. 12729-12734, 2000-11-07 2000, doi: 10.1073/pnas.97.23.12729.
[97] L. Dennerstein, P. Lehert, J. R. Guthrie, and H. G. Burger, "Modeling women's health during the menopausal transition: a longitudinal analysis," (in eng), *Menopause,* vol. 14, no. 1, pp. 53-62, 2007 Jan-Feb 2007, doi: 10.1097/01.gme.0000229574.67376.ba.
[98] Z. A. Al-Safi and A. J. Polotsky, "Obesity and Menopause," *Best Practice & Research Clinical Obstetrics & Gynaecology,* vol. 29, no. 4, pp. 548-553, 2015-05-01 2015, doi: 10.1016/j.bpobgyn.2014.12.002.
[99] C. Bouchard, "Genetic influences on body composition and regional fat distribution," (in eng), *Bol Asoc Med P R,* vol. 83, no. 8, pp. 354-6, Aug 1991.
[100] C. Bouchard, J. P. Després, and P. Mauriège, "Genetic and nongenetic determinants of regional fat distribution," (in eng), *Endocr Rev,* vol. 14, no. 1, pp. 72-93, Feb 1993, doi: 10.1210/edrv-14-1-72.
[101] L. M. Wohlers and E. E. Spangenburg, "17β-estradiol supplementation attenuates ovariectomy-induced increases in ATGL signaling and reduced perilipin expression in visceral adipose tissue," *Journal of Cellular Biochemistry,* pp. n/a-n/a, 2010-01-01 2010, doi: 10.1002/jcb.22553.
[102] S. K. Phillips, K. M. Rook, N. C. Siddle, S. A. Bruce, and R. C. Woledge, "Muscle weakness in women occurs at an earlier age than in men, but strength is preserved by hormone replacement therapy," (in eng), *Clin Sci (Lond),* vol. 84, no. 1, pp. 95-8, Jan 1993, doi: 10.1042/cs0840095.
[103] S. M. Greising, K. A. Baltgalvis, D. A. Lowe, and G. L. Warren, "Hormone Therapy and Skeletal Muscle Strength: A Meta-Analysis," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* vol. 64A, no. 10, pp. 1071-1081, 2009-10-01 2009, doi: 10.1093/gerona/glp082.
[104] L. D. Høeg *et al.*, "Lipid-Induced Insulin Resistance Affects Women Less Than Men and Is Not Accompanied by Inflammation or Impaired Proximal Insulin Signaling," *Diabetes,* vol. 60, no. 1, pp. 64-73, 2011-01-01 2011, doi: 10.2337/db10-0698.
[105] V. Ribas *et al.*, "Impaired oxidative metabolism and inflammation are associated with insulin resistance in ERalpha-deficient mice," (in eng), *Am J Physiol*

Endocrinol Metab, vol. 298, no. 2, pp. E304-19, Feb 2010, doi: 10.1152/ajpendo.00504.2009.

[106] V. Ribas *et al.*, "Skeletal muscle action of estrogen receptor α is critical for the maintenance of mitochondrial function and metabolic homeostasis in females," (in eng), *Sci Transl Med,* vol. 8, no. 334, p. 334ra54, Apr 13 2016, doi: 10.1126/scitranslmed.aad3815.

[107] R. P. D. A. Barros, A. Morani, A. Moriscot, and U. F. Machado, "Insulin resistance of pregnancy involves estrogen-induced repression of muscle GLUT4," *Molecular and Cellular Endocrinology,* vol. 295, no. 1-2, pp. 24-31, 2008-11-01 2008, doi: 10.1016/j.mce.2008.07.008.

[108] P. Garrido, J. Morán, A. Alonso, S. González, and C. González, "17β-Estradiol Activates Glucose Uptake via GLUT4 Translocation and PI3K/Akt Signaling Pathway in MCF-7 Cells," *Endocrinology,* vol. 154, no. 6, pp. 1979-1989, 2013-06-01 2013, doi: 10.1210/en.2012-1558.

[109] R. P. A. Barros, U. F. Machado, M. Warner, and J.-A. Gustafsson, "Muscle GLUT4 regulation by estrogen receptors ERbeta and ER," *Proceedings of the National Academy of Sciences,* vol. 103, no. 5, pp. 1605-1608, 2006-01-31 2006, doi: 10.1073/pnas.0510391103.

