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DEFICIENCY OF ADIPOSE ARYL HYDROCARBON RECEPTOR PROTECTS AGAINST
DIET-INDUCED METABOLIC DYSFUNCTION THROUGH SEXUALLY DIMORPHIC
MECHANISMS

by

Nazmul Haque

B.S, Pharmaceutical Sciences, 2015

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the
Doctor of Philosophy Degree

School of Medicine
in the Graduate School
Southern Illinois University Carbondale
May 2023

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DISSERTATION APPROVAL

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DIET-INDUCED METABOLIC DYSFUNCTION THROUGH SEXUALLY DIMORPHIC
MECHANISMS

by
Nazmul Haque

A Dissertation Submitted in Partial
Fulfillment of the Requirements
for the Degree of
Doctor of Philosophy
in the field of Pharmacology & Neuroscience

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March 16, 2023

AN ABSTRACT OF THE DISSERTATION OF

Nazmul Haque for the Doctor of Philosophy degree in Pharmacology & Neuroscience presented on March 16, 2023, at Southern Illinois University, School of Medicine, Springfield, Illinois.

TITLE: DEFICIENCY OF ADIPOSE ARYL HYDROCARBON RECEPTOR PROTECTS AGAINST DIET-INDUCED METABOLIC DYSFUNCTION THROUGH SEXUALLY DIMORPHIC MECHANISM.

Major professor: Dr. Shelley A. Tischkau

Calorie imbalance due to high energy intake vs. expenditure is considered a major cause of obesity. Molecular mechanisms underlying diet-induced obesity remain unclear due to the complexity of disease progression. The impact of environmental factors contributing to obesity also remains poorly understood. Activation of the xenobiotic sensor, aryl hydrocarbon receptor (AhR), by obesogens may contribute to diet-induced obesity. AhR has the potential to influence lipid metabolism, insulin resistance, and the development of diabetes. Activation of AhR increases the risk of metabolic dysfunction by impairing adipose tissue function. Thus, the hypothesis was that conditional AhR depletion specifically from mature adipose tissue (CadKO) would improve high fat diet (HFD) induced metabolic dysfunction.

Our study suggests CadKO protects mice from HFD (60% calories from fat) induced weight gain. However, the effects were more profound in females compared to males. CadKO female intake significantly less food/calorie and increase energy expenditure (EE) on HFD. No changes in calorie intake and EE were observed among the genotypes in males. Morphology of adipocytes by H&E reveals CadKO improves adipose tissue biology such that females can maintain a lean adipocyte phenotype. This assists in resisting HFD-induced serum leptin rise, as well as promotes maintaining leptin receptor (LepR) expression in the energy regulatory regions of the hypothalamus, suggesting an increased sensitivity to leptin in CadKO female.

Furthermore, estrogen receptor α (ER α), which has anti-obesity effects, was higher in CadKO

female adipose tissue and energy regulatory regions hinting that the sex differences observed may be mediated by differences in estrogen signaling.

Concurrently, exploration of other metabolic functions, such as lipid spillover, beiging of adipose tissue, adipogenesis, lipolysis, and glucose metabolism also demonstrated sexual asymmetry. Depletion of AhR from adipocytes provides female mice with beneficial metabolic parameters mediated by the PPAR-family, HSL, and Fgf21 pathways. Furthermore, the study also demonstrated improved adipose biology in CadKO females as also advantageous for systemic glucose homeostasis. Fasting glucose and glucose tolerance were significantly better in CadKO females under HFD compared to WT. However, contrary to our previous study on global AhR knockout (AhRKO), CadKO male were not protected from HFD-induced changes in systemic glucose tolerance and insulin sensitivity. Although, we did find that CadKO male mice were delayed in disease progression for both obesity and insulin resistance. In males, CadKO ameliorated proinflammatory adipocytokine secretion (such as TNF α , IL1 β , IL6) that facilitates less inflammatory macrophage infiltration into adipose depots.

Altogether, these results indicate AhR deficiency from adipocytes improves overall weight control and systemic glucose homeostasis when faced with HFD challenges in both sexes, but more profoundly in females. Exploration of metabolic functions of tissues demonstrates definite sexual dimorphism. Adipose-specific depletion facilitates the maintenance of a lean phenotype in females that is mediated by healthy adipose-hypothalamic crosstalk. Whereas in males, adipose-specific AhR depletion delays the development of obesity and insulin resistance, due to the maintenance of healthy crosstalk between adipose tissue and the peripheral immune cells.

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DEDICATION

This dissertation is wholeheartedly dedicated to my wonderful parents, my lovely wife, and my baby boy. I hope I made you guys proud with my work.

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TABLE OF CONTENTS

<u>CHAPTER</u>	<u>PAGE</u>
ABSTRACT.....	i
ACKNOWLEDGMENTS	iii
DEDICATION.....	iv
LIST OF TABLES.....	x
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS.....	xiv
CHAPTERS	
CHAPTER 1: BACKGROUND AND SIGNIFICANCE.....	1
1.1 Introduction to obesity and type 2 diabetes	1
1.2 Types of adipose tissue and its role in body energy homeostasis.....	2
1.2.1 Adipose tissue as a multifunctional organ	2
1.2.2 Types of adipose tissue	3
1.2.3 Adipose tissue regulation of systemic energy balance	5
1.2.4 Adipose tissue inflammation and insulin resistance.	9
1.3 Sexual dimorphism in regulation of energy homeostasis	13
1.3.1 Introduction.....	13
1.3.2 Evolutionary survival strategies affect sex-specific changes in adipose tissue function.	13
1.3.3 Sex-based adipose tissue distribution results in sexually dimorphic metabolic diseases.	16
1.3.4 Sex-specific energy regulation by the adipose-hypothalamic axis ..	19

1.3.5 Sexual dimorphism in adipose tissue-mediated inflammation	21
1.4 Aryl hydrocarbon receptor regulation of energy homeostasis.....	22
1.4.1 Xenobiotic stimulation of aryl hydrocarbon receptor signaling.	22
1.4.2 AhR and ER interactions.	26
1.4.3 AhR interactions in sexual and neuroendocrine development.....	29
1.4.4 AhR in adipose tissue pathway to regulate energy homeostasis and inflammation.	30
1.4.5 Metabolic function difference in various AhR depleted mice models.....	33
1.5 Significance.....	34
CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS	36
CHAPTER 3: METHODS AND MATERIALS	43
3.1 Animals and experimental timeline	43
3.2 Chemicals.....	44
3.3 Metabolic chamber.....	44
3.4 Activity behavior monitoring.....	45
3.5 Glucose tolerance test (GTT).....	45
3.6 Histological analysis and Immunohistochemistry	45
3.7 Immunofluorescence.....	46
3.8 Western blot	46
3.9 q-PCR.....	47
3.10 ELISA	47
3.11 Statistical analysis.....	47

CHAPTER 4: RESULTS.....	48
4.1 Specific Aim 1: Establish inducible adipose specific AhR knockout mouse models (CadKO) using Cre-loxp system and characterize them.....	48
4.1.1 Results Subaim 1.1: Construct an inducible knock-out mouse model using the Cre-loxp system that will delete AhR specifically from adipose tissue in a time-dependent manner.	48
4.1.2 Results Subaim 1.2: Evaluating the AhR excision event and functionality in the mouse model.....	51
4.1.3 Summary	53
4.2 Specific Aim 2: Phenotypic investigation of energy and glucose homeostasis in different AhR genotypes after subjecting mice to different diet regimen.....	53
4.2.1 Results Subaim 2.1: Compare weight after subjecting mice to Normal Chow or High Fat Diet.	55
4.2.2 Results Subaim 2.2: Compare calorie intake, metabolic rates, and locomotor activity among various AhR genotypes on high fat diet.	58
4.2.3 Result Subaim 2.3: Compare fasting blood glucose, glucose tolerance and serum insulin level to investigate glucose homeostasis.	62
4.2.4 Summary	65

4.3 Specific Aim 3: Examine molecular changes in adipose tissue in CadKO	
after HFD feeding.	65
4.3.1 Results Subaim 3.1: Compare changes in fat mass and examine	
morphology of WAT.....	66
4.3.2 Results Subaim 3.2: Examine molecular pathways of white	
visceral adipose depot.....	70
4.3.3 Results Subaim 3.3: Examine adipokine secretion by visceral	
adipose depot involving weight and glucose homeostasis.....	73
4.3.4 Results Subaim 3.4: Examine non-shivering thermogenesis in	
adipose depots.....	76
4.3.5 Summary	79
4.4 Specific Aim 4: Explore hepatic steatosis (NAFLD) to examine systemic	
effect CadKO after HFD feeding.....	79
4.4.1 Results Subaim 4.1: Examine weight and morphology of the	
liver tissue.....	81
4.4.2 Results Subaim 4.2: Examine different molecular pathways	
of the liver.	83
4.4.3 Summary	84
4.5 Specific Aim 5: Explore hypothalamic feeding centers to examine	
systemic effect in energy regulation.	85
4.5.1 Results Subaim 5.1: Examine leptin signaling in energy	
regulatory regions of hypothalamus.	86

4.5.2 Results Subaim 5.2: Examine estrogen signaling in energy regulatory regions of the hypothalamus.....	89
4.5.3 Summary	91
CHAPTER 5: DISCUSSION.....	93
5.1 Specific Aim 1: Establish inducible adipose specific AhR knockout mouse models (CadKO) using Cre-loxp system and characterize them.....	93
5.2 Specific Aim 2: Phenotypic investigation of energy and glucose homeostasis in different AhR genotypes after subjecting mice to different diet regimen.....	94
5.3 Specific Aim 3: Examine molecular changes in adipose tissue in CadKO after HFD feeding.	99
5.4 Specific Aim 4: Explore hepatic steatosis (NAFLD) to examine systemic effect CadKO after HFD feeding.....	107
5.5 Specific Aim 5: Examine energy regulation in the hypothalamic feeding centers.	110
CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS.....	115
6.1 Summary	115
6.2 Future Directions	116
REFERENCES	120
VITA.....	168

LIST OF TABLES

<u>TABLE</u>	<u>PAGE</u>
Table 1. Serum measurements from NCD or HFD fed mice.....	112
Table 2. List of Antibodies.	113
Table 3. List of PCR Primers.....	114

LIST OF FIGURES

<u>FIGURE</u>	<u>PAGE</u>
Fig 1. Comparison of the main characteristics of white, beige, and brown adipocytes.....	5
Fig 2. Leptin–melanocortin pathway associated with monogenic obesity through an influence on food intake and energy expenditure..	8
Fig 3. Effects of obesity on adipose tissue leukocyte populations, adipokine secretion, and chronic inflammation	12
Fig 4. Schematic overview of physiological sex differences in energy homeostasis regulation..	14
Fig 5. Schematic overview of pathological sex differences in energy homeostasis regulation....	16
Fig 6. Comparison of roles and function between visceral and subcutaneous adipose tissue.	19
Fig 7. AhR signaling mediated by xenobiotics.....	25
Fig 8. Non-genomic AhR signaling to attenuate ER pathway and to induce inflammation.....	28
Fig 9. AhR involvement in various adipocyte pathways to influence metabolic diseases..	32
Fig 10. Construct of inducible knock-out mouse model using Cre-Loxp system and experimental approach to investigate AhR functionality.....	49
Fig 11. Tomato expression with or without tamoxifen in different adipose depot.....	50
Fig 12. Specificity of <i>Cre recombinase</i> -mediated excision of AhRfx allele upon tamoxifen administration.	52
Fig 13. Schematic representation of experimental design to examine phenotypical comparison between different AhR genotypes and obtain molecular explanation for CadKO mice...	55
Fig 14. Global and adipose-specific AhR deletion protect mice from HFD induced weight gain in both the sexes.....	56
Fig 15. Tamoxifen dose dependent tomato expression and weight difference.....	57

Fig 17. Global and adipose-specific AhR deletion in females increased energy expenditure on HFD.....	60
Fig 18. Adipose-specific AhR deletion protects against decreased locomotor activity on HFD.	61
Fig 19. CadKO improves fasting glucose and systemic glucose tolerance in females on HFD...	63
Fig 20. Global and adipose specific AhR deficiency protects mice from HFD induced hyperinsulinemia.....	64
Fig 21. CadKO reduces adiposity in female mice.	67
Fig 22. CadKO reduces adipocytes size in female SAT but not in male.....	68
Fig 23. CadKO protects adipocyte size in females and HFD-induced presence of crown-like structure in male VAT.	69
Fig 24. CadKO reduces inflammation in male mice by reducing adipocytokine release from VAT.	71
Fig 25. CadKO improved various molecular pathways in VAT from females after HFD.	73
Fig 26. Adipokine secretion by WAT.....	75
Fig 27. CadKO has no effect on non-shivering thermogenesis in BAT.....	77
Fig 28. CadKO female may promote beigeing in white adipose tissue to increase non-shivering thermogenesis.....	78
Fig 29. Liver weight after 15 weeks of HFD.....	81
Fig 30. CadKO protects against HFD-induced hepatic steatosis.....	82
Fig 31. CadKO protects against HFD-induced hepatic steatosis in sexually dimorphic pathway.....	84
Fig 32. CadKO induces satiety response by promoting leptin signaling in female mice when faced with calorie dense diet.....	87

Fig 33. CadKO female resist lowering of leptin receptor expression in liver on HFD. 88

Fig 34. CadKO promotes estrogen signaling in female mice when faced with calorie dense
diet..... 90

Fig 35. Schematic illustration of sex differences in CadKO mice for protection against HFD
induced metabolic dysfunction. 92

LIST OF ABBREVIATIONS

ACC- Acetyl Coenzyme A Carboxylase

AgRP- Agouti Related Protein

AhR- Aryl Hydrocarbon Receptor

AhRKO- Aryl Hydrocarbon Receptor Knockout

AHRR- Aryl Hydrocarbon Receptor Repressor

AKT- Protein Kinase B

AR- Androgen Receptor

ARC- Arcuate Nucleus

ARNT- Aryl Hydrocarbon Receptor Nuclear Translocator

AVPV- Anteroventral Periventricular

BAT- Brown Adipose Tissue

BBB- Blood Brain Barrier

bHLH- basic Helix-Loop-Helix

BMI- Body Mass Index

BNF- Beta Naphthoflavone

CadKO- Conditional Adipose Knockout

CBP- cAMP response element Binding Protein

CCL2- Chemokine Ligand 2

CD- Cluster of Differentiation

CKO- Conditional Knockout

CLS- Crown Like Structure

CNS- Central Nervous System

Cyp- Cytochrome P450
E2- 17 β -Estradiol
EE- Energy Expenditure
EPA- Environmental Protection Agency
ER- Estrogen Receptor
ERE- Estrogen Response Element
ERK- Extracellular Signal
FFA- Free Fatty Acid
FGF21- Fibroblast Growth Factor 21
G6Pase- Glucose 6-phosphatase
GTT- Glucose Tolerance Test
HFD- High Fat Diet
HSL- Hormone Sensitive Lipase
iCKO- inducible Conditional Knockout
IFN- Interferon
IL-1 β - Interleukin-1 beta
IL-6- Interleukin-6
iWAT- inguinal White Adipose Tissue
JNK- c-Jun N-terminal Kinase
Kyn- Kynurenine
LepR- Leptin Receptor
M1- Macrophages 1
M2- Macrophages 2

MAPK- Mitogen-Activated Protein Kinase
MCP1- Monocyte Chemoattractant Protein 1
MCR- Melanocortin Receptor
MSC- Mesenchymal Stem Cell
MSH- Melanocyte Stimulating Hormone
NAFLD- Non-Alcoholic Fatty Liver Disease
NCD- Normal Chow Diet
NPY- Neuropeptide Y
PAS- PER-ARNT-SIM
PCB- Polychlorinated Biphenyls
PER- Period
PI3K- Phosphatidylinositol 3-kinase
POMC- Proopiomelanocortin
PPAR- Peroxisome Proliferator Activated
SAT- Subcutaneous Adipose Tissue
SIM- Single Minded
Srebp1c- Sterol regulatory element binding protein-1c
STAT3- Signal Transducer and Activator of Transcription 3
T2D- Type 2 Diabetes
TCDD- 2,3,7,8 -Tetrachlorodibenzo-p-dioxin
TH1- Type 1 T Helper
TLR4- Toll Like Receptor 4
TNF α - Tumor Necrosis Factor alpha

Trp- Tryptophan

UCP1- Uncoupling protein 1

VAT- Visceral Adipose tissue

VMH- Ventromedial Hypothalamic Nucleus

WAT- White Adipose Tissue

WHO- World Health Organization

WT- Wild Type

XRE- Xenobiotic Response Element

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 Introduction to obesity and type 2 diabetes

The global obesity contributes to millions of deaths each year. Once considered a problem only in developed countries, obesity has grown to epidemic proportions across the globe, particularly in urban settings. Moreover, the obesity problem is not restricted to adults; according to the World Health Organization (WHO), the prevalence of this disease has increased more than 4-fold (from 4% to 18%) globally among children and adolescents from 1975 to 2016.

It has been projected with high predictive accuracy based on current trends by 2030 the incidence of obesity will rise as high as 48.9% and severe obesity will rise as high as 24.2% in the United States. The same study also demonstrated that the demographic categories more likely to be affected by severe obesity will be women, low-income households, and non-hispanic blacks (Ward et al., 2019). Hence, understanding and fighting against obesity is of paramount importance. Obesity is defined as excess accumulation of fat, and is measured by a body mass index (BMI) of 30 or more, according to the WHO. Obesity is a major contributor to mortality as it presents a major risk for several other chronic diseases including type 2 diabetes (T2D), cardiovascular diseases, and cancer. Consumption of a calorie-dense diet and sedentary lifestyles lead to energy imbalances that promote the deposition of fat, which is considered to be a prime reason for obesity.

As obesity contributes majorly to T2D, it is not surprising that patterns of obesity and diabetes prevalence parallel each other across the globe. T2D is associated with significant mortality and morbidity and is a major health concern for the world. Diabetes is a chronic condition in which the body is unable to properly regulate blood sugar levels, leading to high

levels of glucose in the blood. The progression of T2D is characterized by two major phenomena that include insulin resistance and β -cell defect in the pancreas (Ramlo-Halsted & Edelman, 1999). Initially, an overabundance of glucose, from chronic ingestion of an energy-dense diet, leads to tissue insensitivity towards insulin action, which is defined as insulin resistance. The pancreas attempts to compensate by pumping out additional insulin to maintain physiologic blood sugar levels, resulting in hyperinsulinemia. Hence, in the initial stage of insulin resistance (known as pre-diabetic), the plasma glucose level is maintained in the normal range. Information about the pathogenesis and progression of T2D revealed this disease has a prolonged pre-diabetic stage (Ramlo-Halsted & Edelman, 1999). If the phenomenon continues, individuals will eventually develop T2D due to β -cell dysfunction. A better understanding of systemic energy regulation is key to combating these diseases and adipose tissue plays a central role in this regulation.

1.2 Types of adipose tissue and its role in body energy homeostasis

1.2.1 Adipose tissue as a multifunctional organ

Adipose tissue has a multifaceted role in our body, from energy storage to endocrine and immune functions, and its dysfunction can lead to several chronic diseases. Adipose tissue as an energy storage depot has two main roles, which are at the time of feeding and fasting. During the fed state, the main responsibility of the organ is to store energy in the form of triglycerides/lipids. At fasting state, these lipids can be broken down by the process called lipolysis into FFA that can be utilized by the body cells. Adipose tissue also acts as insulation, helping to maintain constant body temperature by regulating EE (Gregory, 1989). The traditional view of adipose tissue as a simple storage organ is, however, outdated and oversimplified. Adipose tissue contains a variety of cells, that includes adipocytes, preadipocytes, immune cells, and others, that are surrounded


by extracellular matrix and vascular networks. Adipose tissue is a special type of multifunctional connective tissue, with endocrine and immune functions that directly regulate many processes, such as energy balance, metabolism, inflammation, and bone metabolism (Frühbeck, 2008; Kershaw & Flier, 2004). The endocrine function of adipose tissue can be categorized into two subgroups: (i) secretion of proteins (adipokines and adipocytokines), and (ii) production of enzymes involved in steroid hormone metabolism. Protein secretion by adipocytes has many metabolic consequences. One example is leptin, which not only controls food intake (suppresses appetite) but also affects lipid metabolism and insulin sensitivity (Cambuli et al., 2008). Adiponectin is another key adipokine that increases insulin sensitivity, suppresses hepatic glucose output, and has anti-inflammatory actions (Dietze-Schroeder et al., 2005). Furthermore, adipocytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF α) promote local and systemic inflammation, recruitment of macrophages to adipose tissue, and insulin resistance through alteration of the insulin signaling pathway.

Adipose tissue not only secretes a large number of adipokines but also expresses a wide range of receptors (such as leptin receptor, adiponectin receptor, estrogen receptor, toll-like receptors, β -adrenergic receptors, etc.) that respond to traditional hormone systems, afferent signals from the brain and even its own proteins. This allows adipose tissue to have systemic (endocrine), and local (paracrine/autocrine) effects. These crucial roles of adipose can be drastically altered by its excess accumulation; changes in adipocyte number or size can have unfavorable metabolic consequences.

1.2.2 Types of adipose tissue

Traditionally, adipose tissue is categorized mainly into two types: lipid-storing white adipose tissue (WAT) and lipid-burning brown adipose tissue (BAT) (Fig. 1). White adipocytes

are large, circular cells containing unilocular lipid droplet with few mitochondria (Frühbeck, 2008). These cells congregate in various sites all over the body and are named based on those locations, which include subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), and inguinal white adipose tissue (iWAT). The location of WAT deposition tells a lot about metabolic health and identity (discussed more in detail in section 1.3.3). BAT, on the other hand, contains polygonal cells with multilocular lipid droplets and numerous mitochondria in the cytoplasm (Giordano et al., 2014). The mitochondria of BAT possess a unique kind of protein known as uncoupling protein 1 (UCP1), which uncouples the production of ATP from fatty acid metabolism and produces heat as a byproduct (Giordano et al., 2014). Active BAT depots are found in a few locations, to include the neck, mediastinum, and paravertebral areas (Rodríguez et al., 2015; van Marken Lichtenbelt et al., 2009). Recently, the recognition of the third type of fat, known as beige or brite (brown in white), has attracted much interest in the metabolic field (Fig. 1). Beige adipocytes arise from endothelial and perivascular cells within WAT, tend to be found more in iWAT, and have a unique gene signature (Cypess et al., 2013). Beige adipocytes express low levels of UCP1 under normal conditions and can increase UCP1 expression through β -adrenergic stimulation after exercise or cold exposure (Wu et al., 2012). This causes an increase in non-shivering thermogenesis through the burning of lipids stored within the WAT depots, hence resulting in loss of adiposity (Bi et al., 2014). Comparison between these different adipose depots is illustrated in Fig 1.



	White Adipocyte	Beige Adipocyte	Brown Adipocyte
Depots (example)	Visceral, Subcutaneous, inguinal	Emerges in white adipose depots with appropriate stimuli	Cervical, supraclavicular, axillary, paraspinal
Appearance	Circular, unilocular	Circular, multilocular	Polygonal, multilocular
Function	Storage of triglycerides, endocrine	Non-shivering thermogenesis, anti-inflammatory	Non-shivering thermogenesis
Uncoupling protein (e.g., UCP1)	Undetectable	Positive after stimulation	Positive
Changes during obesity	Hyperplasia, hypertrophy, immune cell infiltration	Whitening, loss of UCP1 expression	Mostly unaltered

Fig 1. Comparison of the main characteristics of white, beige, and brown adipocytes regarding localization, appearance, main function, uncoupling protein and major changes during obesity.

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1.2.3 Adipose tissue regulation of systemic energy balance

An imbalance between energy intake and expenditure is considered a primary cause of obesity. Hence, understanding energy balance is the key to understanding obesity and its affiliated diseases. Adipose tissue serves a vital function, not only in energy storage but also in its dissipation when needed, which is achieved by crosstalk between fat depots and the central

nervous system (CNS) (Sandoval et al., 2008). As a fast-acting endocrine gland, adipose tissue provides information regarding substrate availability, tissue mass, energy intake, and utilization to the brain. The brain processes these signals and directs peripheral tissues to make necessary changes to maintain energy homeostasis. The neuroendocrine signals for energy balance sent by the brain can be either catabolic or anabolic in nature and can vary under different circumstances.