[110] G. Kolovou, D. Damaskos, K. Anagnostopoulou, and D. V. Cokkinos, "Apolipoprotein E gene polymorphism and gender," (in eng), *Ann Clin Lab Sci,* vol. 39, no. 2, pp. 120-33, 2009.

[111] K. Toda *et al.*, "Alternations in hepatic expression of fatty-acid metabolizing enzymes in ArKO mice and their reversal by the treatment with 17β-estradiol or a peroxisome proliferator," *The Journal of Steroid Biochemistry and Molecular Biology,* vol. 79, no. 1-5, pp. 11-17, 2001-12-01 2001, doi: 10.1016/s0960-0760(01)00135-2.

[112] G. Bryzgalova *et al.*, "Evidence that oestrogen receptor-α plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver," *Diabetologia,* vol. 49, no. 3, pp. 588-597, 2006-03-01 2006, doi: 10.1007/s00125-005-0105-3.

[113] D. B. Lubahn, J. S. Moyer, T. S. Golding, J. F. Couse, K. S. Korach, and O. Smithies, "Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene," *Proceedings of the National Academy of Sciences,* vol. 90, no. 23, pp. 11162-11166, 1993-12-01 1993, doi: 10.1073/pnas.90.23.11162.

[114] A. L. Hevener, Z. Zhou, T. M. Moore, B. G. Drew, and V. Ribas, "The impact of ERα action on muscle metabolism and insulin sensitivity – Strong enough for a man, made for a woman," *Molecular Metabolism,* vol. 15, pp. 20-34, 2018-09-01 2018, doi: 10.1016/j.molmet.2018.06.013.

[115] Z. Zhou *et al.*, "Estrogen receptor α controls metabolism in white and brown adipocytes by regulating," (in eng), *Sci Transl Med,* vol. 12, no. 555, 08 05 2020, doi: 10.1126/scitranslmed.aax8096.

[116] M. E. Olson and J. Bruce, "Ovariectomy, ovariohysterectomy and orchidectomy in rodents and rabbits," (in eng), *Can Vet J,* vol. 27, no. 12, pp. 523-7, Dec 1986.