Body energy regulation starts immediately after a meal. Leptin from adipose tissue acts as a satiety hormone by imparting its action on the leptin receptor (LepR) residing in energy regulatory regions of the brain, specifically the hypothalamic arcuate nucleus (ARC), triggering the melanocortin pathway (Fig. 2) (Cone, 2005). The ARC of the hypothalamus possesses two distinct classes of neurons, which are reciprocally regulated by leptin to reduce appetite and increase EE, through numerous connections with other hypothalamic nuclei, such as the lateral hypothalamus (LH), the paraventricular nucleus (PVN), the ventromedial hypothalamic nucleus (VMH) and the dorsomedial nucleus (DMN) (Cone, 2005). One class of neuron secretes pro-opiomelanocortin (POMC), an anorexigenic peptide, whereas the other secretes orexigenic peptides, neuropeptide-Y (NPY) and agouti-related protein (AgRP). POMC can be post-translationally modified to produce several smaller, biologically active products, including the melanocortin's, α , β , and γ melanocyte-stimulating hormone (MSH) (Bertagna, 1994; Castro & Morrison, 1997). Melanocortin interacts with a family of five G-protein coupled melanocortin receptors (MCRs) (MC1R, MC2R, MC3R, MC4R & MC5R) (Mountjoy et al., 1992; Valverde et al., 1995). MC3R and MC4R, expressed in the ARC, are central to the control of body weight (Cone, 2005). Specifically, activation of these receptors mediates the anorexigenic effects of leptin.

Excess calories can impair the effects of leptin signaling resulting in leptin resistance that can contribute to obesity development (Izquierdo et al., 2019; Myers et al., 2012). There are a number of alterations of molecular mechanisms that lead to leptin resistance. The most common mechanism suggested was the reduced ability of leptin to reach target cells despite high serum leptin levels (Holtkamp et al., 2004), and decreased LepR expression (Izquierdo et al., 2019; Myers et al., 2012). It has been demonstrated that microcapillary vessels at the Blood Brain Barrier (BBB) possess LepR that binds leptin and helps transport it inside the brain (Tartaglia et al., 1995). Hence, reduced LepR expression in the BBB may be one of the reasons for leptin concentrations being high in plasma and low in cerebrospinal fluid. Furthermore, other studies also suggested excessive plasma leptin levels can contribute to decreased BBB permeability (Mantzoros, 1999; Philbrick et al., 2017).

Other hormones also play a big part in energy regulation, such as insulin from the pancreas and 17 β -estradiol (E2) from the ovary in female. Both hormones have a direct effect on the POMC neurons to increase their activity and inhibit the activity of NPY/AgRP neurons, thereby imparting similar effects as leptin (Debons et al., 1970; Stincic et al., 2018). Crosstalk between leptin and insulin signaling in the POMC neurons may integrate their action in anorexigenic neurons, but not in orexigenic neurons (Niswender & Schwartz, 2003; Xu et al., 2005). However, many studies report that the deletion of insulin receptors in the hypothalamic feeding center did not affect feeding or weight (Hill et al., 2008; Könnner et al., 2007; Xu et al., 2005), suggesting central insulin alone may not be sufficient for long-term regulation of energy homeostasis and suggesting that synergistic actions with leptin are important. In females, E2 is involved in food intake, EE, and adipose deposition/distribution; these effects are mediated by ER α within the hypothalamic feeding center and are critical to energy balance (Hammes &

Levin, 2007; Milewicz et al., 2000). After menopause (hypoestrogenic states), decreased activity in the feeding center and a decline in endogenous estrogen protection leads to increased body fat deposition (Augoulea et al., 2005; Carr, 2003; Jasienska et al., 2005).

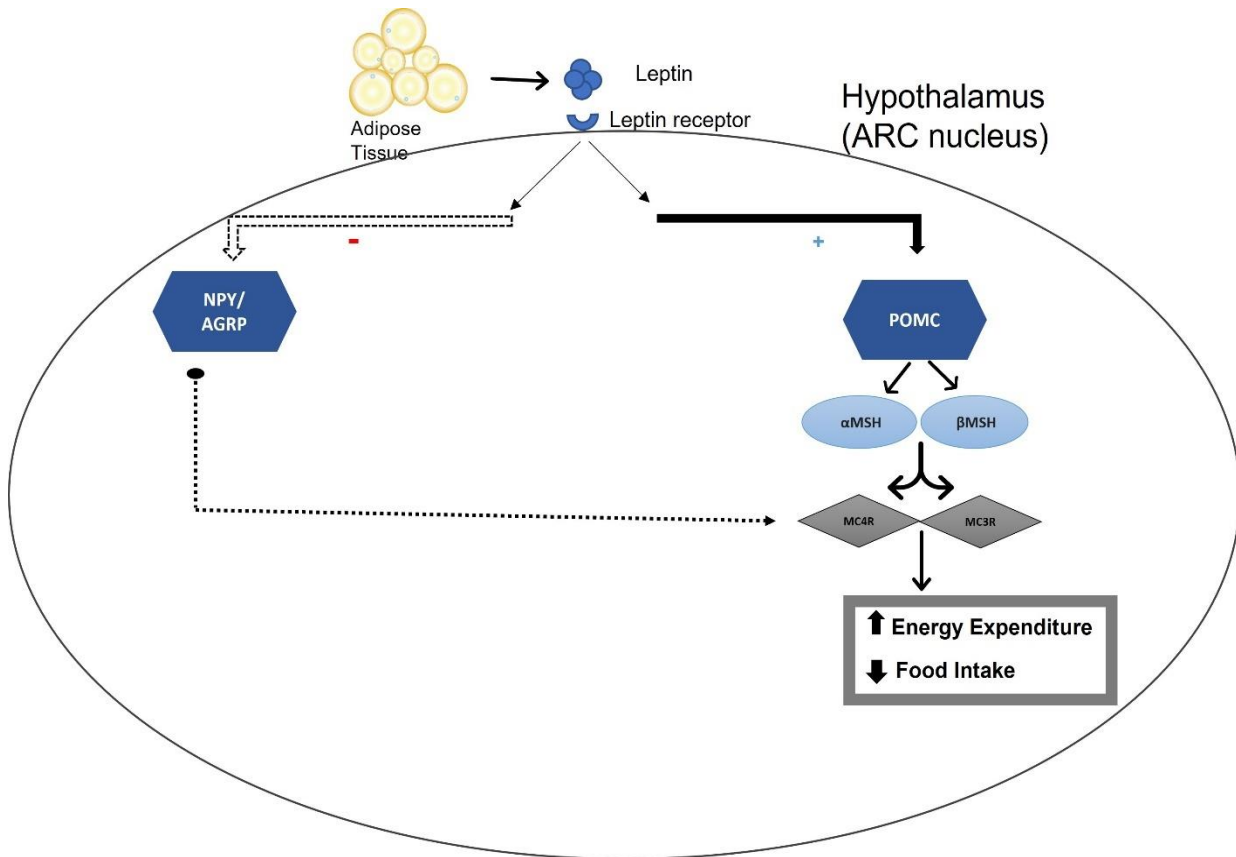


Fig 2. Leptin–melanocortin pathway associated with monogenic obesity through an influence on food intake and energy expenditure. Leptin secreted from adipose tissue binds to the leptin receptor in the hypothalamus. Leptin binding inhibits the neuro-peptide Y/agouti-related protein (NPY/AgRP) production and stimulates pro-opiomelanocortin (POMC) production, which undergoes post-translational modifications to produce peptides such as alpha and beta-melanocyte-stimulating hormone (α - and β -MSH). Alpha and β -MSH bind to melanocortin 3 and melanocortin 4 receptors (MC3R and MC4R) and induce their activity. Increase in the MC3R and MC4R activities results in a decrease in food intake and increase in energy

expenditure. Abbreviations: neuropeptide Y/agouti-related protein: NPY/AgRP; pro-opiomelanocortin: POMC; alpha and beta-melanocyte-stimulating hormone: α - and β -MSH; melanocortin 3 and melanocortin 4 receptors: MC3R and MC4R; ARC: Arcuate Nucleus.

1.2.4 Adipose tissue inflammation and insulin resistance.

Obesity is regarded as a chronic low-grade inflammatory state. WAT itself is an important site for inflammation, which increases considerably during obesity. Through a delicate balance between adipogenesis and apoptosis, adipocyte turnover is maintained. WAT size can be increased through adipogenesis, the production of new adipocytes from preadipocyte differentiation under prolonged calorie excess (Tang et al., 2008). Alternatively, WAT may respond to excess caloric intake by undergoing hypertrophy, which also increases overall fat mass (Arner et al., 2013). If hyperplasia dominates, fat mass does increase, but these depots are filled with numerous small, metabolically healthy adipocytes. Therefore, expansion of adipose tissue size through hyperplasia can help to maintain healthy systemic metabolic function in the face of energy excess. On the contrary, the expansion of adipose tissue through hypertrophy or expansion of existing adipocytes can be pathological, especially when the individual cells reach maximum capacity.

Adipose tissue contains a heterogeneous cell population that includes different leukocyte subsets (Fig 3). Nearly all types of immune cells, such as resident macrophages, mast cells, monocytes, dendritic cells, natural killer cells, B-cells, T-cells, neutrophils, and eosinophils, have been found in adipose tissue (Altintas et al., 2011; Bedford et al., 2006; Deiuliis et al., 2011; Ohmura et al., 2010; Weisberg et al., 2003). Among them, both resident and recruited macrophages are the most abundant and are essential for adipose tissue defense, remodeling, and repair. Macrophages are characterized into two types: proinflammatory M1 (classically

activated) and anti-inflammatory M2 (alternatively activated). Usually, resident macrophages in lean individuals are the M2-macrophage phenotype, and can be differentiated into the classically activated M1-macrophage phenotype as adipose tissue reaches maximum capacity in obesity (Fig 3).

As adipose tissue expands, the population of T-cells changes with a decline in T-reg cells and an increase in CD4⁺ and CD8⁺ T-cells (Feuerer et al., 2009; Nishimura et al., 2009; Pacifico et al., 2006) (Fig 3). This causes a decline in anti-inflammatory cytokines and adipokines, as well as an increase in Type 1 T Helper (TH1) cytokine, Interferon-gamma (IFN γ), that promotes the differentiation of resident M2-macrophages to M1-macrophages (Lumeng et al., 2007; Nishimura et al., 2009). Moreover, the monocyte chemoattractant protein 1/C-C chemokine ligand 2 (MCP1/CCL2), released by adipocytes substantially increases macrophages in WAT because of the recruitment of peripheral blood monocytes (Weisberg et al., 2003). Further expansion of WAT will facilitate an inflammatory environment due to the release of proinflammatory cytokines (IL-6, TNF α , IL-1 β) and adipokines (leptin, resistin) (Fried et al., 1998; Friedman & Halaas, 1998; Hotamisligil et al., 1993; Huang et al., 2014; Netea et al., 2006; Wood et al., 2005). As the expansion of adipocytes is limited by the extracellular matrix, the cells undergo apoptosis and eventually necrosis due to hypoxia resulting from rarefaction (Pasarica et al., 2009). Necrosis of adipocytes driven by obesity can promote peripheral macrophage recruitment in adipose tissue that leads to the formation of crown-like structures (CLS) as M1 macrophages surround the necrotic adipocytes (Fig 3) (Neels & Olefsky, 2006; Sun et al., 2011; Sun et al., 2012). Macrophages are phagocytes that scavenge on dead apoptotic or necrotic adipocytes, alleviating hypoxia conditions and remodeling the extracellular matrix (Chawla et al., 2011). The macrophage markers CD68 and TNF- α in WAT correlate with the

obesity-associated accumulation of adipose tissue macrophages and inflammation (Kern et al., 1995; Weisberg et al., 2003).

Many reports in rodents suggest adipose tissue inflammation can contribute to local and systemic insulin resistance (Hotamisligil et al., 1993; Kanda et al., 2006; Weisberg et al., 2003). However, location/types of adipose tissue expansion can influence the inflammatory state of obese individuals. CLS in obese mice are more prevalent in VAT than in SAT, due to the presence of more macrophages, mast cells, and other immune cells in VAT (Altintas et al., 2011). Studies on targeting inflammation in adipose tissue failed to restore insulin sensitivity. For example, immuno-compromised mice are not protected from systemic insulin resistance by a short-term HFD (Lee et al., 2011). Clinical trials on targeting TNF α have shown little to no beneficial effect on insulin sensitivity (Ofei et al., 1996; Paquot et al., 2000; Stanley et al., 2011; Wascher et al., 2011). These studies do not disprove obesity-mediated inflammation in WAT can cause insulin resistance but bring into question whether anti-inflammation therapy can be an effective strategy in T2D.

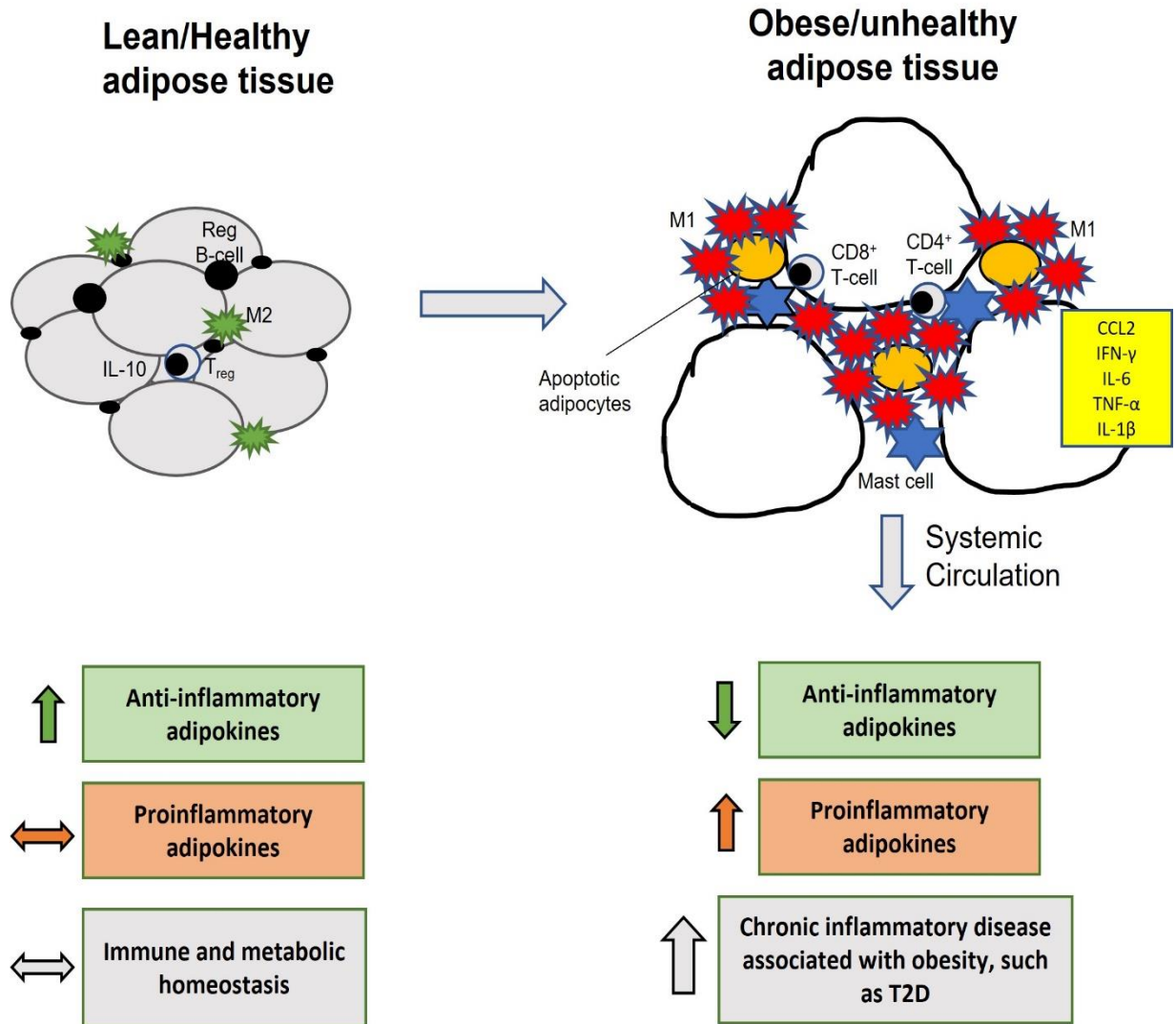


Fig 3. Effects of obesity on adipose tissue leukocyte populations, adipokine secretion, and chronic inflammation. Lean state: anti-inflammatory interleukins, such as IL-4, IL-13, and IL-10 maintain M2 macrophage phenotype with normal metabolic and immune homeostasis. Obese state: hypertrophy promotes necrosis and apoptosis. M1 macrophages engulf necrotic adipocytes forming crown-like structures. Proinflammatory cytokines and adipokines promote inflammation and diseases associated with obesity. Abbreviation: T2D, Type 2 Diabetes; M1,

Macrophages 1; M2, Macrophages 2; IFN γ , Interferon gamma; CCL2, Chemokine Ligand 2; CD- Cluster of Differentiation; IL, Interleukin.
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1.3 Sexual dimorphism in regulation of energy homeostasis

1.3.1 Introduction

Understanding physiological and pathophysiological sex differences is of paramount importance as they might lead to better-targeted therapy. Various aspects of glucose and energy homeostasis are regulated differently in males and females (Fig 4). These differences lead to the development of sex-based metabolic diseases (Fig 5). Obesity and diabetes have a significant impact on both males and females. In the field of endocrinology and metabolism, obesity and T2D has the most documentation on sexual dimorphism. The global prevalence of obesity is higher in women than in men on all continents (Kelly et al., 2008). In contrast, globally, more males are diagnosed with T2D compared to females. In 2014, 14 million more men were reported to be affected with diabetes than women (Whiting et al., 2011). This chapter provides an overview of the fundamental sex differences in glucose and energy homeostasis determined evolutionarily and describes how disruption of homeostasis incurred by excess calories can contribute to sex-based metabolic disease.

1.3.2 Evolutionary survival strategies affect sex-specific changes in adipose tissue function.

Evolutionarily, sex-dependent differences in survival strategies suggest that males prepare for periods of energy absence by increasing food/energy intake to increase fat stores, whereas females react to energy scarcity to survive by decreasing energy expenditure and preserving fat stores (Shi et al., 2007) (Fig 4). These observations led to the idea that females might preferentially store fat in SAT whereas males utilize VAT, because SAT is more adapted

to long term-storage, while VAT is more metabolically active (discussed more in detail in section 1.3.3) (Karastergiou et al., 2012; Palmer & Clegg, 2015) (Fig 4).

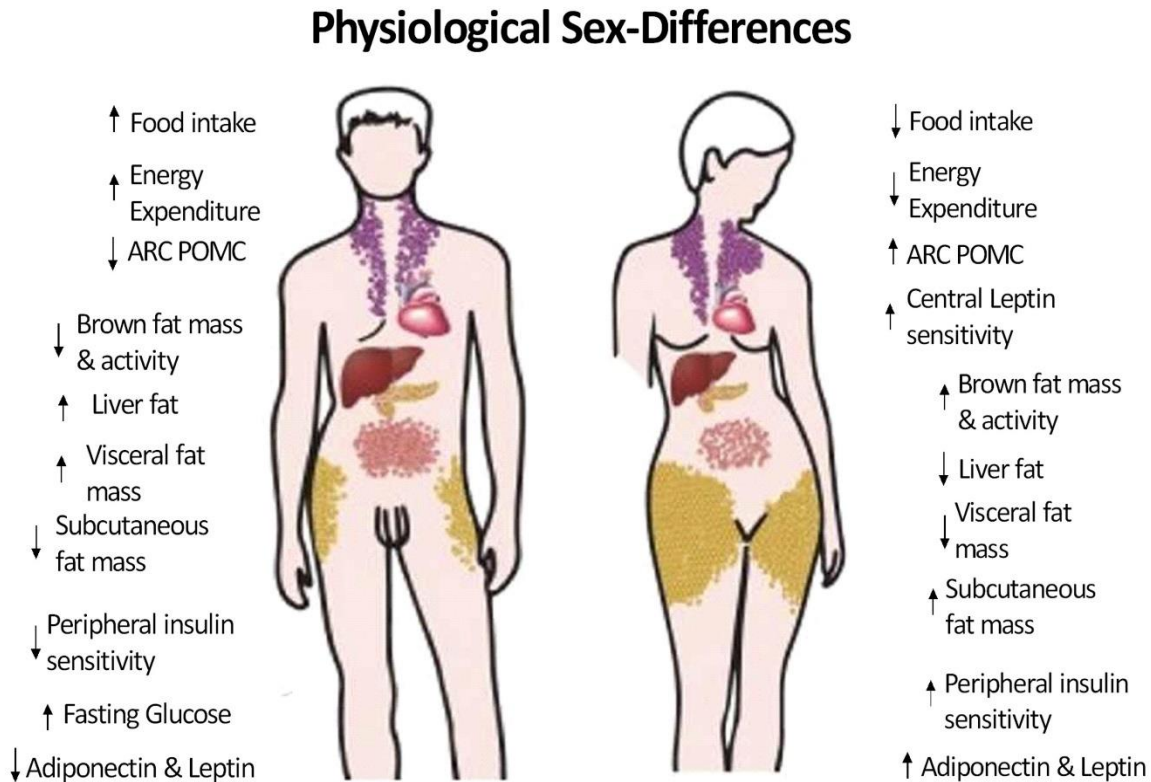


Fig 4. Schematic overview of physiological sex differences in energy homeostasis regulation. Upward or downward arrows indicate higher or lower levels, respectively, of the given phenomena compared to each sex's counterpart. ARC, Arcuate nucleus; POMC, pro-opiomelanocortin. Figure modified from *Kautzky-Willer, Alexandra et al. "Sex and Gender Differences in Risk, Pathophysiology and Complications of Type 2 Diabetes Mellitus." Endocrine reviews vol. 37,3 (2016).*

Energy storage increases only when intake surpasses expenditure. Adaptive thermogenesis, basal metabolism, and physical activity determine an individual's energy expenditure. Sexual dimorphism is not only restricted to energy storage in WAT but is also found in BAT. Females have more active and larger amounts of BAT, as indicated by increased mitochondrial numbers and cristae density, and thus increased capacity for non-shivering thermogenesis (Rodriguez-Cuenca et al., 2002) (Fig 4). Interestingly, during periods of starvation, females have an enhanced capacity to deactivate non-shivering thermogenesis to use energy more efficiently, consistent with the idea that the females' response to low energy availability is reactive (Cardon et al., 1994). Collectively, this information suggests that male and female adipose tissues are very different, and therefore, may communicate with other metabolic tissues in entirely distinctive ways (discussed more in detail in sections 1.3.4 and 1.3.5). Sex differences in the context of physiological and pathophysiological metabolism are illustrated in Figs 4 and 5.

Pathologic Sex-Differences

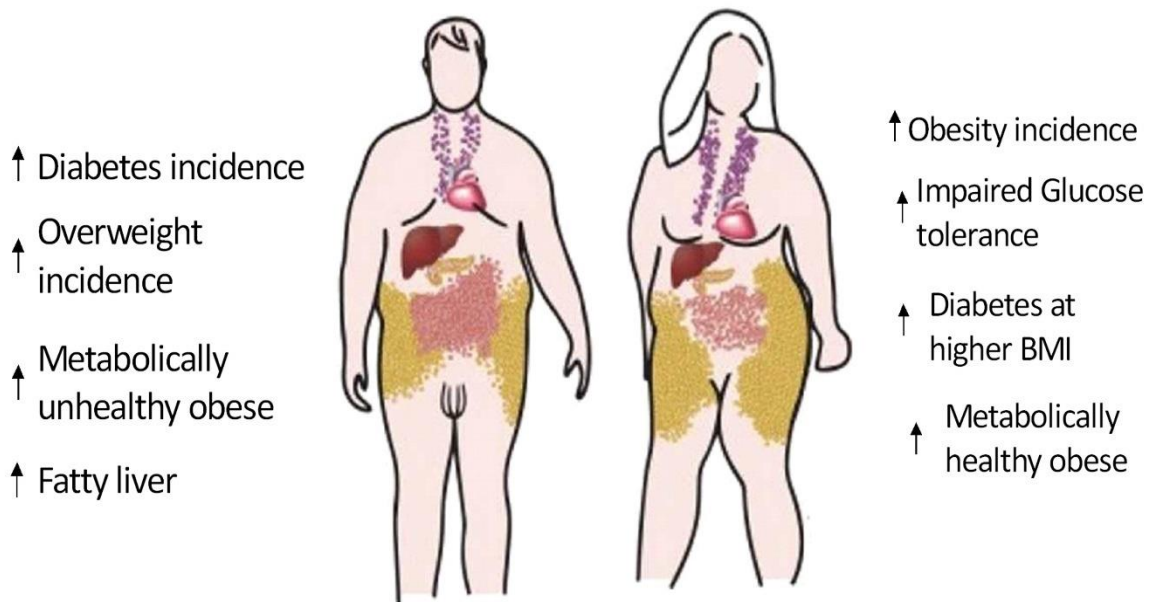


Fig 5. Schematic overview of pathological sex differences in energy homeostasis regulation. Upward or downward arrows indicate higher or lower levels, respectively, of the given phenomena compared to each sex's counterpart. Figure modified from *Kautzky-Willer, Alexandra et al. "Sex and Gender Differences in Risk, Pathophysiology and Complications of Type 2 Diabetes Mellitus." Endocrine reviews vol. 37,3 (2016).*

1.3.3 Sex-based adipose tissue distribution results in sexually dimorphic metabolic diseases.

In response to excess energy, premenopausal women increase gluteo-femoral fat (SAT), promoted by estrogen, that results in a 'pear' shaped feature. Men, on the other hand, accumulate fat mostly in the abdominal or visceral (VAT) depots, resulting in an 'apple' shaped structure (Nielsen et al., 2004; Power & Schulkin, 2008). Functional differences between VAT and SAT contribute to obesity related diseases that differ between the sexes.

VAT is more metabolically and functionally active compared to SAT. Insulin-dependent glucose uptake in VAT adipocytes occurs at a higher rate compared to adipocytes in SAT (Ibrahim, 2010). Moreover, VAT has relatively more capillaries and efferent sympathetic axons per unit volume than SAT (Trayhurn, 2005). Catecholamine-induced lipolysis is higher at the time of intermittent activity and during fight/flight response from VAT to the circulation than SAT (Lönnqvist et al., 1997). Catecholamines trigger lipolysis via β 3-adrenoceptors and inhibit lipolysis via α 2-adrenoceptors (Lafontan & Langin, 1995). In situations of prolonged intake of positive energy, male rats have lower lipolytic capacities as they have a higher α 2/ β 3-adrenoceptor ratio in VAT compared to females (Lladó et al., 2002). Moreover, VAT uptake of triglycerides is greater in males than in females under conditions of excess calorie intake (Nguyen et al., 1996). Storage of excess energy in a fat depot, like VAT, with greater lipolytic capacity that facilitates rapid mobilization of energy makes sense for evolutionarily male roles of hunting, gathering, and protection. Although VAT is metabolically active, they carry a much greater risk for metabolic disorders compared to SAT (Freedland, 2004). VAT is more capable of generating MCP1, pro-inflammatory adipocytokines and is found to have more macrophages, mast cells, other immune cells, and infiltrated immune cells compared to SAT (Altintas et al., 2011; Forouhi et al., 2001; Pou et al., 2007; Weisberg et al., 2003). Adipocytes from VAT are more insulin-resistant than SAT adipocytes (Abate et al., 1996; Frayn, 2000), and the amount of VAT is a critical factor influencing insulin sensitivity (Misra & Vikram, 2003). This increases the risk of systemic metabolic dysfunction, as it is associated with metabolic diseases such as hyperglycemia, hyperinsulinemia, impaired glucose tolerance, insulin resistance and, T2D (Fig 5).

Female SAT has a higher rate of FA uptake and lipogenesis, as well as a lower rate of release compared with males (Kautzky-Willer et al., 2016). SAT is widely distributed within a broad area under the skin is poorly vascularized with sparse sympathetic innervation, and is less metabolically active (Trayhurn, 2005). In contrast, SAT distribution is more metabolically healthy than VAT (Ibrahim, 2010). This is mainly due to SAT's ability to release more metabolically healthy adipokines than unhealthy ones. For example, SAT is the major source of leptin (Wajchenberg, 2000) and adiponectin (Svensson et al., 2014) release, and has less capacity to synthesize and secrete proinflammatory cytokines (Forouhi et al., 2001; Pou et al., 2007) (Fig 4, Fig 6). In humans, adiponectin and leptin levels were higher in women than in men (with adiponectin 18.48 ± 12.77 vs. $7.8 \pm 10.39 \mu\text{g/mL}$, $P < 0.0001$, and leptin 30.77 ± 19.16 vs. 8.66 ± 8.24 ng/mL, $P < 0.0001$) (Awede et al., 2018). As females are the child bearers and are required to breastfeed newborns, there is an evolutionary advantage to SAT deposition due to their ability to efficiently store calories that help to mitigate the energy demands of gestation and lactation. Interestingly also, SAT is more lipolytically active during lactation than VAT in females, which ensures an adequate energy supply during lactation. Furthermore, the accumulation of fat disproportionately in SAT during pregnancy also reduces the risk of metabolic diseases compared to VAT deposition (Kautzky-Willer et al., 2016). Females become more prevalent to metabolic diseases post-menopause, with loss of estrogen production, due to a preferential increase of VAT deposition (Carr & Brunzell, 2004).

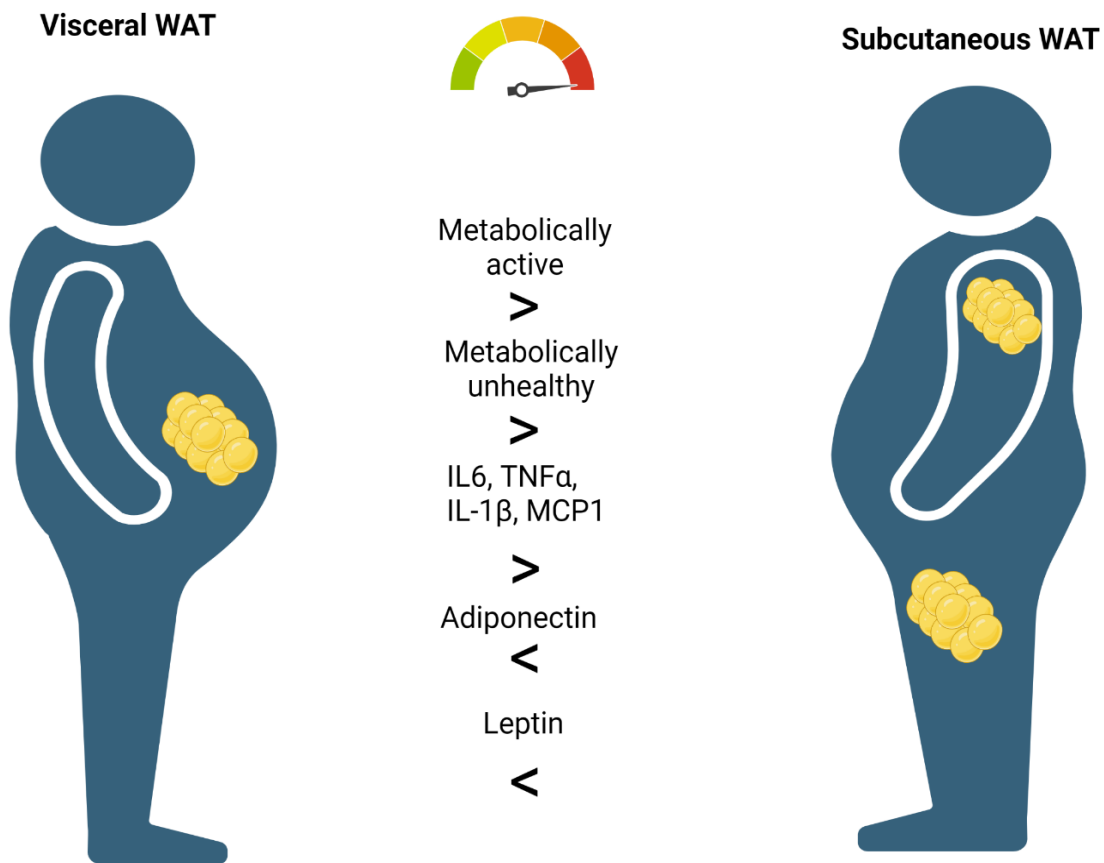


Fig 6. Comparison of roles and function between visceral and subcutaneous adipose tissue. VAT is more metabolically active however unhealthy and opposite in case of SAT, mostly due to the difference in adipokine and adipocytokine release. Abbreviation: VAT, Visceral Adipose Tissue; SAT, Subcutaneous Adipose Tissue; WAT, White Adipose Tissue. Created by Biorender.com.

1.3.4 Sex-specific energy regulation by the adipose-hypothalamic axis

The adipose-hypothalamic axis makes significant contributions to maintaining energy homeostasis by controlling both energy intake and expenditure. Body fat mass can be a classic example of the feedback signals arising from the fat depots that can be sensed by the brain for

the maintenance of body weight. Leptin from WAT circulates in the blood in proportion to fat mass and regulates the set point for body fat stores by informing the central nervous system, acting as an adiposity signal to inhibit food intake (Considine et al., 1996; Sandoval et al., 2008). Circulating leptin concentrations, as well as leptin sensitivity, vary in proportion to fat mass, and sex differences are apparent. Secretion of leptin is highly correlated with amounts of VAT and SAT (Clegg et al., 2003). Leptin is usually secreted more from SAT and is 4 times higher in females than males (Havel et al., 1996). Sex steroids are important regulators of leptin secretion and contribute significantly to sex differences. Androgen decreases leptin concentrations in males (Luukkaa et al., 1998), whereas estrogen increases leptin concentrations in females (Margetic et al., 2002). Female adipose distribution and size also positively correlate with leptin levels, which is not the case for males (Garaulet et al., 2000; Havel et al., 1996). Long-term elevation in leptin, which should occur in response to increased SAT size under conditions of increased adiposity, predicts that females are more susceptible to leptin resistance compared to males. Additionally, the female rat brain is more sensitive to leptin (Clegg et al., 2003).

Sexual asymmetry is also apparent in the organization of the POMC system. Males exhibit decreased POMC neuronal fibers and projections, as well as reduced levels of POMC gene and protein compared to females (Nohara et al., 2011) (Fig 4). Thus, POMC neurons are less active in males, which promotes increased energy intake (Wang et al., 2018). The neonatal testosterone surge in males shapes POMC neuron innervation patterns for hypothalamic feeding circuits (Nohara et al., 2011). Neonatal androgenization of female mice reduces POMC expression and decreases POMC neuronal projections, mimicking a male pattern (Nohara et al., 2011). POMC regulation of BAT that controls the body's non-shivering thermogenesis is also sex-divergent (Burke et al., 2016). Cholinergic preganglionic sympathetic neurons within the

intermediolateral nucleus of the thoracic spinal cord are directly innervated by ARC POMC neurons, and their postganglionic connections innervate BAT (Bamshad et al., 1999; Lowell & Flier, 1997). In the case of orexigenic neuron activity, central administration of AgRP in both males and females induces robust hyperphagia. However, the effects were shorter-lived in males. Although both groups gained similar amounts of weight, females displayed a greater reduction in energy expenditure (Goodin et al., 2008). Moreover, EE in females was normalized upon removal of the gonads without any effects on food intake, suggesting that sex differences generated by AgRP are due to sex hormone-specific changes in energy expenditure (Goodin et al., 2008). NPY also displays sex-dependent differences. Male rats express more NPY mRNA containing neurons in the rostro-caudal ARC compared to females, which stimulates more food intake by inhibiting the melanocortin system (Urban et al., 1993). Testosterone stimulates NPY expression in ARC nuclei in males (Sahu et al., 1989). In females, estradiol inhibits the excitability of the NPY neurons in the hypothalamus and stimulates anorexigenic action (Dhillon & Belsham, 2011; Santollo & Eckel, 2008). Moreover, overexpression of NPY in adrenergic and non-adrenergic neurons in CNS leads to increased fat accumulation in males, but not females (Ruohonen et al., 2008).

The melanocortin system is a target for leptin-activating MC3R & MC4R. Reports demonstrate no sex differences when the agonist (MTII) of MC3&4 receptors is administered over a wide range of doses (Clegg et al., 2003). Thus, the sexual dimorphism of the upstream major mediators of MC3&4 receptors is hypothesized to play the role of such sexual dichotomy.

1.3.5 Sexual dimorphism in adipose tissue-mediated inflammation

Males and females have several differences in innate and adaptive immunity (Klein, 2012). In general, males are more susceptible to infectious diseases (McClelland & Smith, 2011),

whereas females show an increased prevalence of autoimmune diseases (Klein & Flanagan, 2016). Adipocyte hypertrophy driven by chronic energy excess, as occurs under high fat diet (HFD) conditions, leads to macrophage recruitment and promotes adipose tissue inflammation. Previous studies suggest that HFD-induced obesity can promote the profound accumulation of the pro-inflammatory adipose tissue macrophage populations in males, while females show dampened inflammation (Singer et al., 2015; Varghese et al., 2019).

Monocytes recruited from blood to the adipose tissue for macrophage transition are also sexually dimorphic. In males, monocytes respond more robustly to unhealthy metabolic stimuli and are more likely to differentiate into inflammatory macrophages (M1) compared to females (Varghese et al., 2022). Studies have demonstrated females have lower toll-like receptor 4 (TLR4) and lower cytokine secretion (Marriott et al., 2006), whereas males produce more TLR4 pro-inflammatory cytokines such as TNF α , IL6 (Asai et al., 2001; Marriott et al., 2006). Sex steroids are a crucial role in sex differences in inflammatory responses to HFD. Androgen positively correlates to adipose inflammation, whereas estrogen is protective against inflammation in adipocytes. This protective effect is lost after menopause; HFD-induced adipose tissue macrophage infiltration is more prevalent in postmenopausal females with obesity (Pfeilschifter et al., 2002; So et al., 2021).

1.4 Aryl hydrocarbon receptor regulation of energy homeostasis.

1.4.1 Xenobiotic stimulation of aryl hydrocarbon receptor signaling.

An estimated 25-70% of the underlying risk of obesity is genetically based (Cardon et al., 1994; Stunkard et al., 1986), whereas the environment remains critical for 30-75% of cases (Baillie-Hamilton, 2002). Identifying specific genes that affect energy balance in the body remains elusive (Wright & Aronne, 2012). Our lab and others have investigated a metabolic role

for aryl hydrocarbon receptor (AhR), which has been long studied for providing defense against environmental toxicants. The AhR is a member of the bHLH-PAS family that possesses a bHLH/PAS ((basic helix-loop-helix/period (PER), aryl hydrocarbon receptor nuclear translocator (ARNT), and single-minded (SIM)) domain and functions as a transcription factor (Burbach et al., 1992; Ema et al., 1992). The bHLH/PAS proteins contain PAS-A and PAS-B protein-protein interaction domains, which allow the formation of heterodimers that subsequently affect the transcription of target genes. bHLH/PAS superfamily members can affect neurogenesis (Nambu et al., 1991), circadian rhythms (Sassone-Corsi, 1997), hypoxia (Semenza, 1998), and xenobiotic metabolism. AhR has a range of functions, influenced by ligand affinity, cellular context, and other environmental factors.

As a mediator of toxicity to environmental toxicants, AhR can be activated by numerous exogenous ligands. Most toxic contaminants that are AhR ligands are man-made and are produced by various industries, including pesticide, bleaching, wood preservation, metallurgy, and many more. Among the man-made ligands that generate major health concerns are halogenated aromatic hydrocarbons, such as polyhalogenated dibenzodioxins, dibenzofurans, and biphenyls that bind to AhR with high affinity even in the pico-nanomolar range (Denison & Nagy, 2003). Their ubiquitous distribution, lipid solubility, and long half-life promote bioaccumulation of the compounds throughout the food chain by depositing in lipid-heavy tissues such as adipose (Myre & Imbeault, 2014; Pravettoni et al., 2005; Safe, 1990). There they show immense resistance to breakdown. The Environmental Protection Agency (EPA) has recognized a link between increased morbidity rate and level of Polychlorinated Biphenyls (PCBs), which also includes dioxins, in the general US population (Bjerke & Peterson, 1994).

Alarming, people in highly contaminated regions might have surpassed the tolerable dioxin exposure (Muntean et al., 2003).

Naturally occurring and endogenous ligands can also affect AhR signaling and influence physiological function. Some examples of endogenous ligands are arachidonic acid metabolites, tryptophan metabolites, heme metabolites, indigoids, etc. (Heath-Pagliuso et al., 1998; Nguyen & Bradfield, 2008). AhR ligands are also found abundantly in our daily diet. For example, indole metabolites from cruciferous plants and flavonoids from fruits and vegetables are well-known AhR ligands (Denison & Nagy, 2003).

In absence of AhR ligands, AhR is complexed with various cytoplasmic chaperones protein, such as heat shock chaperones (hsp90), immunophilin-like protein X-associated protein 2 (XAP2), and the co-chaperone, p23 (Fig. 7) (Petruilis & Perdew, 2002). Such a complex helps AhR to retain its original conformation which is essential for ligand binding and restricting nuclear translocation. Ligand binding facilitates conformational change allowing the whole AhR complex to locate inside the nucleus, where it binds with the AhR nuclear translocator (ARNT) through PAS domains (Fig. 7) (Gu et al., 2000; Larigot et al., 2018; McGuire et al., 2001). AhR: ARNT complex acts as a transcription factor binding to the xenobiotic response elements (XRE) in target gene promoters (Ko et al., 1996). The *Cytochrome P450* (*Cyp1a1*, *Cyp1b1*), phase I&II metabolizing enzymes, are some of the canonical AhR target genes (Trask et al., 2009). Furthermore, in this process, AhR also targets many non-P450 transcripts that regulate fatty acid and lipid metabolism relevant to obesity and diabetes (Fig. 7) (Lo & Matthews, 2012; Xu et al., 2015). AhR expression is later attenuated by a negative feedback loop facilitated by the AhR repressor gene (AhRR), that targets AhR: ARNT heterodimer (Fig. 7) (Baba et al., 2001; Haarmann-Stemmann & Abel, 2006).

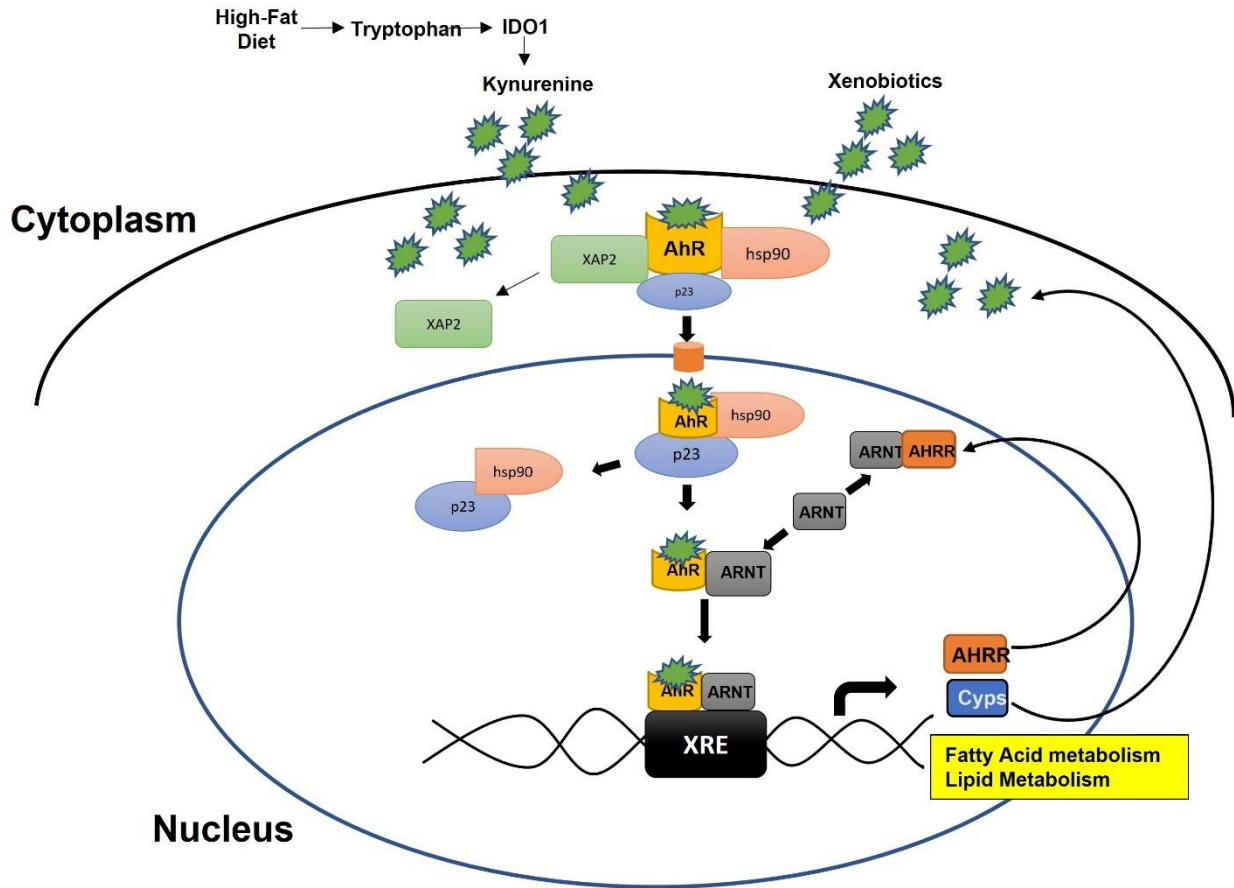


Fig 7. AhR signaling mediated by xenobiotics. Xenobiotics that include various environmental pollutants as well as components of high fat diet, can bind with AhR that is previously complexed with various cytoplasmic chaperones protein to translocate inside the nucleus. Inside the nucleus, AhR dimerizes with ARNT to form AhR:ARNT complex, that acts as transcription factor in xenobiotic response elements (XRE). The *Cytochrome P450* phase I&II metabolizing enzyme, are some of the canonical AhR target genes that helps neutralizing the xenobiotics. AhR also targets many non-P450 transcripts that regulate fatty acid and lipid metabolism relevant to obesity and diabetes. AhR repressor gene (AhRR) is also a target gene which feedback to inhibit AhR:ARNT induce transcription. *Modified from: Tischkau SA.*

Apart from the toxicological effect of AhR, many studies highlight other physiological functions of AhR, mediated through alternative, or non-canonical signaling. This signaling is highly correlated with ligand affinity, cell type, and other environmental factors that may influence other downstream pathways of AhR activation (Boutros et al., 2009; Dere et al., 2011; Lo & Matthews, 2012; Tijet et al., 2006). Moreover, the high promiscuity of AhR is not only limited to ligand binding but also to diverse genomic/non-genomic protein interactions influencing their pathways (Matsumura, 2009; Patel et al., 2009; Tanos et al., 2012). AhR can interact with nuclear receptors, including steroid receptors such as ER and AR. Among them, an extensive study has been conducted on the ER interaction (Fig. 8) (detailed discussion in section 1.4.2). Furthermore, AhR can also interact with many intracellular signaling pathways, such as MAPK (Fig. 8), ERK1/2, p38, PI3K/AKT, and JNK signaling pathway (Banerjee et al., 2016; Tischkau, 2020; Tsai et al., 2014; Wang et al., 2014; Yu et al., 2014). Thus, AhR has a diversified profile to regulate numerous physiological processes, including inflammation (Fig. 8), cell cycle, circadian rhythm, etc. Hence, the evolutionary conservation of AhR to neutralize harmful xenobiotics now raises important questions regarding its function in health and disease due to its ability to bind a variety of man-made, natural, and endogenous compounds indiscriminately.

1.4.2 AhR and ER interactions.

Steroid hormone receptors, including ER α (ER α) and ER β , are nuclear receptors as they act as ligand-dependent transcription factors, similar to AhR. Ligand binding to ER induces homodimerization of the receptor, which facilitates translocation inside the nucleus to act as a

transcription factor in promoter regions of estrogen response elements (ERE) for the regulation of its target genes (Fuentes & Silveyra, 2019). Although the physiological relevance is not well understood, AhR has been shown to play a critical role in female reproduction (Baba et al., 2005). Many reports suggested inhibitory crosstalk between the AhR and ER signaling in different pathways (Fig 8). Data from Matthews et al. revealed that activated AhR can recruit ER α away from ERE to inhibit its pathway and direct it toward AhR-responsive genes (Ahmed et al., 2009; Matthews et al., 2005) (Fig 8). Other studies showed this recruitment occurs through direct protein/protein interactions between ER α and AhR (Ahmed et al., 2009; Beischlag & Perdew, 2005; Ohtake et al., 2003). Matthews et al. also suggested such recruitment can attenuate ER α expression levels to inhibit estrogenic responses (Matthews et al., 2005). Similarly, Wormke et al. also demonstrated that AhR activation reduced ER α protein levels via proteasome activity (Wormke et al., 2003). In addition, AhR: ARNT can also act as a transcriptional repressor of ER α signaling by binding to inhibitory XRE (iXRE), which reduces ER α ability to bind ERE (Safe et al., 1998; Wang et al., 2001) (Fig 8). Moreover, the formation of AhR: ARNT heterodimer reduces ARNT availability to complex with ER α by the process called squelching, which disrupts ER α signaling (Rüegg et al., 2008) (Fig 8). AhR: ARNT can directly bind co-activator proteins, such as p300, and cAMP response element binding protein (CBP), to facilitate squelching (Beischlag & Perdew, 2005; Ma et al., 2009). Overall, AhR can interact with ER α in a multifaceted manner to disrupt ER signaling and such interaction can increase the risk of endocrine disruption.