[117] V. R. Souza, E. Mendes, M. Casaro, A. T. F. B. Antiorio, F. A. Oliveira, and C. M. Ferreira, "Description of Ovariectomy Protocol in Mice," (in eng), *Methods Mol Biol,* vol. 1916, pp. 303-309, 2019, doi: 10.1007/978-1-4939-8994-2_29.
[118] J. P. G. Camporez *et al.*, "Cellular Mechanism by Which Estradiol Protects Female Ovariectomized Mice From High-Fat Diet-Induced Hepatic and Muscle Insulin Resistance," *Endocrinology,* vol. 154, no. 3, pp. 1021-1028, 2013-03-01 2013, doi: 10.1210/en.2012-1989.
[119] M. L. Misso, Y. Murata, W. C. Boon, M. E. E. Jones, K. L. Britt, and E. R. Simpson, "Cellular and Molecular Characterization of the Adipose Phenotype of the Aromatase-Deficient Mouse," *Endocrinology,* vol. 144, no. 4, pp. 1474-1480, 2003-04-01 2003, doi: 10.1210/en.2002-221123.
[120] M. L. Misso, K. N. Hewitt, W. C. Boon, Y. Murata, M. E. Jones, and E. R. Simpson, "Cholesterol feeding prevents adiposity in the obese female aromatase knockout (ArKO) mouse," (in eng), *Horm Metab Res,* vol. 37, no. 1, pp. 26-31, Jan 2005, doi: 10.1055/s-2005-861028.
[121] K. L. Britt, E. R. Simpson, and J. K. Findlay, "Effects of phytoestrogens on the ovarian and pituitary phenotypes of estrogen-deficient female aromatase knockout mice," (in eng), *Menopause,* vol. 12, no. 2, pp. 174-85, Mar 2005, doi: 10.1097/00042192-200512020-00012.
[122] M. R. Iñigo *et al.*, "Estrogen receptor-α in female skeletal muscle is not required for regulation of muscle insulin sensitivity and mitochondrial regulation," *Molecular Metabolism,* vol. 34, pp. 1-15, 2020-04-01 2020, doi: 10.1016/j.molmet.2019.12.010.
[123] R. Ye *et al.*, "Impact of tamoxifen on adipocyte lineage tracing: Inducer of adipogenesis and prolonged nuclear translocation of Cre recombinase," *Molecular Metabolism,* vol. 4, no. 11, pp. 771-778, 2015-11-01 2015, doi: 10.1016/j.molmet.2015.08.004.
[124] M. E. E. Jones, K. J. Mcinnes, W. C. Boon, and E. R. Simpson, "Estrogen and adiposity—Utilizing models of aromatase deficiency to explore the relationship," *The Journal of Steroid Biochemistry and Molecular Biology,* vol. 106, no. 1-5, pp. 3-7, 2007-08-01 2007, doi: 10.1016/j.jsbmb.2007.05.029.
[125] A. K. Aladhami *et al.*, "Macrophage tumor necrosis factor-alpha deletion does not protect against obesity-associated metabolic dysfunction," *The FASEB Journal,* vol. 35, no. 7, 2021-07-01 2021, doi: 10.1096/fj.202100543rr.
[126] R. T. Enos *et al.*, "Influence of dietary saturated fat content on adiposity, macrophage behavior, inflammation, and metabolism: composition matters," *Journal of Lipid Research,* vol. 54, no. 1, pp. 152-163, 2013-01-01 2013, doi: 10.1194/jlr.m030700.
[127] R. T. Enos, K. T. Velázquez, and E. A. Murphy, "Insight into the impact of dietary saturated fat on tissue-specific cellular processes underlying obesity-related diseases," *The Journal of Nutritional Biochemistry,* vol. 25, no. 6, pp. 600-612, 2014-06-01 2014, doi: 10.1016/j.jnutbio.2014.01.011.
[128] R. T. Enos, K. T. Velázquez, J. L. Mcclellan, T. L. Cranford, M. D. Walla, and E. A. Murphy, "Lowering the dietary omega-6: omega-3 does not hinder nonalcoholic fatty-liver disease development in a murine model," *Nutrition*

Research, vol. 35, no. 5, pp. 449-459, 2015-05-01 2015, doi: 10.1016/j.nutres.2015.04.003.

[129] R. T. Enos *et al.*, "A Low Dose of Dietary Quercetin Fails to Protect against the Development of an Obese Phenotype in Mice," *PLOS ONE,* vol. 11, no. 12, p. e0167979, 2016-12-13 2016, doi: 10.1371/journal.pone.0167979.

[130] R. T. Enos *et al.*, "High-fat diets rich in saturated fat protect against azoxymethane/dextran sulfate sodium-induced colon cancer," (in eng), *Am J Physiol Gastrointest Liver Physiol,* vol. 310, no. 11, pp. G906-19, 06 01 2016, doi: 10.1152/ajpgi.00345.2015.

[131] K. T. Velázquez *et al.*, "miR155 deficiency aggravates high-fat diet-induced adipose tissue fibrosis in male mice," *Physiological Reports,* vol. 5, no. 18, p. e13412, 2017-09-01 2017, doi: 10.14814/phy2.13412.

[132] E. M. Morris *et al.*, "Difference in Housing Temperature-Induced Energy Expenditure Elicits Sex-Specific Diet-Induced Metabolic Adaptations in Mice," *Obesity,* vol. 28, no. 10, pp. 1922-1931, 2020-10-01 2020, doi: 10.1002/oby.22925.

[133] A. I. Mina, R. A. LeClair, K. B. LeClair, D. E. Cohen, L. Lantier, and A. S. Banks, "CalR: A Web-Based Analysis Tool for Indirect Calorimetry Experiments," (in eng), *Cell Metab,* vol. 28, no. 4, pp. 656-666.e1, 10 02 2018, doi: 10.1016/j.cmet.2018.06.019.

[134] J. Vandesompele *et al.*, "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes," (in eng), *Genome Biol,* vol. 3, no. 7, p. RESEARCH0034, Jun 18 2002, doi: 10.1186/gb-2002-3-7-research0034.