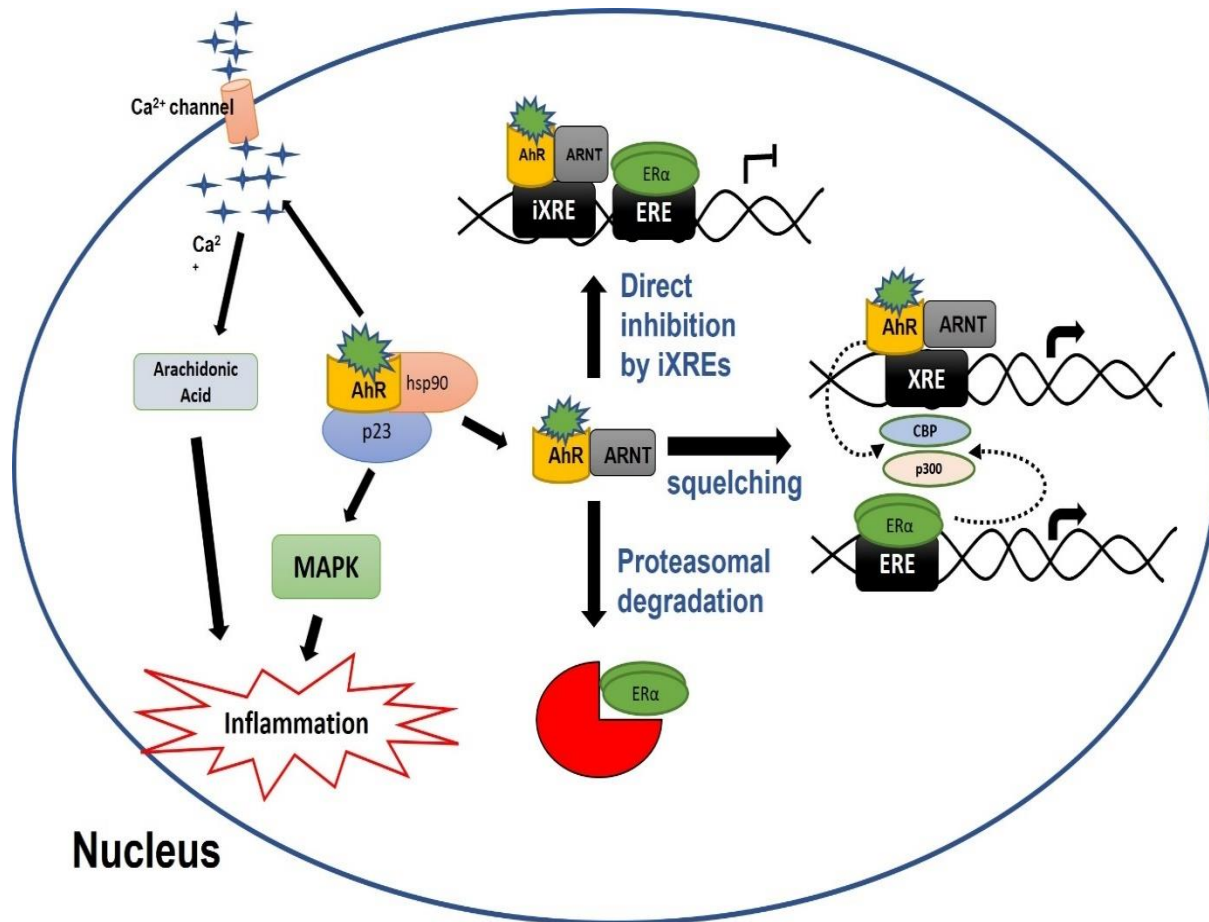


Fig 8. Non-genomic AhR signaling to attenuate ER pathway and to induce inflammation. AhR signaling has been reported to reduce ER activity through several mechanisms: direct inhibition by AhR:ARNT heterodimer through binding to inhibitory XRE (iXRE) present in ER target genes; sequestration of shared coactivators; proteasomal degradation of ER. AhR can also mediate inflammation by allowing influx of Ca^{2+} ions or through release from the endoplasmic reticulum, inducing arachidonic acid production and hence inflammation. AhR can also interact to increase MAPK activity that also promotes inflammation. Modified from: Tischkau SA. Mechanisms of circadian clock interactions with Aryl hydrocarbon receptor signaling. Eur

J Neurosci. 2020 and Matthews J, Gustafsson JA. Estrogen receptor and aryl hydrocarbon receptor signaling pathways. Nucl Recept Signal. 2006

1.4.3 AhR interactions in sexual and neuroendocrine development.

Emerging evidence suggests that early life exposure to environmental toxicants can have long-lasting impacts on development and health. bHLH-PAS family proteins are crucial regulators of the hypothalamus, and neuroendocrine development (Hosoya et al., 2001; Petersen et al., 2000; Shearman et al., 1999). Dioxins may accumulate during prenatal and postnatal periods via the placenta and breast milk (Kreuzer et al., 1997; Lai et al., 2004; Patandin et al., 1999). With a half-life that is roughly decades, the chances of staying in tissue from birth to adult to activate AhR chronically are high. Dioxin burdens in the perinatal stage can lead to irreversible changes in brain development that become apparent in adulthood. Therefore, understanding how these toxicants impact development is critical. Reproductive development appears sensitive to toxicant exposure. Prenatal exposure to the potent AhR agonist, 2,3,7,8 - Tetrachlorodibenzo-p-dioxin (TCDD) feminizes male rats (Bjerke et al., 1994; Bjerke & Peterson, 1994; Gray Jr et al., 1995; Mably et al., 1992). TCDD-treated male mice exhibit gonadotropin secretion patterns similar to females and decreased plasma androgen levels (Mably et al., 1992). Feminization of the preoptic area of the hypothalamus may contribute to this feminization (Petersen et al., 2006). Prenatal exposure to TCDD in females leads to reproductive dysfunction in adulthood, including complications in the estrus cycle, ability to achieve and maintain pregnancy, and sometimes infertility (Abbott et al., 1999; Gray Jr et al., 1995; Gray & Ostby, 1995). Altered gonadotropin release patterns due to TCDD-induced alterations of the POA are considered to be the reason for diminished reproductive capacity.

The link between AhR and sex steroid receptor pathways prompted the exploration of AhR expression in known sexually dimorphic areas of the brain. Interestingly, AhR and ARNT expression is sexually dimorphic in the hypothalamus, particularly in the POA, which is important for sex behaviors (Gorski et al., 1978; Simerly et al., 1985). Overlap with regions high in ER (Shughrue et al., 1997), may explain why the alteration of gonadal hormones due to TCDD exposure has sex-specific effects. Similarly, interactions between AhR and ER may affect the function of other hypothalamic nuclei, including the anteroventral periventricular (AVPV), arcuate (ARC), and ventromedial (VMH). Interestingly, these regions regulate both sexual behavior and energy homeostasis (Fetissov et al., 2004; Gray & Brooks, 1984; Liu & Shi, 2015; Petersen & Barraclough, 1989). Manipulation of sex hormones and their receptors during development can lead to permanent changes in neuronal connectivity and functions, and exogenous AhR ligands can interact with these events, leading to irreversible changes.

1.4.4 AhR in adipose tissue pathway to regulate energy homeostasis and inflammation.

One of the noticeable symptoms of a high/lethal dose of TCDD, a potent agonist of AhR, is the wasting syndrome characterized by profound weight loss due to reduced food intake (Kelling et al., 1985; Seefeld et al., 1984). Surprisingly, low/sublethal doses of TCDD not only failed to give similar effects, but they also impose hyperphagia and weight gain (Seefeld et al., 1984). Combined with HFD, TCDD can induce obesity in female mice in a dose-dependent manner (Zhu et al., 2008). Overall, these studies indicate the magnitude of AhR activation may play a role in energy regulation which might be different between sexes.

Epidemiological studies link AhR activation by persistent organic pollutants (POPs) to insulin resistance (Fierens et al., 2003; Henriksen et al., 1997; Lee et al., 2006; Magliano et al., 2021). Targeted studies revealed that AhR can modulate gene expression of metabolic pathways,

including those that regulate blood glucose, lipid, and energy homeostasis (Boverhof et al., 2006; Fletcher et al., 2005; Kurachi et al., 2002; Sato et al., 2008). The highly lipophilic nature of POPs suggests a connection with adipose tissue. In addition, various dietary fats and fat derivatives (McMillan & Bradfield, 2007), which can act as ligands for the AhR, deposit in adipose tissue. There they can chronically activate AhR to affect its biology for an indefinite amount of time as they possess very long half-lives. For example, environmental toxicants such as POPs and PCBs were reported to promote adipose tissue inflammation and impair glucose homeostasis (Baker, Karounos, et al., 2013; Baker et al., 2015; Lee et al., 2006). In contrast, administration of an AhR antagonist (resveratrol) prevents impairment of glucose homeostasis and promotes insulin sensitivity in adipose tissue (Baker, English, et al., 2013). In addition, another study that was conducted in human adipose-derived stem cells and in vivo mice revealed AhR ligands significantly induced inflammatory cytokines (IL-8), macrophage chemoattractant (MCP1) as well as Cyp1b1 in adipose tissues, which was reduced by pretreatment of AhR antagonist (α -NF) (Kim et al., 2012). Moreover, several other studies on TCDD-mediated AhR activation demonstrated alteration of many key adipose pathways, such as inhibition of fatty acid synthesis (Lakshman et al., 1988; Lakshman et al., 1989), adipogenesis (Alexander et al., 1998). Cyp1b1 has been reported to play a pivotal role in promoting obesity and fatty liver disease (Li et al., 2014). Disruption of Cyp1b1 expression in transgenic Cyp1b1 deficient mice that were fed an HFD reduces obesity and improves glucose tolerance compared to WT mice (Liu et al., 2015). Cyp1b1 expression is more prevalent in adipose tissue on AhR activation and hints towards a potential mechanism for such metabolic disruption (Ellero et al., 2010). Some studies suggest Cyp1b1 could be attributed to metabolizing steroid hormones and lipid-modulating adipose tissue biology (Donovan et al., 2013; Vasiliou & Gonzalez, 2008).

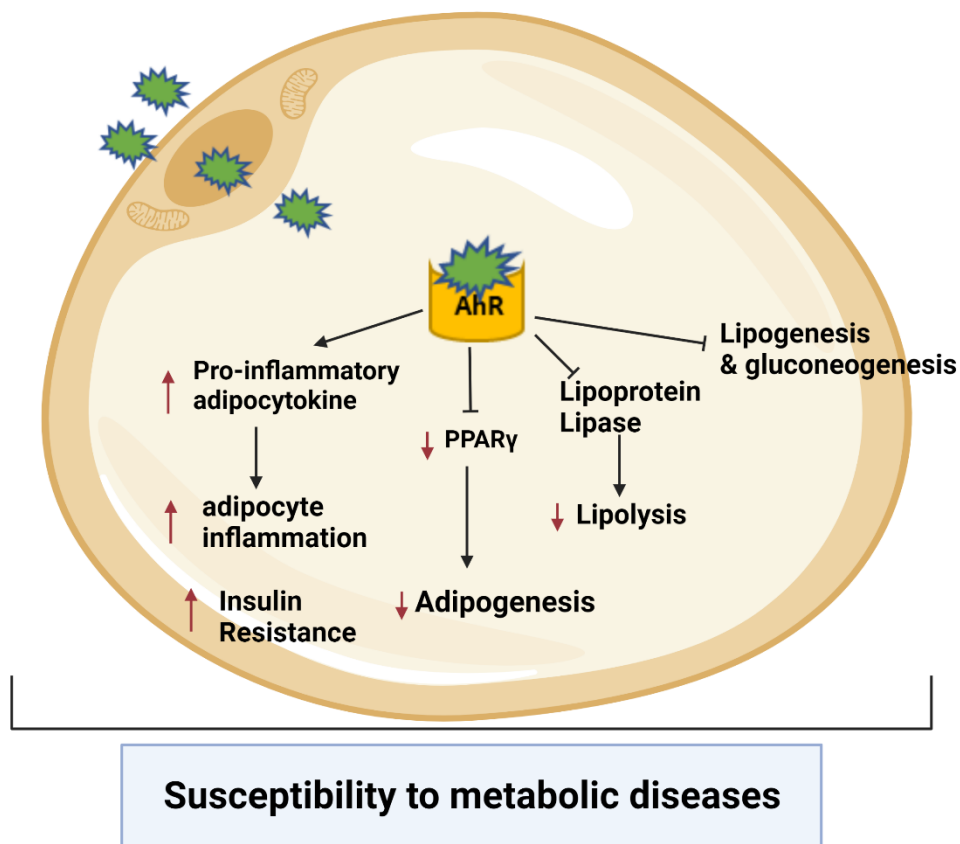


Fig 9. AhR involvement in various adipocyte pathways to influence metabolic diseases.

Agonist of AhR (metabolite of HFD, dioxins) activates the receptor and evokes toxicological as well as many biological effects. These agonists can bioaccumulate within adipose tissues and influence its pathways. Among them, AhR can regulate adipogenesis by regulating PPAR signaling pathway, inflammation by increasing proinflammatory adipocytokine release, inhibition of lipolysis by dysregulation of lipoprotein lipase activity, and other pathways to promote metabolic diseases. Created by Biorender.com.

High-fat diet (HFD) also can mediate similar effects in adipose tissue compared to environmental toxicants. It is now well-established that global AhR deficiency protects mice from HFD-induced obesity (Kerley-Hamilton et al., 2012; Xu et al., 2015). Under HFD,

tryptophan metabolites that act as AhR ligands are increased, which activates AhR and promotes an obese phenotype (Manzella et al., 2018). The tryptophan (Trp) metabolite, kynurenine (Kyn), is an example of a fat derivative that activates AhR to promote an obese phenotype (Huang et al., 2022). White adipose tissue (WAT) is a primary site for Kyn metabolism, which is elevated in the circulation of obese subjects. Excessive Kyn promotes AhR activity to activate the AhR/Stat3/IL-6 pathway in adipocytes and mediate the development of obesity and insulin resistance (Huang et al., 2022). Moreover, Xu et al from the Tischkau lab also revealed AhR as an important regulator of WAT adipogenesis and function as well as BAT thermogenesis (Xu et al., 2015). This study prompts for further study in adipose tissue in Tischkau Lab. Khazaal (Khazaal and Tischkau, unpublished) on adipocytes derived from mesenchymal stem cells (MSC: Mesenchymal stem cells) and 3T3L1 (pre-adipocytes) revealed that AhR activation disrupted various adipocyte functions in a way that could promote metabolic diseases. Khaazal showed activation of AhR by BNF impairs adipogenesis, and proposed this can lead to adipocyte hypertrophy and inflammation leading to insulin resistance during obesity. As an extension of these studies, the current study explored the specific role of AhR by depleting the gene in mature adipose tissue using an inducible Cre-loxp system.

1.4.5 Metabolic function difference in various AhR depleted mice models.

Previous studies from various labs, including ours, demonstrated that genetic depletion or pharmacological inhibition of AhR promotes healthy metabolic function. Male mice with germline depletion of AhR (AhRKO) have increased metabolic function marked by enhanced glucose, heterozygous AhR^{+/-}, or the presence of a congenic low-affinity AhR variant (B6.D2) protects from HFD-induced obesity and metabolic dysfunction (Kerley-Hamilton et al., 2012; Xu et al., 2015). Moreover, AhR antagonists (α -Naphthoflavone and CH-223191) can prevent HFD-

induced metabolic dysfunction and can improve function in metabolically impaired, obese mice (Moyer et al., 2016). These studies indicate that systemic depletion or inhibition of AhR enhances metabolic function. However, these studies lacked the specificity and understanding of the importance of AhR within individual tissues.

AhR is highly expressed in both liver and adipose tissue, both of which are important for the regulation of systemic metabolism and responses to HFD. Tissue-specific depletion can assist in understanding the contribution of AhR within a specific tissue to overall metabolic function. In comparison to AhRKO (global knockout), tissue-specific depletion has yielded different, and sometimes contradictory, results. Specific, gestational deletion of AhR from the liver exacerbates metabolic disease conditions, such as hepatic steatosis, under HFD, whereas conditional knockout from the adult liver helps ameliorate the pathology (Girer et al., 2019; Wada et al., 2016). Molecular studies substantiate these findings, where HFD treatment of animals with gestational excision showed augmented or unchanged gene expression related to various metabolic processes; there was an increase in lipogenesis and inflammation, and no differences in fatty acid uptake, β -oxidation, or gluconeogenesis. On the other hand, adult CKO of AhR from the liver demonstrated significantly less weight gain and adiposity from HFD. These animals had increased respiratory capacity of BAT and WAT, due to more production of FGF21 by the liver. Adipose-specific ablation of AhR during the gestational period also produces results that conflict with global germline depletion, where the group found augmented development of obesity (Baker et al., 2015).

1.5 Significance

Obesity and its associated diseases, such as diabetes, can develop through sex-specific mechanisms manifested by dissimilar gene expressions in metabolic tissues. AhR affects energy

homeostasis and gene expression patterns in many metabolic tissues including adipose tissue, liver, and brain, which also display differences between sexes. AhR contributes to sex-specific differences in a complex manner. AhR is ubiquitously present in adipose tissues and can influence its biology. Inhibition of adipogenesis from pre-adipocytes, impediment of lipolysis to reduce the release of free fatty acid (FFA), alteration of adipokine secretion, and promotion of pro-inflammatory cytokines release are some of the effects reported upon AhR activation in adipose (Fig 9) (Baker, Karounos, et al., 2013; Khazaal et al., 2023; Shimba et al., 2003; Shimba et al., 1998). Because adipose tissue function is critical to systemic metabolic function, and AhR influences adipose tissue biology, a better understanding of the specific role of AhR in adipose tissue is important. However, the effects of AhR deletion appear obfuscated by the timing of AhR deletion. Therefore, an inducible, tissue-specific depletion of AhR allows for better control of the timing of AhR depletion to understand the specific function of AhR in adipose tissue, in mediating systemic responses to HFD-induced metabolic dysfunction. In this study, the deletion of AhR in mature adipocytes of adult mice protects them from HFD-induced obesity and metabolic dysfunction in a sexually asymmetric way. Females are found to have robust protection from both weight gain and insulin resistance. Depletion in males remains protective but is less profound. These results highlight the possibility of therapeutically targeting AhR signaling in adipose tissue to combat obesity and insulin resistance in both sexes.

CHAPTER 2

HYPOTHESIS AND SPECIFIC AIMS

Adipose tissue plays a key role in regulating body weight and metabolism. Since AhR ligands are lipophilic and can cause chronic AhR activation in adipose tissue, AhR regulation in this tissue function has the potential to impact systemic metabolic homeostasis. Preliminary studies on AhR activation in preadipocytes (3T3-L1) and mature adipocytes indicate disruption of various metabolic pathways in adipose tissue, such as adipogenesis, and lipolysis. In contrast, global AhR deficiency improves metabolic parameters, for example in lipid and glucose metabolism. This study was undertaken to explore the contribution of AhR within adipose tissue to the regulation of systemic metabolism under conditions known to precipitate obesity and downstream metabolic pathology. Another key focus of this study was to investigate sex differences. There are fundamental sex differences in the progression of obesity and diabetes mediated by adipose tissue, and AhR signaling can be sex-biased. Thus overall, this study focuses on AhR regulation of adipose tissue in the development of obesity and metabolic dysfunction and variation between sexes. Mechanistically, this proposal examined the *overarching hypothesis that adipose-specific deletion of AhR will improve metabolic parameters in mice when challenged with a high fat diet.*

The overall hypothesis was tested using the following specific aims:

Specific Aim 1: Establish inducible adipose specific AhR knockout mouse models (CadKO) using Cre-loxp system and characterize them.

Hypothesis: Tamoxifen treatment will induce Cre-recombinase to deplete AhR specifically from mature adipocytes driven by adiponectin promoter.

Rationale: Previous studies have explored the role of AhR in mediating the systemic response to diet-induced obesity using different AhR-depleted mice (e.g., global; germline liver, and adipose-specific depletion) but not from matured adipocytes. Data from these labs and others support adipose tissue has a crucial site of AhR function to regulate metabolism. Hence, the current study tested the specific role of AhR in mature adipose tissue mediating local and systemic effects in diet-induced metabolism. To ensure time-specific depletion of AhR only from mature adipocytes, this study utilized a tamoxifen-responsive, adipose-specific AhR depletion.

Subaim 1.1: Construct inducible knock-out mouse model using Cre-loxp system that will delete AhR specifically from adipose depot in a time dependent manner.

The development of conditional knockout of AhR specifically from fat tissue using Cre-loxp system linked to a Rosa26loxP-STOP-loxPtdTomato reporter (Adiponectin-CreERTM::AhR^{loxP/loxP}::Tomato⁺ mice), provided an innovative approach for our study. To date, no lab has investigated metabolism with inducible conditional AhR-specific deletion from adipose tissue. This model specifically targets adipose tissue using adiponectin as a promoter and removes floxed exon 2 of AhR. The Rosa26loxP-STOP-loxPtdTomato reporter was incorporated to demonstrate recombination events. This allowed more control in these experiments since we could examine the AhR effects specifically in mature adipose tissue in adult mice.

Subaim 1.2: Evaluating the AhR excision event and functionality in the mouse model.

To examine the excising of AhR specific to adipose tissue in Adiponectin-CreERTM::AhR^{loxP/loxP}::Tomato⁺ mice with or without tamoxifen, multiplex PCR was used in different metabolic tissues (different adipose depot, liver, and muscle). Finally, to verify the functionality of the truncated AhR protein, downstream genes of AhR (Cyp1b1) were examined by qPCR after administering AhR agonist β -naphthoflavone (BNF).

Specific Aim 2: Phenotypic investigation of energy and glucose homeostasis in different AhR genotypes after subjecting mice to different diet regimen.

Hypothesis: Global and adipose-specific AhR deficiency will improve phenotypic parameters for weight and glucose homeostasis under high fat diet.

Rationale: This aim examined phenotypic differences by examining various metabolic characteristics. Data from this aim should provide the framework to direct molecular studies for the rest of the study. For example, indirect calorimetry patterns can complement thermogenesis gene/protein expression data to elucidate metabolic rate in mice. Two different AhR genotypes were used for this aim. Apart from CadKO, AhR global knockout (AhRKO) mice were used to compare the phenotypic differences between the models.

Subaim 2.1: Compare weight after subjecting mice to Normal Chow or High Fat Diet.

Body weight of all the genotype mice were measured each week throughout 12 week of normal chow (NCD) or high fat diet (HFD) regimen.

Subaim 2.2: Compare calorie intake, metabolic rates, and locomotor activity among various AhR genotypes.

The food weight of all the genotype mice was measured each week throughout 12 weeks of the NCD or HFD regimen and total calorie intake was measured at the end of the diet regimen. At week 13 of HFD, animals were singly housed for indirect calorimetry studies using the Oxymax Indirect Calorimetry System/Metabolic Cage from Columbus Instruments (CLAMS, Columbus, OH). During the subsequent week (week 14), activity behavior was monitored using infrared beam detectors to investigate locomotion.

Subaim 2.3: Compare fasting blood glucose level, glucose tolerance and serum insulin level to investigate glucose homeostasis.

Glucose Tolerance tests (GTT) were performed at week 15 to investigate both fasting blood glucose level and glucose tolerance. Blood was collected by cardiac puncture and serum insulin level was quantified by ELISA.

Specific Aim 3: Examine molecular changes in adipose tissue in CadKO after HFD feeding.

Hypothesis: Adipose specific AhR deficiency will improve adipocyte phenotype to improve diet-induced adipose hypertrophy, inflammation, and thermogenesis.

Rationale: Morphology and different molecular pathways in adipocytes, such as adipogenesis, lipolysis, and lipogenesis can affect the biology of adipocytes to have a systemic consequence and is crucial to examine. Moreover, examining various adipokine (i.e., leptin and adiponectin) and adipocytokine (i.e., TNF α , IL1 β , and IL6) levels in serum and adipose tissue can help us understand the inflammatory condition of the tissue, to determine whether they are metabolically healthy or unhealthy.

Activated BAT expends excess energy through the production of heat through the process called non-shivering thermogenesis under certain stimuli that increases β -adrenergic signaling. Browning of white adipose tissue is another key phenomenon for non-shivering thermogenesis that can be stimulated by similar as well as different stimuli compared to BAT thermogenesis. Hence, examining the gene expression of these processes can provide a molecular explanation for the metabolic rate differences.

Subaim 3.1: Compare changes in fat mass and examine morphology of WAT.

To determine percentage body fat, mice were weighed before dissection and gonadal fat pad (surrogate of VAT) and subcutaneous fat pad were weighed. Tissues collected were fixed in 4% paraformaldehyde for 24 hours, then processed for hematoxylin & eosin (H&E) staining.

Subaim 3.2: Examine molecular pathways of white visceral adipose depot.

Tissues collected were snap-frozen in liquid nitrogen. RNA was extracted to prepare cDNA followed by real-time PCR to quantify the transcript level of different gene expressions involving pathways such as adipogenesis, lipolysis, and lipogenesis. For protein detection at the subcellular level, a western blot was utilized.

Subaim 3.3: Examine adipokine secretion by adipose depot involving weight and glucose homeostasis.