[135] M. Van Sinderen *et al.*, "Sexual dimorphism in the glucose homeostasis phenotype of the Aromatase Knockout (ArKO) mice," *The Journal of Steroid Biochemistry and Molecular Biology,* vol. 170, pp. 39-48, 2017-06-01 2017, doi: 10.1016/j.jsbmb.2016.05.013.

[136] A. Amano *et al.*, "Abnormal lipid/lipoprotein metabolism and high plasma testosterone levels in male but not female aromatase-knockout mice," *Archives of Biochemistry and Biophysics,* vol. 622, pp. 47-58, 2017-05-01 2017, doi: 10.1016/j.abb.2017.03.007.

[137] M. L. Van Sinderen *et al.*, "Effects of Estrogens on Adipokines and Glucose Homeostasis in Female Aromatase Knockout Mice," *PLOS ONE,* vol. 10, no. 8, p. e0136143, 2015-08-28 2015, doi: 10.1371/journal.pone.0136143.

[138] J. E. Bader *et al.*, "Repeated clodronate-liposome treatment results in neutrophilia and is not effective in limiting obesity-linked metabolic impairments," (in eng), *Am J Physiol Endocrinol Metab,* vol. 316, no. 3, pp. E358-E372, 03 01 2019, doi: 10.1152/ajpendo.00438.2018.

[139] L. R. Nelson and S. E. Bulun, "Estrogen production and action," *Journal of the American Academy of Dermatology,* vol. 45, no. 3, pp. S116-S124, 2001-09-01 2001, doi: 10.1067/mjd.2001.117432.

[140] J. Cui, Y. Shen, and R. Li, "Estrogen synthesis and signaling pathways during aging: from periphery to brain," *Trends in Molecular Medicine,* vol. 19, no. 3, pp. 197-209, 2013-03-01 2013, doi: 10.1016/j.molmed.2012.12.007.

[141] K. Subbaramaiah *et al.*, "Obesity Is Associated with Inflammation and Elevated Aromatase Expression in the Mouse Mammary Gland," *Cancer Prevention Research,* vol. 4, no. 3, pp. 329-346, 2011-03-01 2011, doi: 10.1158/1940-6207.capr-10-0381.
[142] P. Bhardwaj *et al.*, "Estrogens and breast cancer: Mechanisms involved in obesity-related development, growth and progression," *The Journal of Steroid Biochemistry and Molecular Biology,* vol. 189, pp. 161-170, 2019-05-01 2019, doi: 10.1016/j.jsbmb.2019.03.002.
[143] K. M. Mair, R. Gaw, and M. R. Maclean, "Obesity, estrogens and adipose tissue dysfunction – implications for pulmonary arterial hypertension," *Pulmonary Circulation,* vol. 10, no. 3, p. 204589402095201, 2020-07-01 2020, doi: 10.1177/2045894020952023.
[144] "The 2012 Hormone Therapy Position Statement of The North American Menopause Society," *Menopause,* vol. 19, no. 3, pp. 257-271, 2012-03-01 2012, doi: 10.1097/gme.0b013e31824b970a.
[145] S. Gaillard and V. Stearns, "Aromatase inhibitor-associated bone and musculoskeletal effects: new evidence defining etiology and strategies for management," *Breast Cancer Research,* vol. 13, no. 2, p. 205, 2011-04-01 2011, doi: 10.1186/bcr2818.
[146] T. Hyder, C. C. Marino, S. Ahmad, A. Nasrazadani, and A. M. Brufsky, "Aromatase Inhibitor-Associated Musculoskeletal Syndrome: Understanding Mechanisms and Management," (in eng), *Front Endocrinol (Lausanne),* vol. 12, p. 713700, 2021, doi: 10.3389/fendo.2021.713700.
[147] M. Iwata *et al.*, "A novel tetracycline-responsive transgenic mouse strain for skeletal muscle-specific gene expression," *Skeletal Muscle,* vol. 8, no. 1, 2018-12-01 2018, doi: 10.1186/s13395-018-0181-y.
[148] E. S. Díaz-Cruz, Y. Sugimoto, G. I. Gallicano, R. W. Brueggemeier, and P. A. Furth, "Comparison of Increased Aromatase versus ERα in the Generation of Mammary Hyperplasia and Cancer," *Cancer Research,* vol. 71, no. 16, pp. 5477-5487, 2011-08-15 2011, doi: 10.1158/0008-5472.can-10-4652.
[149] S. Virtue and A. Vidal-Puig, "GTTs and ITTs in mice: simple tests, complex answers," (in eng), *Nat Metab,* vol. 3, no. 7, pp. 883-886, 07 2021, doi: 10.1038/s42255-021-00414-7.
[150] S. E. Bulun and E. R. Simpson, "Aromatase Expression in Women's Cancers," in *Advances in Experimental Medicine and Biology*: Springer New York, 2008, pp. 112-132.
[151] G. S. Chetrite, J. Cortes-Prieto, J. C. Philippe, F. Wright, and J. R. Pasqualini, "Comparison of estrogen concentrations, estrone sulfatase and aromatase activities in normal, and in cancerous, human breast tissues," *The Journal of Steroid Biochemistry and Molecular Biology,* vol. 72, no. 1-2, pp. 23-27, 2000-01-01 2000, doi: 10.1016/s0960-0760(00)00040-6.
[152] S. E. Bulun, D. Chen, I. Moy, D. C. Brooks, and H. Zhao, "Aromatase, breast cancer and obesity: a complex interaction," *Trends in Endocrinology & Metabolism,* vol. 23, no. 2, pp. 83-89, 2012-02-01 2012, doi: 10.1016/j.tem.2011.10.003.