Blood collected by cardiac puncture and serum protein level of leptin and adiponectin was quantified by ELISA. Tissues collected were snap-frozen in liquid nitrogen. RNA was extracted to prepare cDNA followed by real-time PCR to quantify the transcript level of adiponectin gene expression.

Subaim 3.4: Examine non-shivering thermogenesis by different depot of adipose.

Some portions of white (VAT) and brown (BAT) adipose tissues were snap-frozen in liquid nitrogen for RNA extraction and real-time PCR to quantify the transcript levels of different genes involved in the non-shivering thermogenesis pathways. For protein detection, some portions of the tissue were fixed with 4% paraformaldehyde for 24 hours and processed to perform immunofluorescence for UCP1.

Specific Aim 4: Explore hepatic steatosis (NAFLD) to examine systemic effect CadKO after HFD feeding.

Hypothesis: Adipose specific AhR deficiency will exert systemic effect and protect mice from diet induced hepatic steatosis/NAFLD.

Rationale: Aim 3 explored how adipose-specific AhR depletion affects adipose tissue biology in both sexes to explain some of the phenotypic differences observed in HFD. This aim focused on how the alteration of adipose biology can have a systemic effect. As the liver plays a central role

in metabolism, we examined NAFLD in liver tissue to obtain a molecular explanation for the systemic effect in CadKO mice. Moreover, NAFLD is correlated to adipose tissue inflammation and plays a pivotal role in insulin resistance, hence can suggest these disease conditions.

Morphology of the liver to investigate lipid spillover and different molecular pathways of the liver can suggest the magnitude and mechanism of steatosis respectively that can be different in CadKO mice. Moreover, processes like gluconeogenesis and lipogenesis help control glucose and lipid fluxes during fasting and feeding. Examining gene levels of such processes can help in deducing metabolic aspects of mice related to HFD-induced insulin resistance, hyperglycemia, and hyperinsulinemia.

Subaim 4.1: Examine weight and morphology of liver tissue.

Mice were dissected to collect whole liver organs and weighed. Some portions of the organ were fixed in 4% paraformaldehyde for 24 hours, then processed for hematoxylin & eosin (H&E) staining.

Subaim 4.2: Examine different molecular pathways of the liver.

Some portions of liver tissues collected were snap-frozen in liquid nitrogen. RNA was extracted to prepare cDNA followed by real-time PCR to quantify the transcript levels of different gene expressions involving pathways such as hepatic steatosis, lipogenesis, and gluconeogenesis.

Specific Aim 5: Explore hypothalamic feeding centers to examine systemic effect in energy regulation.

Hypothesis: Adipose specific AhR deficiency will exert systemic effect to improve crosstalk between adipocytes and hypothalamic energy regulatory regions under HFD.

Aim 2 demonstrated CadKO females consume fewer calories when presented with HFD compared to their WT counterparts. Adipose tissue can maintain systemic energy homeostasis by communicating with the brain regarding substrate availability, tissue mass, energy intake, and utilization via the afferent pathway. The brain responds to direct the periphery by making necessary changes to maintain the body homeostasis including energy balance via the efferent pathway. Leptin and estrogen are two key messengers to propagate this afferent signaling. Thus, this aim examined the serum protein and receptor level for these two important hormones in energy regulation. Increased receptor levels would be considered indicative of intact, healthy signaling.

Subaim 5.1: Examine Leptin signaling in energy regulatory regions of hypothalamus.

The brain was collected and fixed in 4% paraformaldehyde for 24 h and then transferred into 20% sucrose. Third ventricle (3V), Arcuate (ARC), and ventromedial hypothalamic (VMH) nucleus of the hypothalamus were located and collected as coronal sectioning to perform immunofluorescence using leptin receptor (LepR) antibody. Blood was collected by cardiac puncture and serum leptin level was quantified by ELISA.

Subaim 5.2: Examine estrogen signaling in energy regulatory regions of hypothalamus.

The brain was collected and fixed in 4% paraformaldehyde for 24 h and then transferred into 20% sucrose. Third ventricle (3V), Arcuate (ARC), and ventromedial hypothalamic (VMH) nucleus of the hypothalamus were located and collected as coronal sectioning to perform immunofluorescence using Estrogen Receptor-alpha ($ER\alpha$) antibody. Blood collected by cardiac puncture and serum 17- β estradiol (E2) level was quantified by ELISA.

CHAPTER 3

METHODS AND MATERIALS

3.1 Animals and experimental timeline

All experiments were approved by the *Institutional Animal Care and Use Committee* at Southern Illinois University School of Medicine and performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Experiments utilized 8-week-old mice. AhR flox (AhR^{fx/fx} strain designation AhR^{tm3.1Bra/J}) and Adiponectin-Cre with modified estrogen receptor (strain designation B6.FVB-Tg (Adipoq-Cre^{ER})1Evdr/J) were purchased from the Jackson Laboratory (Bar Harbor, ME). Adiponectin-CreER^{T2}, linked to a Rosa²⁶loxP-STOP-loxP-tdTomato reporter, were crossed with AhR^{fx/fx} mice to generate Adipoq-CreER^{T2}::AhR^{fx/fx}::Tomato⁺ mice that can be used as a tamoxifen-inducible AhR knockout mouse model. These mice were administered a single dose of tamoxifen (T5648; Sigma-Aldrich), either 75 or 150mg/kg, by intraperitoneal (IP) injection for two consecutive days to induce gene deletions. Mice were rested for 1-week to allow for tamoxifen metabolism. Multiplex PCR was used to analyze *AhR*^{fx} excision with forward primers 4062 (5-GTCACTCAGCATTACACTTTCTA) and 4064 (5-CAGTGGGAATAAGG CAAGAGTGA) in combination with the reverse primer 4088 (5-GGTACAAGTGCACATGC CTGC). Primers for the *AhR*^{fx}-excised allele (4062/4088) amplified a 180-bp band, whereas primers for the *AhR*^{fx}-unexcised allele (4064/4088) produced a 140-bp band.

Male and female Adiponectin-CreER^{T2}::AhR^{fx/fx}::Tomato⁺ with (CadKO) or without (WT) tamoxifen and global AhR null (AhRKO) mice were exposed to either a standard rodent chow (NCD, Lab diet formula 5001, Cat# 1319, St Luis, MO) or a 60% kcal high-fat diet (HFD, Research Diets #D12079B, #D12492, New Brunswick, NJ, USA) for 15 weeks. All mice were

housed on corn cob bedding in a pathogen-free climate and temperature-controlled facility. At week 13, animals were singly housed for indirect calorimetry studies using the Oxymax Indirect Calorimetry System/Metabolic System from Columbus Instruments (CLAMS, Columbus, OH) in which zirconia and infrared sensors are used to record O₂ and CO₂, respectively (detailed below). During the subsequent week (week 14), activity behavior was monitored using infrared beam detectors (Minimitter, Bend, OR) (detailed below). Glucose Tolerance tests (GTT) were performed (detailed below) at week 15.

All animals were sacrificed by cervical dislocation to collect gonadal WAT (a VAT depot), SAT, BAT, hypothalamus, liver, muscle, and serum. All tissues were snap-frozen in liquid nitrogen. Some portions of liver, VAT, and SAT were fixed in 4% paraformaldehyde for 24 hours, then processed for staining as detailed below.

3.2 Chemicals

All chemicals were prepared and stored according to manufacturer recommendations unless otherwise noted. Tamoxifen (Cat: T5648), Sodium chloride (Cat: 7647-14-5), Sucrose (Cat: 57-50-1), Sodium phosphate, Dibasic, Anhydrous (Cat: 7558-79-4), Potassium chloride (Cat: 7447-40-7), Dextrose (Cat: 50-99-7), Bovine serum albumin (Fraction V) (Cat: 9048-46-8) were obtained from Thermo Fisher Scientific (Waltham, MA). Porcine Insulin (Cat: 12584-58-6), Potassium phosphate (Cat: 7778-77-01) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Triton X-100 (Cat: 807426), Sodium borohydride (Cat: 102894) were obtained from MP Biomedicals (Santa Ana, CA).

3.3 Metabolic chamber

Total and resting metabolic rates were measured by indirect calorimetry using the Oxymax Indirect Calorimetry System/Metabolic System from Columbus Instruments (CLAMS,

Columbus, OH, USA). Mice were individually housed in respiratory chambers. All comparisons were based on mice studied simultaneously in eight different respiratory chambers connected to the same O₂ and CO₂ sensor to minimize the effects of environmental variation and instrument calibration. Mice had free access to food and water and were adapted to metabolic cages for 24 h before data collection. Gas samples were collected and analyzed every 5 min per animal; hourly averages were calculated. Output data include O₂ consumption (VO₂) (ml kg⁻¹ per minute), CO₂ production (VCO₂) (ml kg⁻¹ per minute), respiratory quotient (RQ = VCO₂/VO₂) and heat production (heat = CV × VO₂; CV = 3.815+(1.232 × RQ)). Columbus Instruments Equations for Energy Expenditure provides details (<http://www.colinst.com>).

3.4 Activity behavior monitoring

Animals assigned to behavior monitoring were housed in cages within the light chambers fitted with infrared activity detectors (Minimitter, Bend, OR). Actiview software was used to collect activity data into 6 min bins. Data were analyzed using Clocklab software (Actimetrics, Evanston, IL). The activity was calculated from actogram counts/minute.

3.5 Glucose Tolerance Test (GTT)

After 15 hours of fasting a drop of blood from the snipped tail were used to test the glucose concentration using a Contour Next EZ Glucometer (Parsippany, NJ) and mice were injected intraperitoneally with a bolus of 20% glucose at a dose of 1g/kg of body weight. Blood glucose was subsequently measured at 15, 30, 60, 90- and 120-minutes post-injection.

3.6 Histological analysis and Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E) and CD68 antibody at the Histology Department of Memorial Medical Center, Springfield IL. Quantification of adipocyte size was done on H&E-stained

sections using ImageTool software. Liver, VAT, and SAT were fixed in PBS + 10% formalin for 24 h, then stored in 70% ethanol. Single-blind, randomized images were obtained using a Nikon Eclipse E-600 microscope equipped with an Olympus-750 video camera system. Lipid droplets and adipocyte size were quantified using Image J Software 1.48 (RRID: SCR_003070) by an experimenter blinded to experimental conditions.

3.7 Immunofluorescence

Adipose and hypothalamus tissues were fixed in 4% paraformaldehyde for 24 h and then transferred into 20% sucrose in 0.1M phosphate buffer for 24 h prior to sectioning. 20 μ m adipose and hypothalamus sections (containing the ARC and VMH) were collected serially using a cryostat (Model HM525 NX, ThermoFisher Scientific). Sections were permeabilized with PBST (0.1M PBS with 0.25% TritonX-100), washed, treated with sodium borohydride in PBS (1mg/ml) for antigen retrieval, washed, blocked using 10%, normal goat serum/1% BSA for 1 hour, and then incubated overnight in primary antibody (listed in table 2) at 4°C. Sections were then washed PBST, incubated in secondary antibody (listed below and dilution) for 2 hours, washed and cover slipped using ProLong™ Gold antifade reagent with DAPI. Images were captured using Nikon Eclipse E-600 microscope equipped with an Olympus-750 video camera system. Staining intensities were measured using National Institute of Health Image J Software 1.48 (RRID:SCR_003070). Staining intensity was obtained after background staining was subtracted from mean intensities. Antibody for immunofluorescence are listed in table 2.

3.8 Western Blot

Adipose (VAT & SAT), liver, and muscle tissue were homogenized in tissue protein extraction TPER lysis buffer and centrifuged at 12,000 g for 15 minutes at 4°C. Protein was quantified using the BSA assay. Proteins were separated using SDS-page and transferred onto a

nitrocellulose membrane. Membranes were blocked for 1 hour with 5% serum BSA and incubated at 4°C overnight with primary antibodies (listed in table 2). Membranes were washed with TBST and incubated with secondary antibodies for 1 hour at room temperature. Bands corresponding to the protein of interest were scanned using LI-COR Odyssey. Beta-actin was used as the loading control. Antibodies for western blot is listed in table 2.

3.9 q-PCR

Total RNA was extracted using Trizol and cDNA was synthesized. SYBR green-based real-time reverse transcriptase PCR was carried out on AB 1 step one plus real-time PCR system. Values for genes of interest were normalized using B₂M as the housekeeping gene and the relative levels of mRNA were determined using the $\Delta\Delta C_t$ method. Primers sequence for real time PCR is listed in table 3.

3.10 ELISA

Blood samples were collected at sacrifice. Serum leptin (90030, Crystal Chem), insulin (90080, Crystal Chem), adiponectin (KMP0041, Novex) and 17 β -estradiol (ab108667, Abcam) were measured by ELISA according to the manufacturer's instructions. Triglycerides were quantified using triglycerides liquid reagent set (Pointe Scientific INC. Cat# T7532-120).

3.11 Statistical Analysis

Data are presented as mean \pm SEM. The rate of change of metabolic rate was calculated utilizing linear regression in GraphPad Prism Software V6.0. As mentioned in the text, ANCOVA was utilized to determine if two slopes were different from one another. A one-way or two-way ANOVA with Tukey's post hoc tests was utilized to identify significant differences between groups, where appropriate. P values of less than 0.05 were considered statistically significant.

CHAPTER 4

RESULTS

4.1 Specific Aim 1: Establish inducible adipose specific AhR knockout mouse models (CadKO) using Cre-loxp system and characterize them.

Many AhR ligands, including metabolites of HFD, are lipophilic and accumulate in adipose tissue to chronically activate AhR. Previous data from the Tischkau lab support adipose tissue as a crucial site of AhR function to regulate metabolism. Moreover, previous studies using different AhR-depleted mouse models (e.g., global; germline liver, and adipose-specific depletion) have established a role for AhR in mediating systemic metabolism on HFD. These studies also support AhR's crucial role in adipose tissue biology (Baker, Karounos, et al., 2013; Khazaal et al., 2023; Shimba et al., 2003; Shimba et al., 1998). The field still lacks AhR-specific role in matured adipocytes and how this affects them systemically, which is the aim of the current study. To ensure time-specific depletion of AhR only from mature adipose tissue, the present study utilized a tamoxifen-responsive adipose-specific AhR deletion.

Hypothesis: Tamoxifen treatment will induce Cre-recombinase to deplete AhR specifically from mature adipocytes driven by adiponectin promoter.

4.1.1 Results Subaim 1.1: Construct an inducible knock-out mouse model using the Cre-loxp system that will delete AhR specifically from adipose tissue in a time-dependent manner.

Floxed AhR (AhR^{fx/fx} strain) and Adiponectin-Cre with modified estrogen receptor (Adiponectin-CreER^{T2}), that were linked to a Rosa²⁶loxP-STOP-loxP-tdTomato reporter, crossed together to generate Adiponectin-CreER^{T2}::AhR^{fx/fx}::Tomato^{+/+} mice that can be used as a tamoxifen-inducible AhR knockout mouse model (Fig 10, left panel).

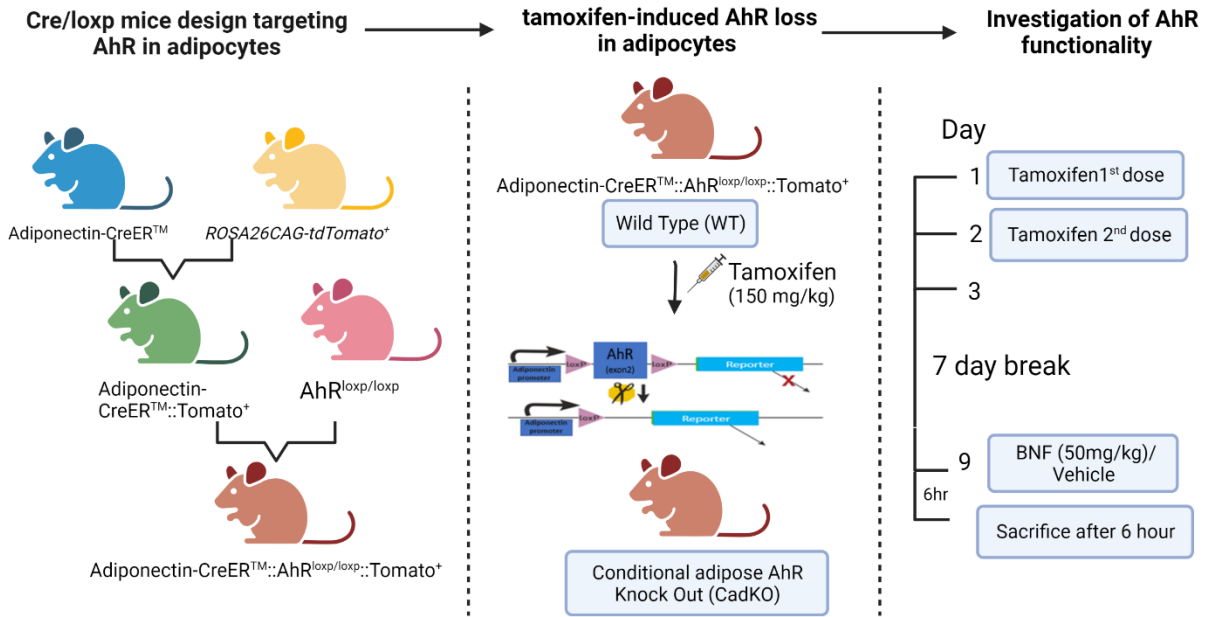


Fig 10. Construct of inducible knock-out mouse model using Cre-Loxp system and experimental approach to investigate AhR functionality. Left and Middle Panels: Schematic representation of tamoxifen inducible Cre/loxP mice design and its reporter (tomato) construct to target AhR specifically from adipose tissue driven by adiponectin promoter. Right lane: Experimental plan to investigate AhR loss and its functionality by injecting its agonist BNF (50mg/kg) after tamoxifen (75 or 150mg/kg) injection for two consecutive days. Created by BioRender.com.

The inclusion of the *Rosa26^{loxP-STOP-loxP-tdTomato}* reporter allowed visualization of *Cre-recombinase* activity in tissues. After administration of tamoxifen, (75 or 150 mg/kg), by intraperitoneal (IP) injection for two consecutive days to induce gene deletions (Fig 10, middle panel), tissues were processed and examined for expression of the *tdTomato* reporter. High expression for *tdTomato* was observed in the tamoxifen-treated groups (75mg/kg and 150mg/kg) in different adipose depots (visceral, subcutaneous, brown) compared to the non-treated group

suggesting high *Cre-recombinase* activity (Fig 11). However, we observed robust *tdTomato* expression in the 150mg/kg treatment group compared to the 75 mg/kg, suggesting a dose-dependent effect in the *Cre-recombinase* activity (Fig 11).

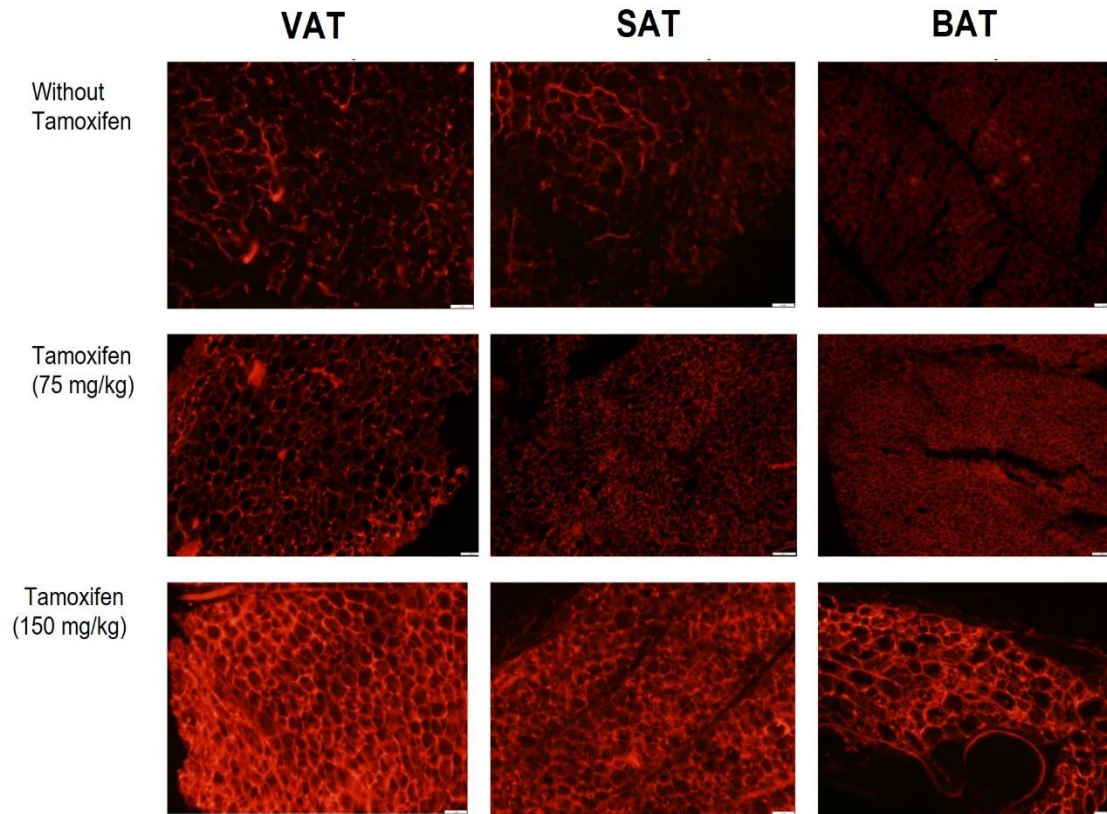


Fig 11. Tomato expression with or without tamoxifen in different adipose depot. Upper panel: fluorescent microscopy to examine tomato expression of visceral (VAT), subcutaneous (SAT) and brown (BAT) adipose tissue cluster from left to right without tamoxifen administered mice. Middle panel: fluorescent microscopy to examine tomato expression of VAT, SAT, and BAT cluster from left to right with 75mg/kg tamoxifen administered mice. Lower panel: fluorescent microscopy to examine tomato expression of VAT, SAT, and BAT cluster from left to right with 150mg/kg tamoxifen administered mice.

4.1.2 Results Subaim 1.2: Evaluating the AhR excision event and functionality in the mouse model.

Multiplex PCR was used to analyze *AhR^{fx}* excision events with forward primers 4062 and in combination with the reverse primer, 4064 and 4088 in different metabolic tissues (adipose depots, liver, and muscle). Primers for the *Ahr^{fx}*-excised allele (4062/4088) amplified a 180-bp band, whereas primers for the *Ahr^{fx}*-unexcised allele (4064/4088) produced a 140-bp band (Fig 12.a). The unexcised allele (140-bp) was present in all the tissues for tamoxifen treatment (CadKO) and without treatment (WT) mice (Fig 12.b). Whereas, tamoxifen treatment (CadKO) generated the excised allele (180-bp), which was not seen without the treatment (WT) mice (Fig 12.b), suggesting successful excision events when tamoxifen was administered. Next, to examine AhR functionality in white adipose depot (VAT was used as a representative) after the excision, downstream AhR gene (*Cyp1b1*) expression was investigated after 6-hr of AhR agonist (BNF) treatment (Fig 10, right panel). *Cyp1b1* mRNA expression was minimal similar to the vehicle-treated group in VAT of CadKO male and female mice in response to the AhR agonist, BNF (50mg/kg) (Fig 12.c).

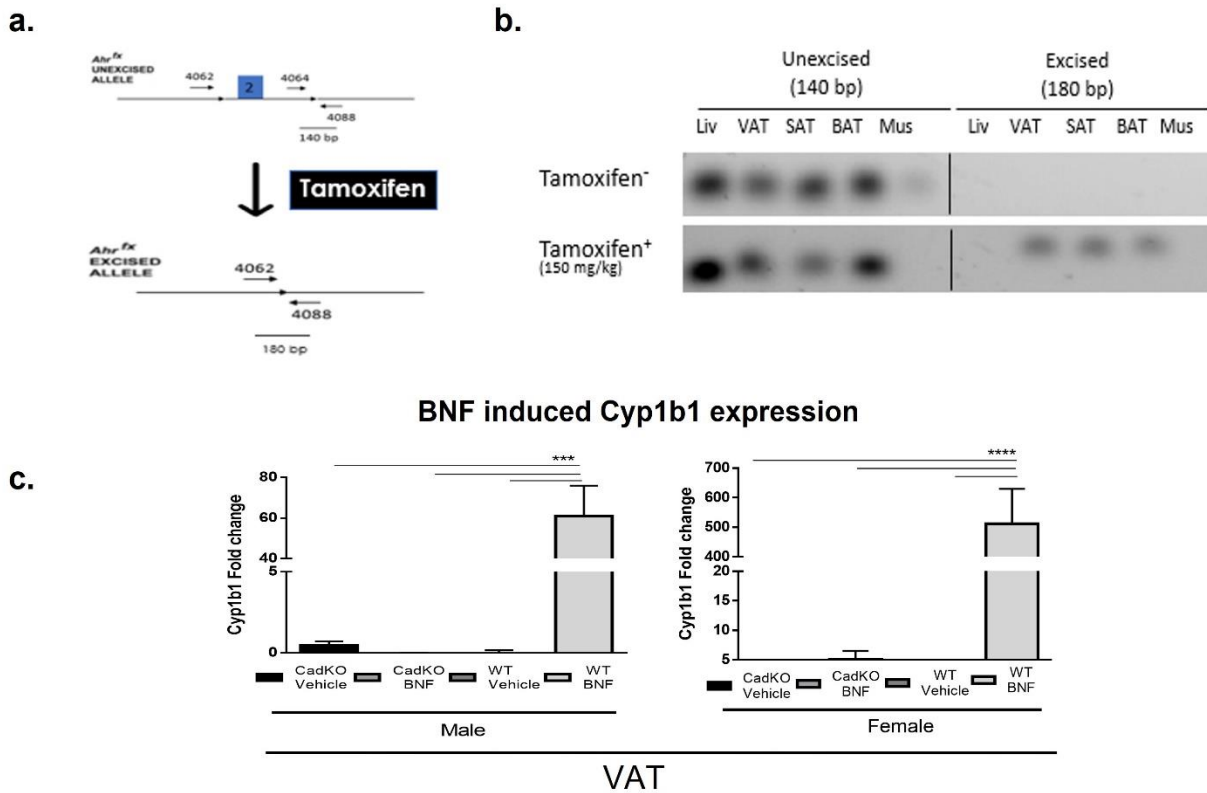


Fig 12. Specificity of *Cre recombinase*-mediated excision of AhRfx allele upon tamoxifen administration. (a) Diagrammatic representation of the AhR^{fx}-unexcised and the AhR^{fx}-excised alleles. Solid lines represent the fragment sizes generated by PCR amplification of the AhR^{fx}-unexcised and AhR^{fx}-excised allele using the forward primers (4062 and 4064) and the reverse primer (4088). (b) Specificity of excised events was determined by genotyping for both the unexcised (left panel) and excised (right panel) alleles in genomic DNA of various tissues collected from AdiponectinCre^{ERT2}:AhR^{fx} mice with (upper panel) or without (lower panel) tamoxifen. Liv: Liver; VAT: Visceral Adipose Tissue; SAT: Subcutaneous Adipose Tissue; BAT: Brown Adipose Tissue; Mus: Muscle. (c) VAT were harvested, and total RNA extracted to quantify mRNA expression level by real-time PCR analysis. Level of Cyp1b1 (downstream of AhR gene) mRNA expression in VAT upon BNF or Vehicle treatment in male (left) and

female(right) CadKO and WT mice. PCR data were normalized against the amount of B2M. n=4-5 for each group by one-way ANOVA with Tukey's post hoc comparison. ***p<0.001, ****p<0.0001 by one-way ANOVA with Turkey's post hoc comparison. p values of less than 0.05 were considered statistically significant.

4.1.3 Summary

Collectively, these data examined successful tamoxifen inducible *Cre-recombinase* activity and validate the depletion of AhR that compromised AhR functionality in *Adiponectin-CreERT2:AhR^{loxP/loxP}* mouse.

4.2 Specific Aim 2: Phenotypic investigation of energy and glucose homeostasis in different AhR genotypes after subjecting mice to different diet regimen.

Body weight is maintained by homeostatic control of energy balance involving regulation of energy intake and output/expenditure. Coordination among multiple organs regulates feeding behavior, energy expenditure (EE) and physical activity that contributes in maintaining energy balance (Broberger, 2005). Furthermore, glucose (body primary fuel source) is also maintained by a delicate balance by the body as its level is critical for mammalian life. Glucose homeostasis is disrupted during metabolic dysfunction when serum glucose burden leads to β -cell exhaustion under constant pressure that leads to T2D. Insulin, produced by the pancreas, is a key hormone that helps to transports glucose inside the cells and lower its serum level. Altered levels of blood glucose are good indicators of T2D. Fasting glucose, glucose tolerance and serum insulin level are some of the diagnostic tools used for detecting the early stage of T2D. Sex differences exist across multiple organs in the regulation of energy and glucose homeostasis.

The AhR was implicated in the regulation of energy and glucose metabolism and this study examined different AhR genotypes in such regulation. Apart from CadKO, AhR global

knockout (AhRKO) mice were incorporated to compare the phenotypic differences between animals where AhR was only knocked out in adult adipose tissue (CadKO) and those where AhR was depleted in the germline (AhRKO). Phenotypic investigation of energy and glucose homeostasis by examining various metabolic characteristics provides a solid framework and direction for molecular studies. Male and female Adiponectin-CreER^{T2}::AhR^{fx/fx}:: Tomato⁺ with (CadKO) or without (WT) tamoxifen and global AhR null (AhRKO) mice were exposed to either a standard rodent chow (NCD) or a 60% kcal high-fat diet (HFD) for 15 weeks (Fig 13). Body and food consumed weight per week measured for 12 weeks. Total calorie intake was measured at the end of week 12 (Fig 13). During the subsequent week (week 13), animals were singly housed for indirect calorimetry studies using the Oxymax Indirect Calorimetry System/Metabolic Cage in which zirconia and infrared sensors are used to record O₂ and CO₂ to examine energy expenditure (Fig 13). At week 14, activity behavior was monitored using infrared beam detectors (Fig 13). Glucose Tolerance tests (GTT) were performed at week 15 (Fig 13). A similar experiment was conducted previously on AhRKO male mice by our lab. The data acquired in the present study were compared with that previous study where applicable.

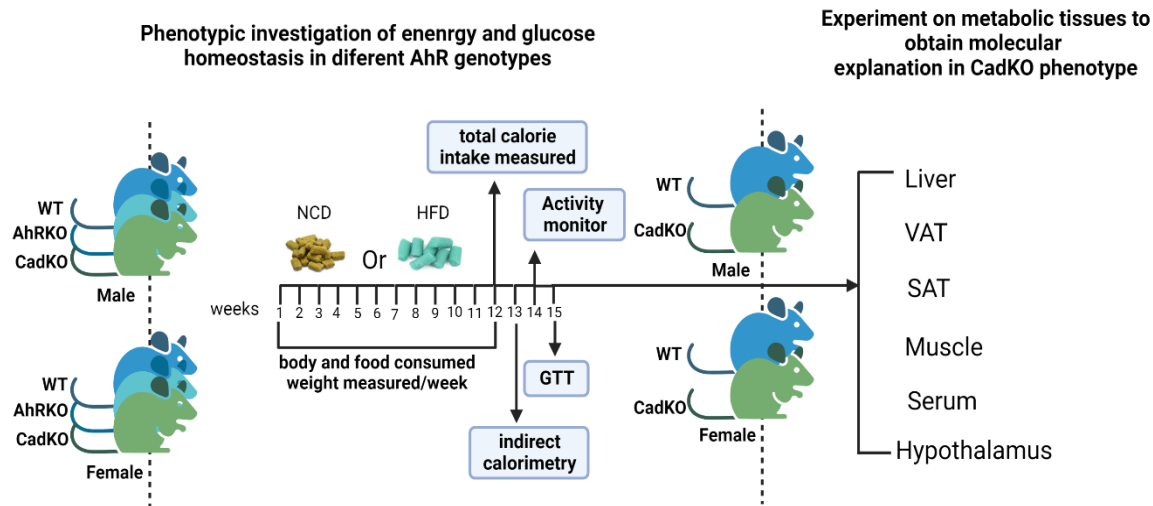


Fig 13. Schematic representation of experimental design to examine phenotypical comparison between different AhR genotypes and obtain molecular explanation for CadKO mice.

Abbreviation: NCD, Normal Chow Diet; HFD, High Fat Diet; GTT, Glucose Tolerance Test.

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Hypothesis: Global and adipose-specific AhR deficiency will improve phenotypic parameters for weight and glucose homeostasis under high fat diet.

4.2.1 Results Subaim 2.1: Compare weight after subjecting mice to Normal Chow or High Fat Diet.

Previous studies from our lab indicate global depletion of AhR (AhRKO) protects male mice from HFD-induced obesity and metabolic dysfunction (Jaeger et al., 2017; Xu et al., 2015). This study explored and compared the effects of adipose-specific deletion of AhR (CadKO) in diet-induced metabolic dysfunction with AhRKO, including both sexes. Weight gain within sex was not different among the genotypes throughout NCD regimen (Fig 14.a, b). AhRKO and

CadKO males gained less weight than WT on the HFD, but more weight than all genotypes on NCD with significant differences apparent at week 4 (Fig 14.a). Interestingly, the statistical significance between WT and CadKO males was reduced at week 11 and was no longer different at week 12. In contrast, AhRKO males maintained the robust significant difference throughout the HFD regimen.

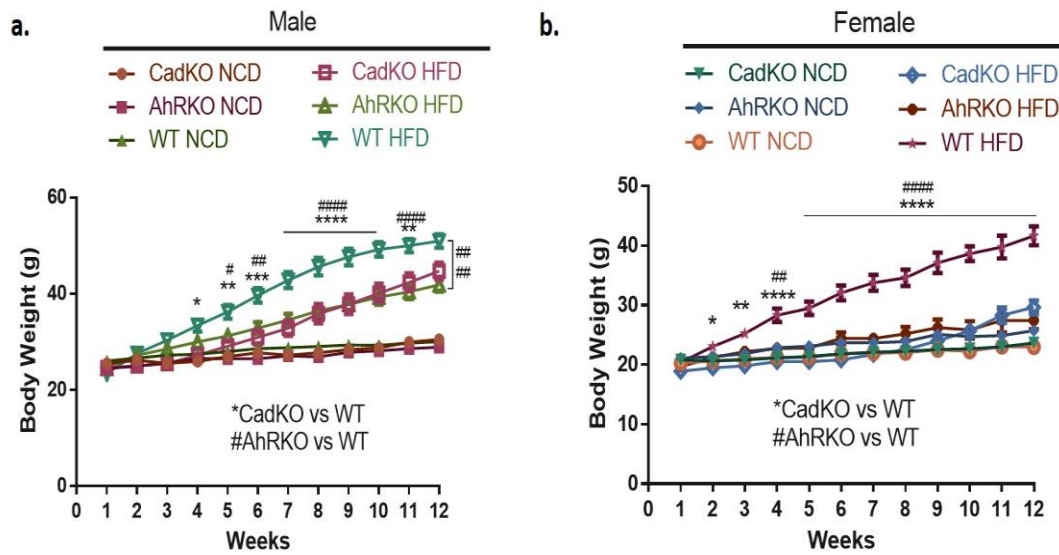


Fig 14. Global and adipose-specific AhR deletion protect mice from HFD induced weight gain in both the sexes. (a, b) Body weight gain in AhRKO (n=8), CadKO (n=8) and WT (n=8) mice for 12 weeks of NCD and HFD in male (a) and female (b). */#p<0.05, **/##p<0.01, ***/###p<0.001, ****/####p<0.0001 by 2-way ANOVA with Turkey's post hoc comparison. p values of less than 0.05 were considered statistically significant. Two-independent study (n=4, for each group). Abbreviation: NCD, Normal Chow Diet; HFD, High Fat Diet.

Depletion of AhR on the other hand had a greater protective effect from HFD-induced weight gain in females. CadKO and AhRKO females weighed less than WT as early as week 2

and differences were maintained throughout the experiment. Interestingly, HFD-fed female AhRKO and CadKO weighed the same as NCD-fed mice of all genotypes (Fig 14.b). Therefore, the removal of AhR in females completely negated HFD-induced weight gain. Changes in weight gain in CadKO mice on HFD were also tamoxifen dose-dependent, suggesting higher dose may excise AhR from greater numbers of adipocytes exerting a greater effect on weight homeostasis (Fig 15.a, b).

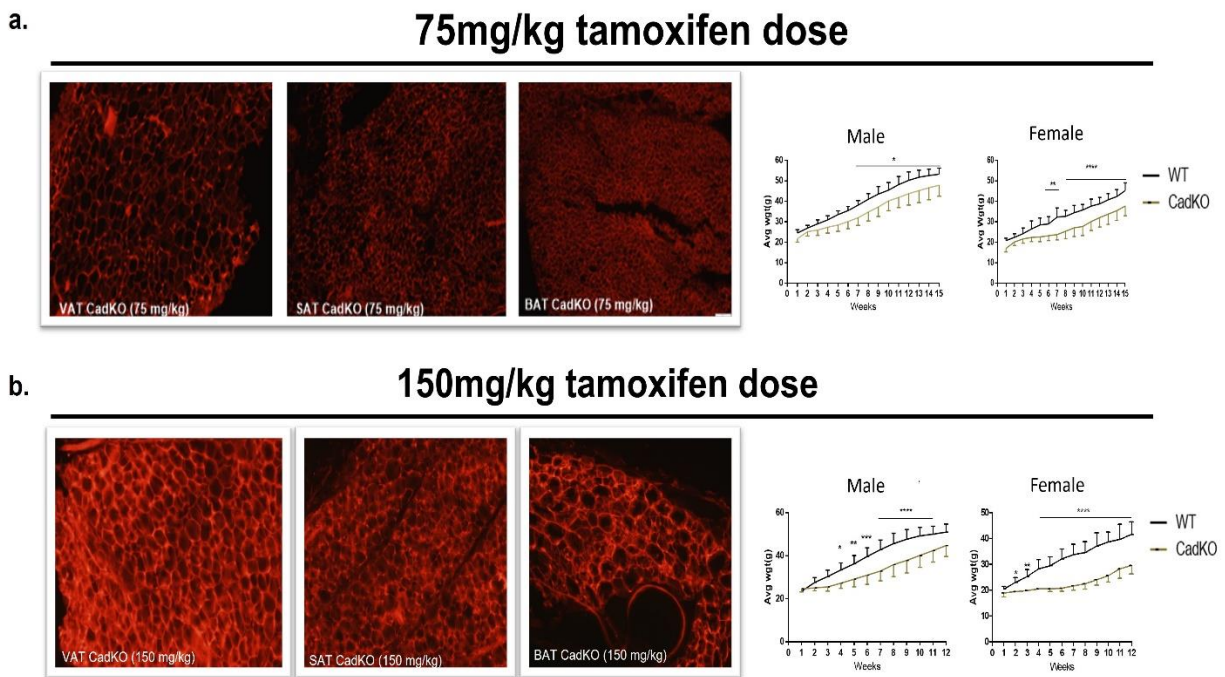


Fig 15. Tamoxifen dose dependent tomato expression and weight difference. (a) Fluorescent microscopy to determine tomato expression of different adipocytes (visceral, subcutaneous, brown: from left to right), and weight difference between WT (n=8) and CadKO (n=8) mice when administered 75mg/kg of tamoxifen on HFD. (b) Fluorescent microscopy to determine tomato expression of different adipocytes (visceral, subcutaneous, brown: from left to right), and weight difference between WT (n=8) and CadKO (n=8) mice when administered

150mg/kg of tamoxifen on HFD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by one-way ANOVA with Turkey's post hoc comparison. p values of less than 0.05 were considered statistically significant. Abbreviation: VAT, Visceral Adipose Tissue; SAT, Subcutaneous Adipose Tissue; BAT, Brown Adipose Tissue; HFD, High Fat Diet.

4.2.2 Results Subaim 2.2: Compare calorie intake, metabolic rates, and locomotor activity among various AhR genotypes on high fat diet.

To examine differences in weight gain, various metabolic parameters were examined. 12 weeks net calorie intake was similar in males of all genotypes on both diets (Fig 16.a). No significant difference was detected in caloric intake or total food consumption throughout the HFD regimen for males (Fig 16.a, b). However, both the CadKO and AhRKO females on HFD consumed similar amounts of calories as NCD females of all genotypes and were significantly lower compared to WT (Fig 16.a). WT females also consumed significantly more amount of food and calories on HFD throughout the experiment (Fig 16.a, c).

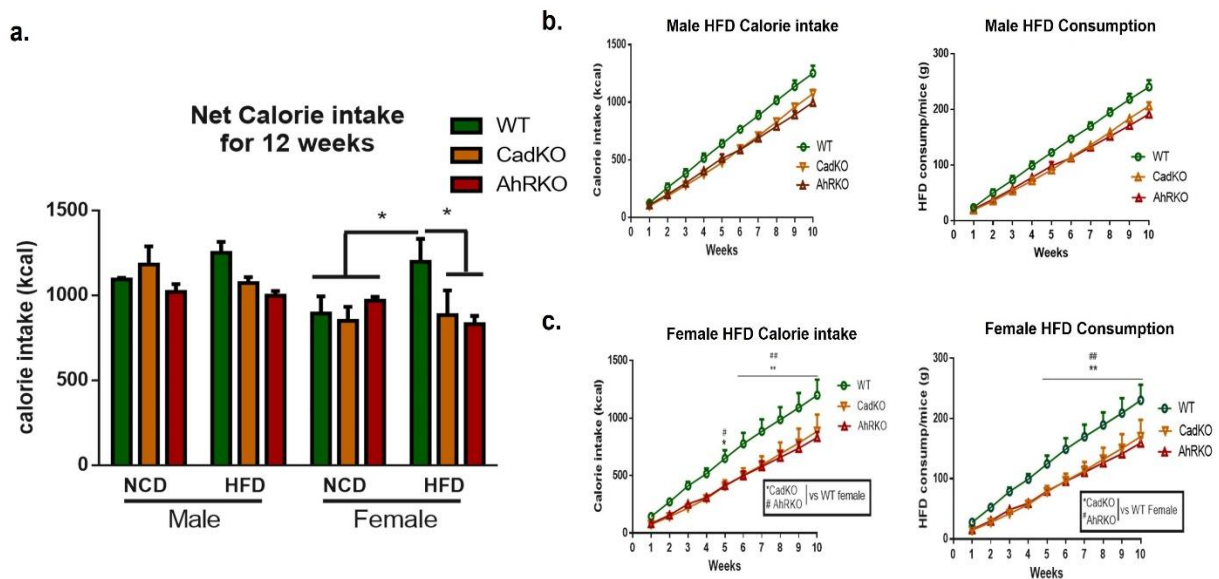


Fig 16. Global and adipose-specific AhR deletion reduces calorie intake on HFD in females.

(a) Total average kilocalories consumed by AhRKO (n=4), CadKO (n= 4) and WT (n=4) mice after 12 weeks of NCD (3.36kcal/g) and HFD (5.21 kcal/g). (b, c) Progressive calorie intake and food consumption in male (b) and female (c). */#p<0.05, **/##p<0.01 by 2-way ANOVA with Turkey's post hoc comparison. p values of less than 0.05 were considered statistically significant. Abbreviation: NCD, Normal Chow Diet; HFD, High Fat Diet.

Indirect calorimetry suggested that mass independent EE, indicated by similar regression lines, was not different among all male genotypes (Fig 17.a). Similarly, daily overall vO₂, vCO₂ and mass-dependent EE were unchanged in CadKO and AhRKO males (Fig 17.c-e) In contrast, both AhRKO and CadKO females displayed higher mass-independent EE on HFD, indicated by separate regression lines (Fig 17.b). Daily overall vO₂, vCO₂ and mass-dependent EE were also significantly higher in CadKO and AhRKO females (Fig 17.c-e), indicating higher metabolic activity in female animals where AhR has been depleted. Respiratory quotient was in the range of 0.7-0.8 among all genotypes suggesting the use of mostly fat as a fuel source on HFD (Fig 17.f). Overall, these data suggest protection against HFD-induced weight gain by AhR depletion occurs through sex-specific mechanisms.

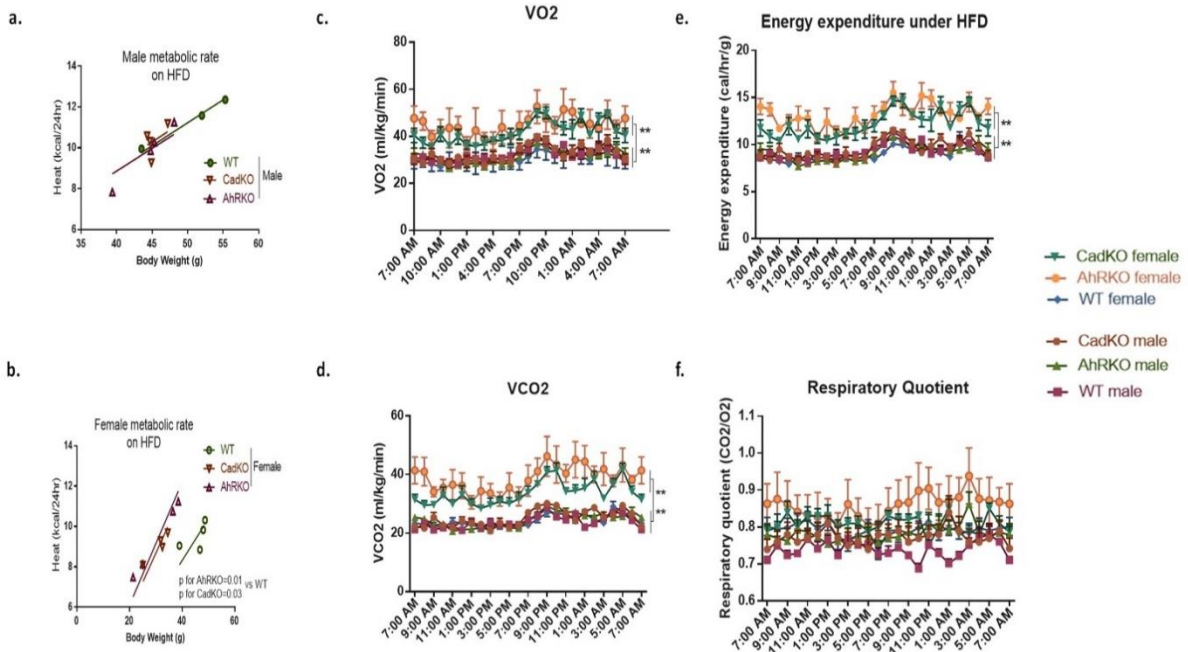


Fig 17. Global and adipose-specific AhR deletion in females increased energy expenditure on HFD. (a, b) Total mass-independent metabolic rates of AhRKO, CadKO and WT mice were measured at week 13 of HFD diet using the Oxymax Indirect Calorimetry System/Metabolic Cage from Columbus Instruments (CLAMS) for 24 hours in male (a) and female (b) mice. (c-f) Summary of 24hr VO₂ intake (c) and CO₂ expelled (d) were used to calculate energy expenditure (e) and respiratory quotient (f). Please refer to Columbus Instruments\Equations for Energy Expenditure and Respiratory Quotient calculation details (<http://www.colinst.com/brief.php?id=61>). n=4, ****p<0.00001 by 2-way ANOVA with Turkey's post hoc comparison. ANCOVA was utilized to determine if two slopes were different from one another (a, b), p values of less than 0.05 were considered statistically significant.

Next, we examined locomotor activity to investigate the influence of physical activity on energy output. WT mice displayed less overall activity on HFD compared to NCD (Fig 18). However, activity of AhRKO and CadKO animals on HFD was maintained at levels similar to NCD. No sex differences in activity were observed.

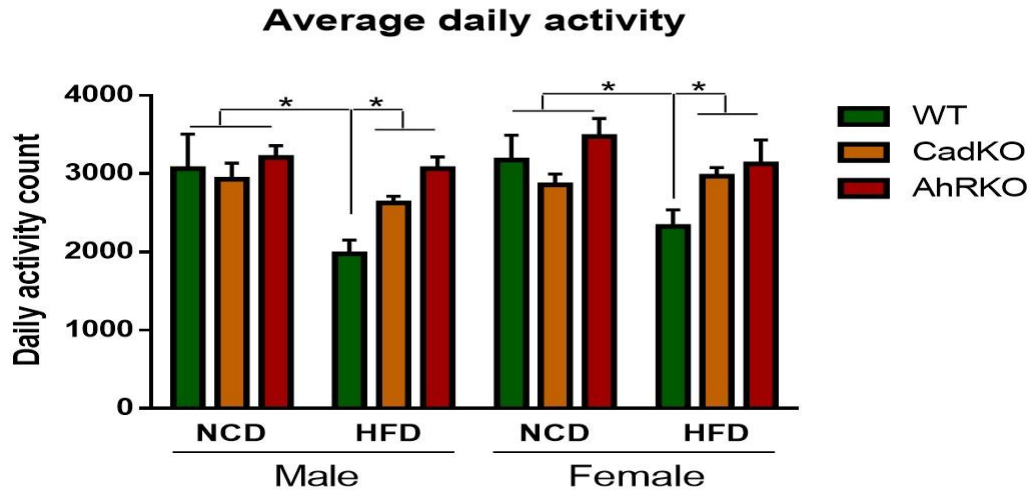


Fig 18. Adipose-specific AhR deletion protects against decreased locomotor activity on HFD.