[153] X. Wang, E. R. Simpson, and K. A. Brown, "Aromatase overexpression in dysfunctional adipose tissue links obesity to postmenopausal breast cancer," *The Journal of Steroid Biochemistry and Molecular Biology,* vol. 153, pp. 35-44, 2015-09-01 2015, doi: 10.1016/j.jsbmb.2015.07.008.
[154] Z. Peng, X. Li, S. Mäkelä, H. K. Väänänen, and M. Poutanen, "Skeletal Changes in Transgenic Male Mice Expressing Human Cytochrome P450 Aromatase," *Journal of Bone and Mineral Research,* vol. 19, no. 8, pp. 1320-1328, 2004-08-01 2004, doi: 10.1359/jbmr.040510.
[155] U. Mandava, N. Kirma, and R. R. Tekmal, "Aromatase overexpression transgenic mice model: cell type specific expression and use of letrozole to abrogate mammary hyperplasia without affecting normal physiology," *The Journal of Steroid Biochemistry and Molecular Biology,* vol. 79, no. 1-5, pp. 27-34, 2001-12-01 2001, doi: 10.1016/s0960-0760(01)00133-9.
[156] E. A. Musgrove and R. L. Sutherland, "Biological determinants of endocrine resistance in breast cancer," *Nature Reviews Cancer,* vol. 9, no. 9, pp. 631-643, 2009-09-01 2009, doi: 10.1038/nrc2713.
[157] M. Droog, K. Beelen, S. Linn, and W. Zwart, "Tamoxifen resistance: From bench to bedside," *European Journal of Pharmacology,* vol. 717, no. 1-3, pp. 47-57, 2013-10-01 2013, doi: 10.1016/j.ejphar.2012.11.071.
[158] H. Hua, H. Zhang, Q. Kong, and Y. Jiang, "Mechanisms for estrogen receptor expression in human cancer," *Experimental Hematology & Oncology,* vol. 7, no. 1, 2018-12-01 2018, doi: 10.1186/s40164-018-0116-7.
[159] L. Flanagan, K. V. Weelden, C. Ammerman, S. P. Ethier, and J. Welsh, "SUM-159PT cells: a novel estrogen independent human breast cancer model system," *Breast Cancer Research and Treatment,* vol. 58, no. 3, pp. 193-204, 1999-12-01 1999, doi: 10.1023/a:1006331716981.
[160] Y.-W. Zhang *et al.*, "Acquisition of estrogen independence induces TOB1-related mechanisms supporting breast cancer cell proliferation," *Oncogene,* vol. 35, no. 13, pp. 1643-1656, 2016-03-31 2016, doi: 10.1038/onc.2015.226.
[161] A.-H. Capietto *et al.*, "Novel ERα positive breast cancer model with estrogen independent growth in the bone microenvironment," *Oncotarget,* vol. 7, no. 31, pp. 49751-49764, 2016-08-02 2016, doi: 10.18632/oncotarget.10443.