Locomotor activity (n=7-8) as determined under normal light-dark cycle after 12 weeks of NCD or HFD using infrared beam interruption sensor. Daily activities were measured by summation of both day and night activity during the period of lights on (7am to 7pm) and lights off (7pm to 7am) respectively and averaged taken from 7 days data. *p<0.05, by 2-way ANOVA with Turkey's post hoc comparison. p values of less than 0.05 were considered statistically significant. Abbreviation: NCD, Normal Chow Diet; HFD, High Fat Diet.

4.2.3 Result Subaim 2.3: Compare fasting blood glucose, glucose tolerance and serum insulin level to investigate glucose homeostasis.

To investigate whether protection from weight gain in CadKO is concurrent with improved glucose homeostasis, fasting glucose was measured and subsequent glucose tolerance was examined by GTT. Genotype or sex did not affect fasting glucose under NCD (Fig 19.a). HFD on the other hand increased fasting glucose in WT males and females. As expected, based on previous study (Xu et al., 2015), AhRKO males had decreased fasting glucose on HFD. However, fasting glucose in HFD-fed CadKO males was similar to WT. In contrast, both the AhRKO and CadKO females exhibited significantly lower fasting glucose on HFD than WT (Fig 19.a).

AhRKO and CadKO males showed significantly better glucose tolerance than WT on NCD (Fig 19.b, d). Glucose tolerance in HFD-fed males was not different among genotypes, although AhRKO was nearly significant ($p=0.07$) (Fig 19.e, g). GTT was not different among female genotypes on NCD (Fig 19.c, d), nevertheless, both AhRKO and CadKO females displayed significantly better glucose tolerance on HFD compared to WT (Fig 19.f, g).

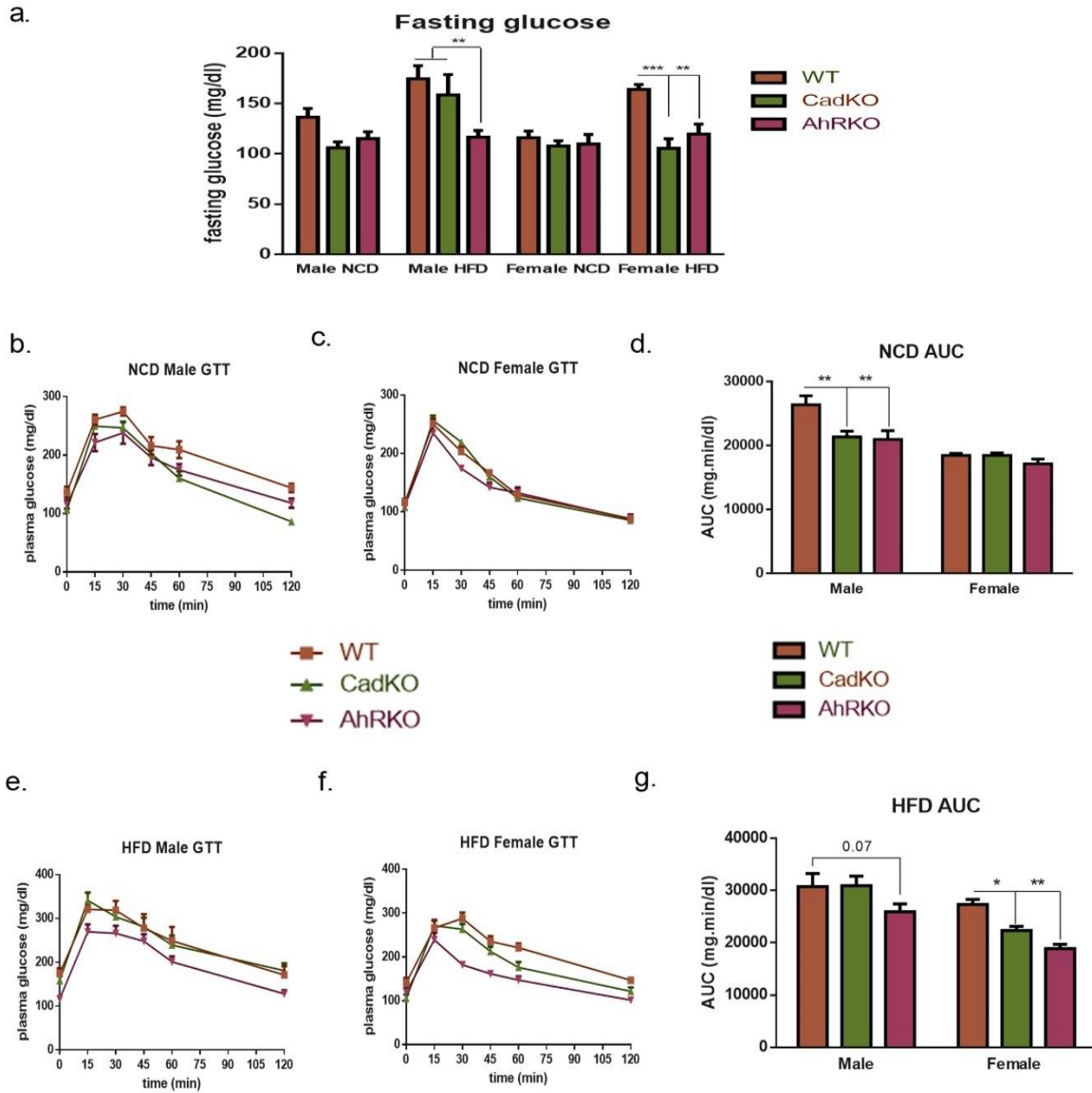
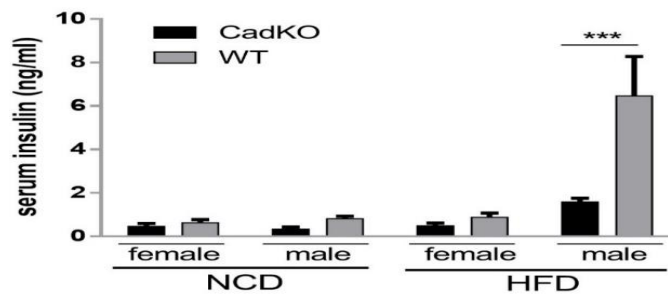


Fig 19. CadKO improves fasting glucose and systemic glucose tolerance in females on HFD. (a) 15 hours fasted male and female blood glucose levels were obtained from tail vein of WT (n=8-10), CadKO (n=8-10) and AhRKO (n=8-10) mice after 15 weeks of NCD and HFD fed. Two-independent study (n=4-5, for each group). (b-g) GTT after 15 weeks of NCD (b, c) and HFD (e, f) for male (b, e) and female (c, f) WT, CadKO and AhRKO mice. (d, g) Area under

the curve (AUC) for each time point were measured to obtain glucose sensitivity for NCD (d) and HFD (g) groups. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ by 2-way ANOVA with Turkey's post hoc comparison. p values of less than 0.05 were considered statistically significant.

As glucose homeostasis strongly correlates with insulin secretion, we measured serum insulin level. Insulin levels in WT males were substantially increased by HFD compared to CadKO suggesting HFD-induced hyperinsulinemia (Fig 20.a). Previous study conducted in the Tischkau lab revealed similar observations in AhRKO males (Fig 20.b). In contrast, insulin was unchanged with HFD feeding in females of both genotypes similar as NCD (Fig 20.a).

a.



b.

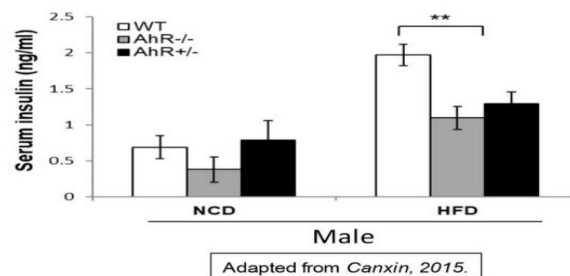


Fig 20. Global and adipose specific AhR deficiency protects mice from HFD induced hyperinsulinemia. (a) Serum insulin concentration was assessed for CadKO and WT male and female mice by ELISA commercial kit after 15 weeks of NCD (n=5-6) and HFD (n=8-10). (b)

Figure adapted from Canxin et al, 2015. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by 2-way ANOVA with Turkey's post hoc comparison. p values of less than 0.05 were considered statistically significant.

4.2.4 Summary

The present study demonstrated AhRKO and CadKO protect mice from HFD-induced weight gain in a sex-specific manner. In males, depletion of AhR in adipose tissue alone does not afford the same levels of protection as global depletion, as shown by weight gain, metabolic parameters, and glucose homeostasis. CadKO males, however, are protected from HFD-induced hyperinsulinemia, suggesting less β -cell load, which can delay the onset of T2D. Female AhR deficiency on the other hand reduced calorie consumption and increased metabolic rates on HFD. This study also suggests that depletion of AhR from adipose tissue alone is sufficient to provide widespread protection as global knockout in females from HFD-induced changes not only in weight but also in glucose homeostasis (demonstrated by improved fasting and glucose tolerance).

4.3 Specific Aim 3: Examine molecular changes in adipose tissue in CadKO after HFD feeding.

Adipose tissue is a critical regulator of body weight and glucose homeostasis through changing the physiological dynamic locally and systemically. Not only does WAT store and release energy depending on metabolic demand, but also releases numerous proteins that have a significant impact on endocrinology and systemic metabolism (Frühbeck, 2008; Kershaw & Flier, 2004). Moreover, brown and beige adipose tissue dissipates energy as a form of heat to maintain normothermia as well as to counteract obesity. Therefore, the role of adipose tissue in obesity and insulin resistance is extremely crucial. AhR activation can contribute to metabolic disorders, as it can regulate various genes associated with weight and glucose homeostasis. AhR

is abundantly expressed in adipose tissue and its activation has a significant impact on adipose tissue biology, including the processes of adipogenesis, lipolysis, and mediation of inflammation (McMillan & Bradfield, 2007; Sayed et al., 2022). Thus, the present aim examined the contribution of AhR in changing the local physiology of adipose tissue in quest of obtaining a molecular explanation for the phenotypical differences observed in Aim 2.

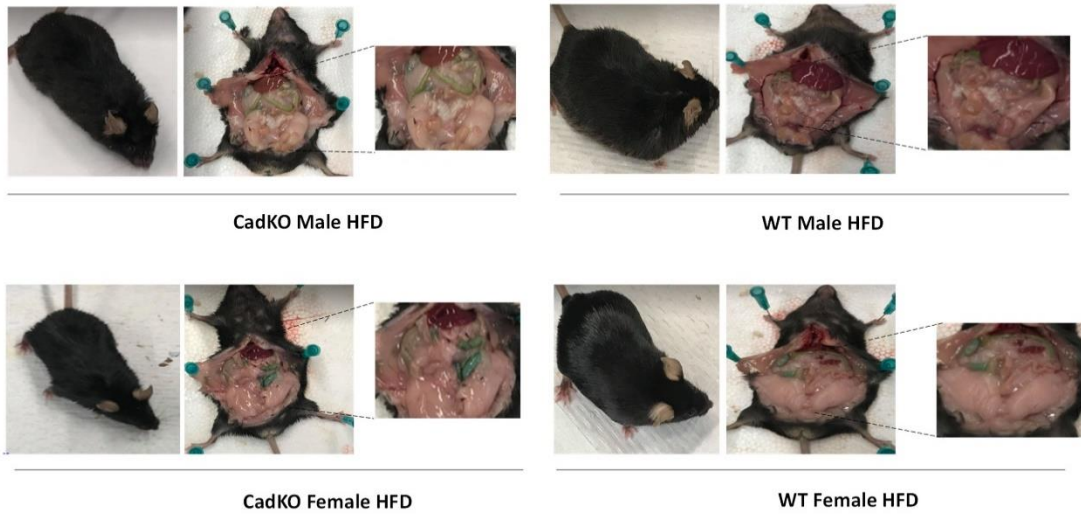
Hypothesis: Adipose specific AhR deficiency will improve adipocyte phenotype to improve diet-induced adipose hypertrophy, inflammation, and thermogenesis.

4.3.1 Results Subaim 3.1: Compare changes in fat mass and examine morphology of WAT.

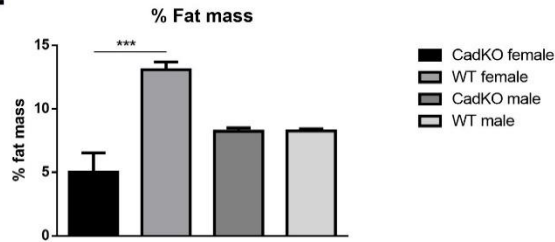
Changes in adipose tissues were explored in HFD-fed animals to explore mechanisms underlying the phenotypic changes. In males, gross morphology, and overall fat mass reveals CadKO did not protect from the overall accumulation of body fat (Fig 21.a, b). Exploration of individual fat depots reveals that VAT weight was similar for both WT and CadKO males (Fig 21.c). However, CadKO males were protected from increased SAT mass on HFD (Fig 21.c). As males tend to accumulate fat in VAT before SAT, this may suggest that VAT was saturated, while SAT was not.

In females, gross morphology and overall fat mass were significantly reduced in HFD-fed CadKO females (~5% fat mass) compared to HFD-fed WT females (~13% fat mass) (Fig 21.a, b). As expected, both white adipose depots (VAT and SAT) were significantly higher in WT females (VAT= 8.5%, SAT= 4.6%) compared to CadKO (VAT= 3.5%, SAT=2.9%) (Fig 21.c).

a.



b.



c.

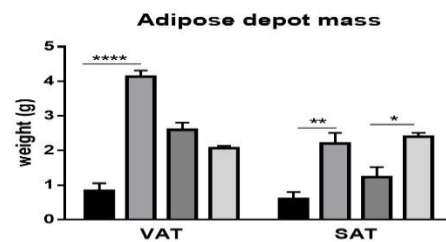


Fig 21. CadKO reduces adiposity in female mice. (a) Body weight appearance and gross morphology revealing adiposity after 15 weeks of HFD in CadKO and WT for both the sex. (b, c) % Fat mass, Visceral and subcutaneous fat pad mass (VAT and SAT) in CadKO (n=3) and WT (n=3) fed HFD for 15 weeks. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA with Turkey's post hoc comparison. p values of less than 0.05 were considered statistically significant.

Next, the morphology of adipose depots was examined by performing H&E staining. In males, HFD increased adipocyte size in SAT for the majority of the adipocytes and had a broader distribution of sizes in both the WT and CadKO group (Fig 22.a.b). Similar size distribution was

observed in WT females under HFD (Fig 22.a, b). In contrast, CadKO females had an increased percentage of smaller adipocytes under HFD, similar to the lean phenotype (Fig 22.a, b).

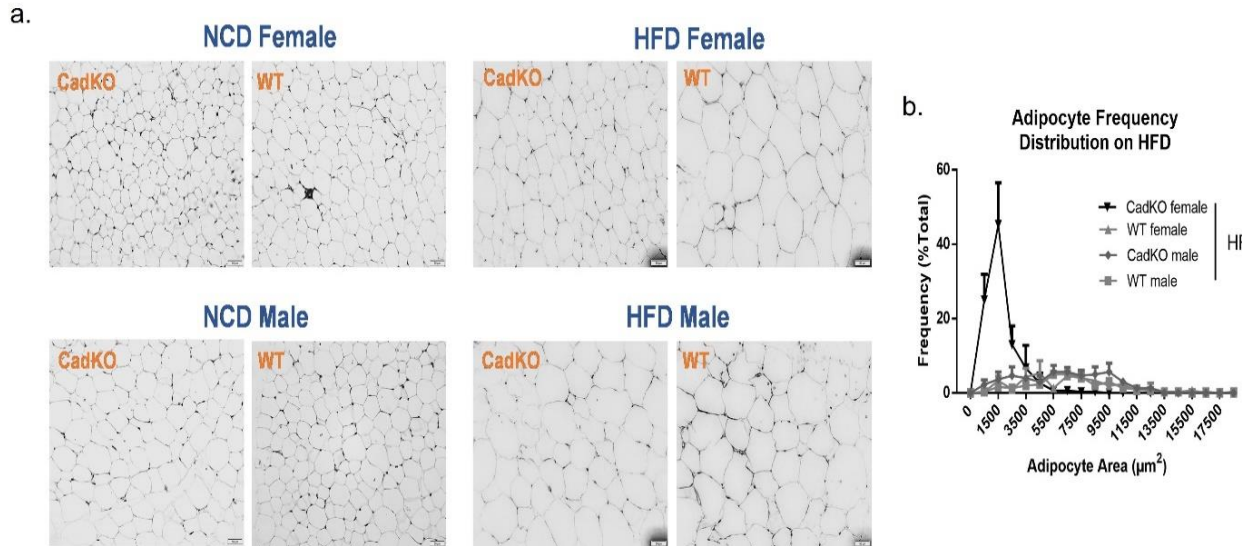


Fig 22. CadKO reduces adipocytes size in female SAT but not in male. (a) Histological data (H&E staining) obtained on subcutaneous adipose tissue of the CadKO and WT male and female mice after 15 weeks of NCD (n=1) and HFD (n=3-4). Scale bar: 50μm. (b) Adipocyte area frequency distribution plotted for CadKO and WT group in male and female on HFD.

Adipocyte size distribution in VAT was similar to SAT for both males and females (Fig 23.a, b). Moreover, H&E staining of VAT in HFD-fed WT males was significant for the presence of crown-like structures (CLS), thought to represent mononuclear cell infiltration. Immunohistochemistry of CD68 highlights the presence of cytoplasmic macrophages, which were not observed in CadKO males (Fig 23.b). CLS around dead adipocytes and macrophage infiltration is typical in obese metabolically unhealthy individuals. CLS was not observed on SAT in any of the groups (Fig 22). As changes in VAT is associated with poor metabolic health,

and alteration of its biology contributes to HFD-induced metabolic disease, subsequent sub-aims focused on VAT.

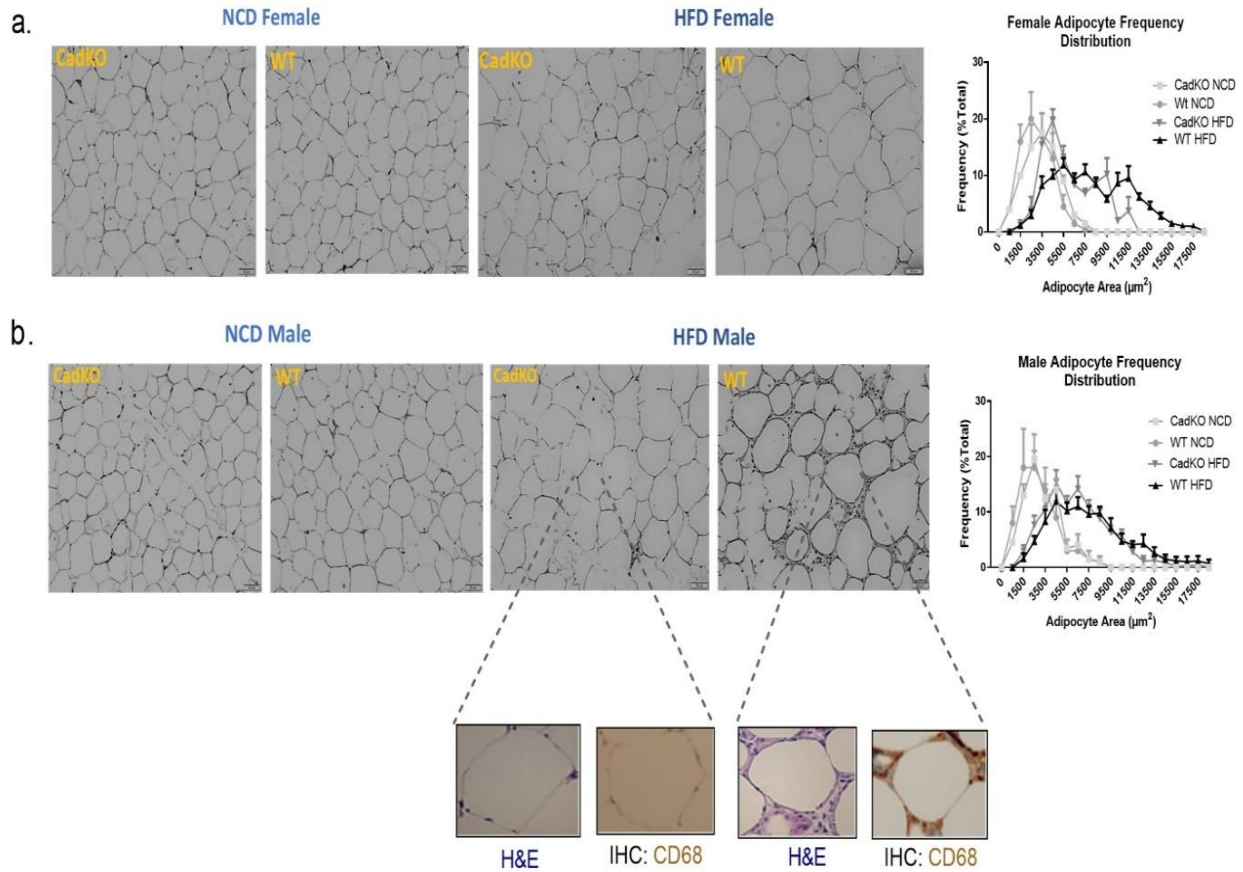


Fig 23. CadKO protects adipocyte size in females and HFD-induced presence of crown-like structure in male VAT. (a, b) Histological data (H&E staining) obtained in VAT of CadKO and WT females (a) and males (b) mice after 15 weeks of NCD (n=3) and HFD (n=3-4). Scale bar:50 μ m. Adipocyte area frequency distribution plotted for CadKO and WT group in females (a) and males (b). Immunohistochemistry (IHC) of CD68 antibody, marker for male adipose tissue macrophages on HFD.

4.3.2 Results Subaim 3.2: Examine molecular pathways of white visceral adipose depot.

As inflammation in adipose tissue is congruent with the presence of CLS, we investigated the inflammatory pathways of VAT. Transcript levels of proinflammatory cytokines (TNF α and IL1 β) were examined via qPCR. Gene expression profiles represented as a ratio of levels in HFD: NCD groups to accentuate changes associated with diet (value >1 represents an increase in HFD compared to NCD). TNF α expression was increased by HFD in WT males (HFD/NCD=3.1) but was not changed by HFD in CadKO males (HFD/NCD=1.4) (Fig 24.a). Similarly, IL1 β transcript level was increased by HFD in WT males (HFD/NCD=5.5) compared to CadKO males (HFD/NCD=1.1) (Fig 24.a). In contrast, HFD neither significantly elevated TNF α (HFD/NCD=1.4) nor IL1 β (HFD/NCD=1.8) in WT females (Fig 24.a). A similar observation was also noted for TNF α (HFD/NCD=0.8) and IL1 β (HFD/NCD=1.5) in CadKO females (Fig 24.a).

Next, Stat3/IL6 pathway was investigated, as activation of AhR by fat derivatives within the HFD, e.g., kynurenine (Kyn), has been reported to promote this pathway in WAT, inducing an obese phenotype (Huang et al., 2022). We observed Stat3/IL6 pathway attenuation in VAT for both the sexes of CadKO mice (Fig 24.b), revealing another pathway by which AhR may influence metabolic function.

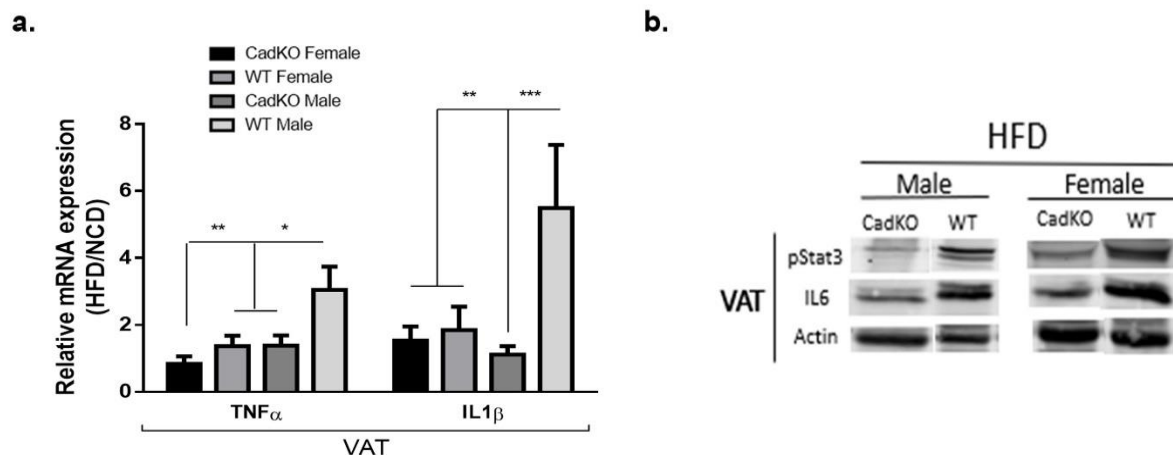


Fig 24. CadKO reduces inflammation in male mice by reducing adipocytokine release from VAT. (a) Total RNA was isolated from VAT to investigate mRNA levels of TNF α and IL1 β by real-time PCR on HFD male and female for CadKO (n=4-6) and WT (n=4-6) mice. Each gene was normalized against the amount of housekeeping gene, Actin. Data are expressed as the ratio of levels in HFD to NCD animals to accentuate changes associated with diet. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Turkey's post hoc multiple comparisons test. (b) After 15 weeks of HFD feeding, VAT were harvested, and western-blot was performed to assess HFD induced phospho-Stat3-IL6 pathway. β -actin were used to normalize. n=2-3 for each group. Representative blots were shown for each group.

Other key genes involving various metabolic pathways were also examined in VAT. PPAR γ , an indicator of adipogenesis, was not altered by HFD in WT (HFD/NCD=1.6) and CadKO (HFD/NCD=1.2) males (Fig 25). However, HFD significantly increased the transcript level for CadKO (HFD/NCD=4.7) compared to WT (HFD/NCD=1.7) in females (Fig 25).

Other key process, lipolysis (breakdown of lipid), in WAT were investigated by examining rate-limiting enzyme, *Hsl*, expression level. *Hsl* transcript levels were reduced by

HFD in WT (HFD/NCD=0.4) and CadKO (HFD/NCD=0.5) males (Fig 25). In contrast, HFD increased *Hsl* expression in both WT (HFD/NCD=1.9) and CadKO (HFD/NCD=2.7) females, which was significantly higher compared to males (Fig 25).

Lipogenesis (synthesis of lipid) is another critical pathway in WAT, determining HFD induced metabolic diseases. *Srebp1c*, a rate-limiting enzyme for lipogenesis, expression was reduced by HFD in WT (HFD/NCD=0.5) but in comparison increased significantly in CadKO male (HFD/NCD=1.3) (Fig 25). Whereas *Srebp1c* transcript level was reduced by HFD on both WT (HFD/NCD=0.2) and CadKO (HFD/NCD=0.4) genotypes for female (Fig 25).

To investigate the sexual dichotomy, we investigated ER α expression, as activated AhR attenuates ER α expression. ER α expression was reduced by HFD in males for both WT (HFD/NCD=0.4) and CadKO (HFD/NCD=0.6) genotypes (Fig 25). In females, HFD only reduced ER α in WT (HFD/NCD=0.6), but not in CadKO (HFD/NCD =1.1) (Fig 25), suggesting ER α signaling may have a crucial role in sexual dimorphism.

Overall, these data suggest adipose-specific deletion of AhR provides a mechanism to reduce adiposity in female mice and inflammation in male mice.

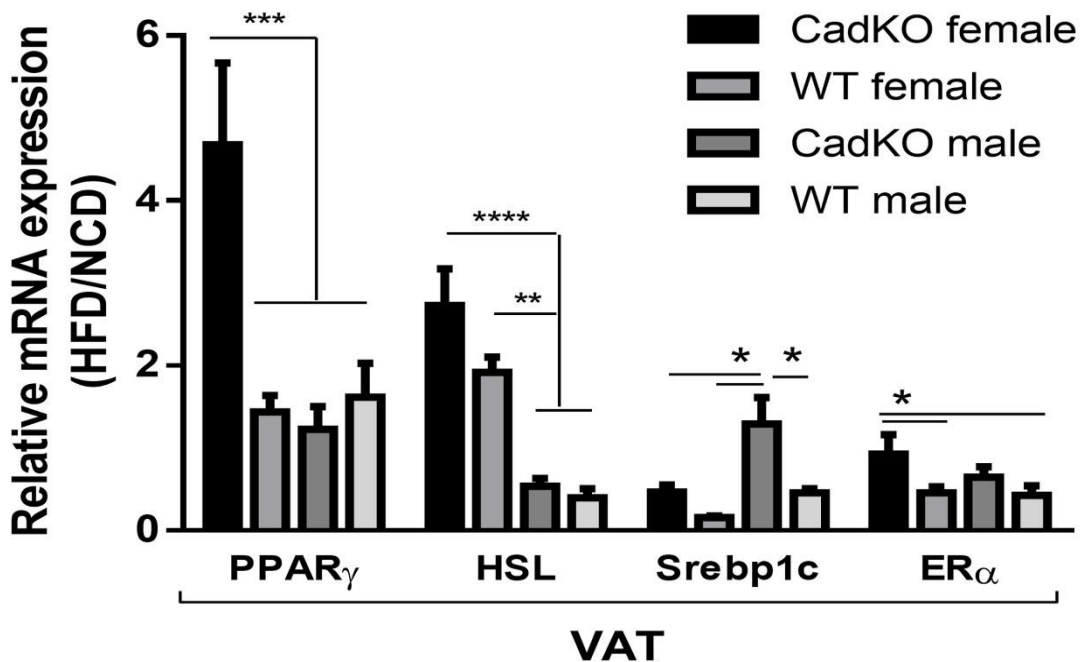


Fig 25. CadKO improved various molecular pathways in VAT from females after HFD. Total RNA was isolated from VAT to investigate mRNA levels of PPAR γ , HSL, Srebp1c and ER α by real-time PCR for CadKO (n=4-6) and WT (n=4-6) mice. Each gene was normalized against the amount of housekeeping gene, Actin. Data are expressed as the ratio of levels in HFD to NCD animals to accentuate changes associated with diet. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA with Turkey's post hoc multiple comparisons test. p values of less than 0.05 were considered statistically significant.

4.3.3 Results Subaim 3.3: Examine adipokine secretion by visceral adipose depot involving weight and glucose homeostasis.

To address whether CadKO affects the endocrine function of WAT in response to HFD, serum adipokine levels were examined. Circulating leptin provides information about the amount of fat stores and the possibility of leptin resistance in the body. Serum leptin was not different

across sex or genotype on NCD, but significantly elevated by HFD in all groups (Fig 26.a). No significant differences were noted within the male groups on HFD, however, leptin levels in female were significantly higher in HFD-fed WT compared to the CadKO (Fig 26.a).

Moreover, we examined another key adipokine, adiponectin, that reduces inflammation and improves insulin sensitivity. Adiponectin levels are typically higher in individuals with high metabolically healthy body fat and are lower in individuals that are obese or have unhealthy body fat. Female mice demonstrated significantly higher adiponectin compared to males on normal diet (Fig 26.b). Males on HFD for both the genotypes increased fat mass (Fig 21) but failed to increase serum adiponectin level suggesting an unhealthy metabolic condition (Fig 26.b). On the other hand, fat mass and serum adiponectin level were significantly higher in WT females compared to CadKO females (Fig 26.b), suggesting the adipose tissue in both the female groups was maintained in a healthy state. When adiponectin release was normalized to fat mass CadKO females on HFD showed considerably higher adiponectin release (39.1mg/ml/g) compared to the other groups (WT female=17.8, WT male=18.5, CadKO male=17mg/ml/g) (Fig 26.c). Next, the adiponectin expression in adipocytes was investigated. Similarly, the transcript level of adiponectin expression was elevated in CadKO female adipocytes on HFD (HFD/NCD=2.9) compared to WT female (HFD/NCD=0.9), WT male (HFD/NCD=1.4) and CadKO male (HFD/NCD=1.2) (Fig 26.d).

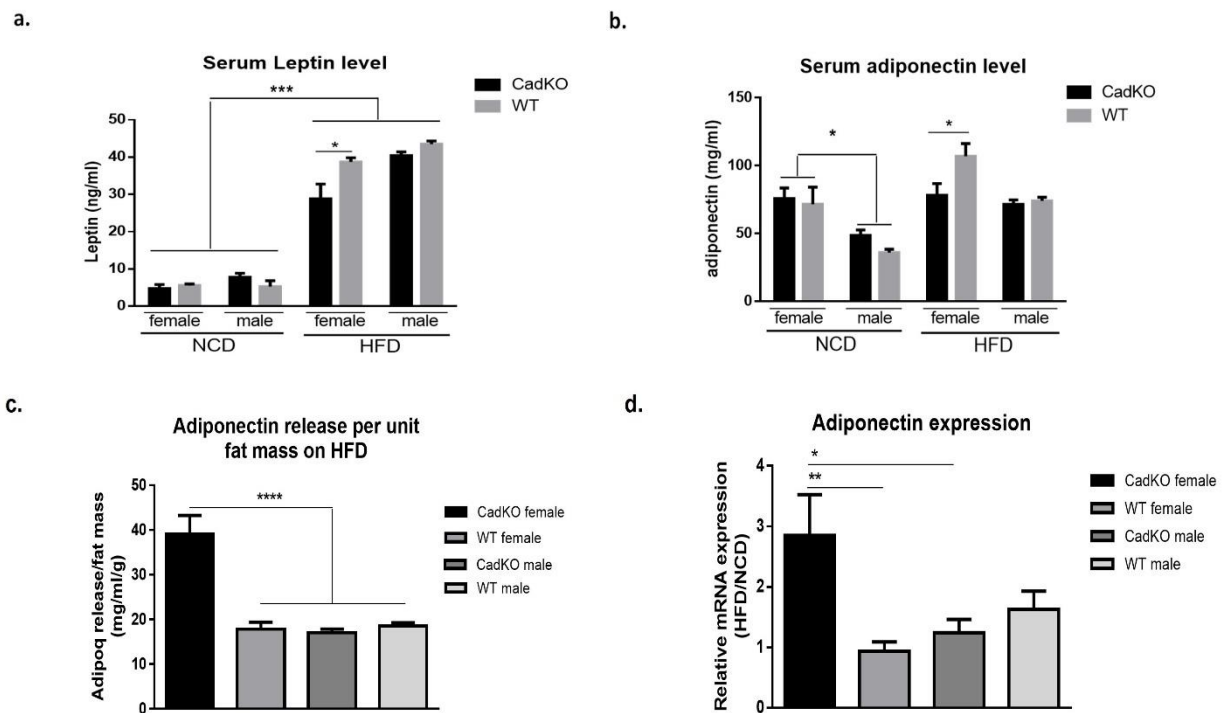


Fig 26. Adipokine secretion by WAT. (a, b) Blood was collected from male and female mice groups at week 15 of NCD (n=5-6) or HFD (n=8-10). Serum leptin and adiponectin were measured by ELISA. * $p < 0.05$, *** $p < 0.001$ by 2-way ANOVA with Turkey's post hoc comparison. (c) Bar graph representing adiponectin release per unit fat mass on HFD. (d) Total RNA was isolated from VAT to investigate mRNA levels of adiponectin by real-time PCR on male and female for CadKO (n=4-6) and WT (n=4-6) mice. Each gene was normalized against the amount of housekeeping gene, Actin. Data are expressed as the ratio of levels in HFD to NCD animals to accentuate changes associated with diet. * $p < 0.05$, ** $p < 0.01$, by one-way ANOVA with Turkey's post hoc multiple comparisons test. p values of less than 0.05 were considered statistically significant.

4.3.4 Results Subaim 3.4: Examine non-shivering thermogenesis in adipose depots.

To evaluate potential mechanisms underlying differences in EE (Fig 17), BAT non-shivering thermogenesis was examined. Non-shivering thermogenesis in BAT is usually stimulated to maintain thermal homeostasis in response to stimuli such as cold. This is achieved with the help of thermogenic gene uncoupling protein 1 (UCP1). The primary function of UCP1 is to conduct proton across the inner mitochondrial membrane, which facilitates uncouple oxidative phosphorylation from ATP synthesis and dissipating heat. HFD did not affect transcript or protein levels of UCP1 in any of the groups (Fig 27.a, b). Body temperatures were also not different in any of the groups (Fig 27.c), suggesting that CadKO had no effect on body temperature or in the thermogenesis pathway of BAT.

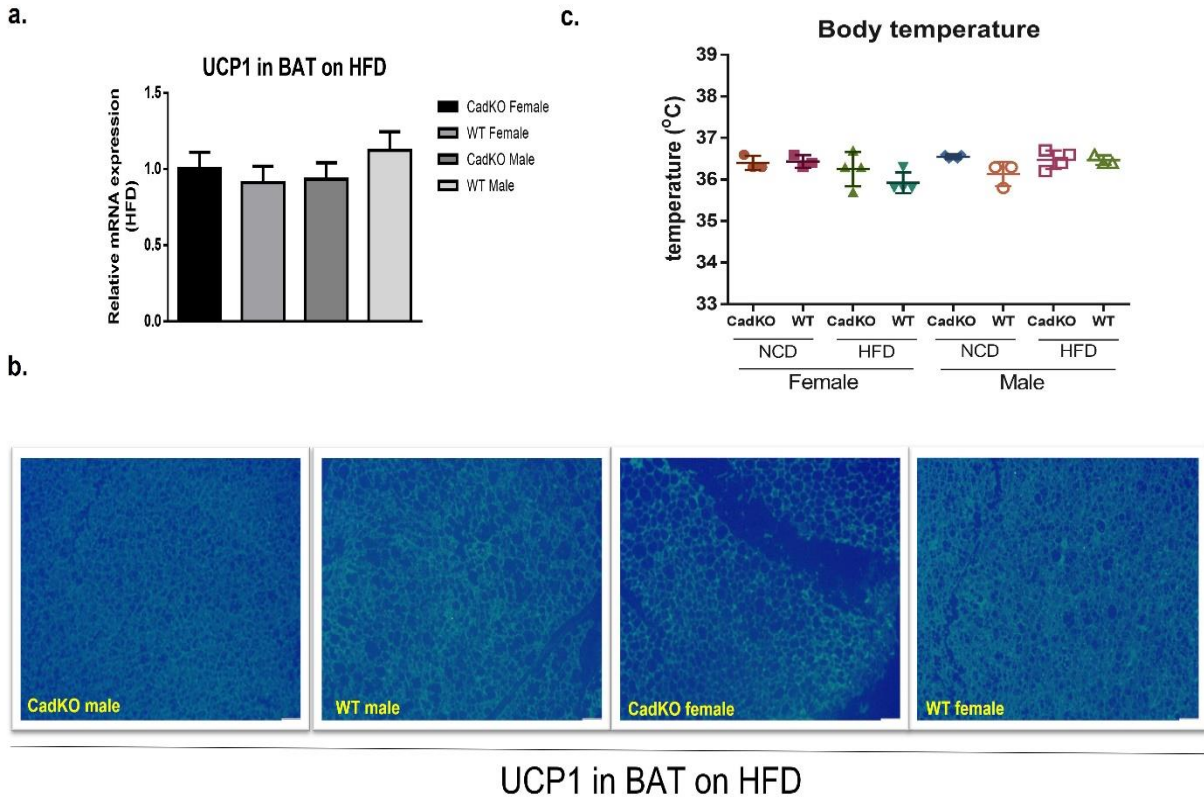


Fig 27. CadKO has no effect on non-shivering thermogenesis in BAT. (a) Total RNA was isolated from brown adipose tissue (BAT) to investigate mRNA levels of Ucp1 respectively by real-time PCR on HFD male and female for CadKO (n=4-6) and WT (n=4-6) mice. Each gene was normalized against the amount of housekeeping gene, Actin. (b) Body temperature at room temperature of CadKO and WT after 15 weeks of NCD and HFD for both sexes. (c) Immunofluorescence images of BAT UCP1 expression in CadKO and WT mice after 15 weeks of HFD. Scale bar:100 μ m

Next, we examined beiging (browning of white adipocytes) in WAT as it can also increase body EE. Chronic cold exposure is regarded as a primary stimulus for beiging. During the time of cold exposure, the liver releases a key hormone called Fgf21, which activates the sympathetic nervous system leading to BAT thermogenesis and beiging. Fgf21 can also be released in an autocrine/paracrine fashion by the WAT (Abu-Odeh et al., 2021) to promote WAT beiging facilitated by PPAR γ (Lo & Sun, 2013). The impact of HFD on beiging is not clearly understood. However, a previous study suggests HFD can positively regulate the process as dietary fat can increase sympathetic outflow in rats by increasing norepinephrine turnover (Schwartz et al., 1983).

As CadKO female on HFD maintained a lean adipocyte phenotype (Fig 23) and increased PPAR γ expression in VAT (Fig 25), we used these data as a hint to explore the beiging process in VAT. Fgf21 gene expression in the liver on HFD was not different between the sexes of CadKO and WT groups (Fig 28.a). Unaltered liver Fgf21 and BAT UCP1 expression (Fig 27) suggest that the liver Fgf21-induced BAT thermogenesis pathway was not affected by CadKO in either sex. In VAT, HFD had little effect on Fgf21 in WT males (HFD/NCD=1.3), however CadKO increased Fgf21 expression (HFD/NCD=1.9) (Fig 28.c). Similar result was noted in

female groups, as HFD significantly elevated Fgf21 expression in CadKO (HFD/NCD=2.4) compared to WT(HFD/NCD=0.96) (Fig 28.c). Finally, HFD induces transcript level of UCP1 in VAT for all the groups (Fig 28.c). In males, we found no significant difference between WT (HFD/NCD=2.1) and CadKO (HFD/NCD=1.8) on HFD (Fig 28.c). However, in females CadKO significantly increased UCP1 transcript (HFD/NCD= 7.2) compared to WT (HFD/NCD=1.3) (Fig 28.c). Similarly, protein for UCP1 was robustly expressed in CadKO female compared to other groups on HFD revealed by immunofluorescence (Fig 28.d). Overall, these data suggest AhR-specific deletion from adipose tissue in females may promote beiging in response to HFD to enhance metabolic rate and protect against diet-induced weight gain.

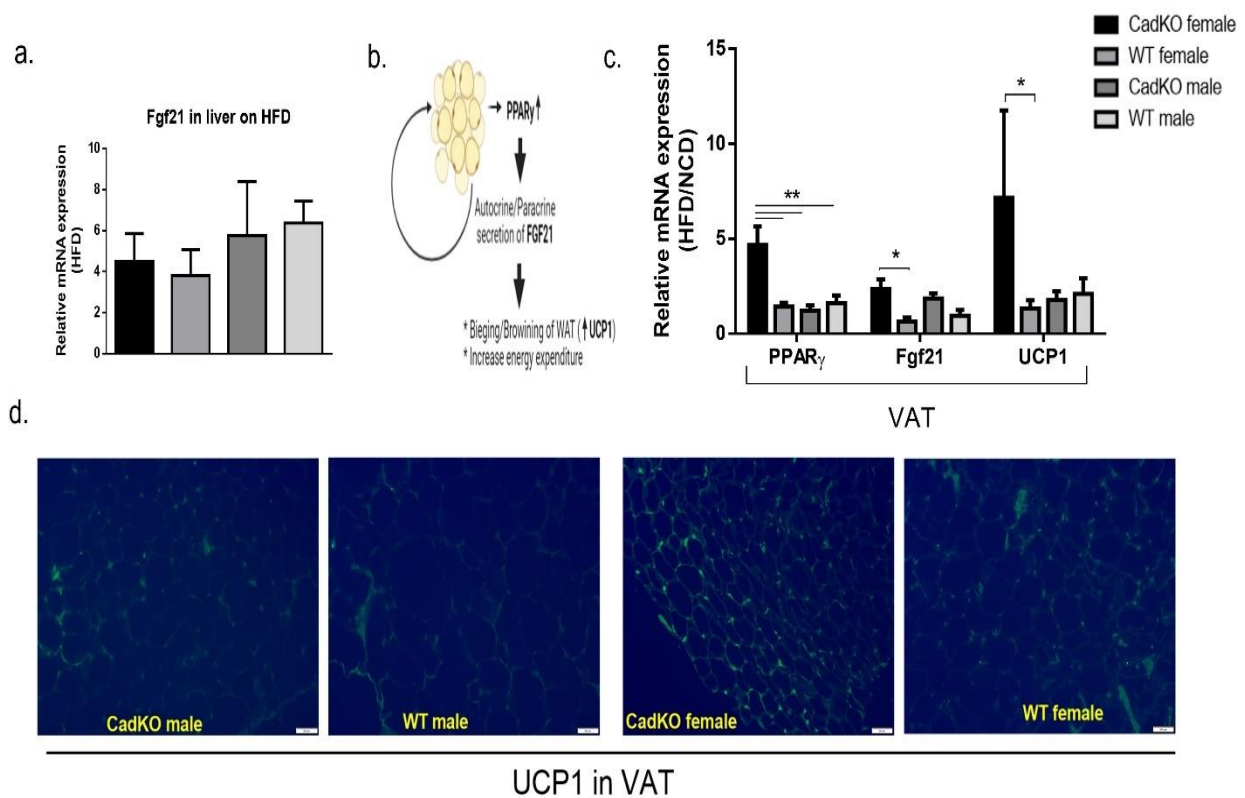


Fig 28. CadKO female may promote beiging in white adipose tissue to increase non-shivering thermogenesis. (a) Total RNA was isolated from liver to investigate mRNA levels Fgf21 by real-time PCR on HFD male and female for CadKO (n=5-6) and WT (n=5-6) mice. Each gene

was normalized to Actin. (b) Illustrative diagram of induce expression of the key modulator of beiging, PPAR γ and Fgf21, to promote differentiation from white to brite adipocytes and thus increasing Ucp1 level. (c) Total RNA was isolated from visceral adipose tissue (VAT) to investigate mRNA levels of PPAR γ , Fgf21 and Ucp1 respectively by real-time PCR on HFD male and female for CadKO (n=3-6) and WT (n=4-6) mice. Each gene was normalized against the amount of housekeeping gene, Actin. Data are expressed as the ratio of levels in HFD to NCD animals to accentuate changes associated with diet. (d) Immunofluorescence images of VAT Ucp1 expression in CadKO and WT mice after 15 weeks of HFD. Scale bar:50 μ m. *p<0.05, **p<0.01, by one-way ANOVA with Turkey's post hoc multiple comparisons test. p values of less than 0.05 were considered statistically significant.

4.3.5 Summary

These data demonstrate the improvement of adipose tissue physiology for CadKO mice in a sexually dimorphic manner that promotes maintaining healthy phenotype. In males, CadKO provides a mechanism to improve adipose biology that presents as reduced secretion of proinflammatory adipocytokines on HFD, such as TNF α , IL1 β , IL6, thus reducing inflammation. In females, CadKO prevents HFD-induced adiposity to preserve a lean adipose phenotype and promote healthy metabolic pathways, such as adipogenesis, lipolysis, and beiging. We also observed healthy adipokine signaling, such as adiponectin and leptin, in CadKO females, which likely promotes a healthy weight and glucose homeostasis.

4.4 Specific Aim 4: Explore hepatic steatosis (NAFLD) to examine systemic effect CadKO after HFD feeding.

Aim 3 explored how adipose-specific AhR depletion affects adipose tissue biology in both sexes to explain some of the phenotypic differences observed in HFD. This aim focused on

determining whether the alteration of adipose biology can exert a systemic effect. As the liver plays a central role in metabolism, we examined liver tissue to obtain a molecular explanation for systemic effects in CadKO mice. The phenotypic data presented in this study clearly demonstrate that AhR depletion from adipose tissue has sex-specific effects on systemic metabolism in the face of HFD. The coordinated function of metabolic tissues such as the liver, muscle and adipose helps maintain normal blood glucose (Petersen & Shulman, 2018). Failure in one tissue can influence the function of others and contribute to the development of systemic pathology. For example, the deleterious effects of high levels of dietary fat on adipose tissue can lead to tissue inflammation and insulin resistance (Kahn et al., 2019). Insulin resistance makes it increasingly more difficult to maintain healthy blood glucose levels. Initially, the body combats difficulty with glucose uptake by increasing pressure on pancreatic beta cells to produce more and more insulin, resulting in hyperinsulinemia with relatively normal blood glucose. Excess insulin can stimulate the liver to produce and store more fat, thus contributing to the accumulation of fat in the liver resulting in a form of hepatic steatosis known as non-alcoholic fatty liver disease (NAFLD) (Chen et al., 2017). Hepatic steatosis is a pathological condition in the liver causing inflammation and scarring. If the condition is not treated it can lead to liver fibrosis, cirrhosis and even liver failure. Moreover, there are sex differences in the prevalence and severity of NAFLD. Overall, both the prevalence and severity of NAFLD is increased in males (Lonardo et al., 2019). AhR signaling can regulate the accumulation of fat in the liver and the development of NAFLD (Carambia & Schuran, 2021). The present study examined whether specific deletion of AhR from mature adipose tissue can contribute to the development of NAFLD.

NAFLD, one form of hepatic steatosis, is a common condition in a fat-dense diet. Hence, this specific aim examined how AhR deficiency specifically from adipose tissue may affect the

development of hepatic steatosis induced by HFD. The data were compared with a previous study on AhRKO males where applicable.

Hypothesis: Adipose specific AhR deficiency will exert systemic effect and protect mice from diet induced hepatic steatosis/NAFLD.

4.4.1 Results Subaim 4.1: Examine weight and morphology of the liver tissue.

NAFLD is a common cause of an enlarged liver and heightened liver weight. HFD increased liver mass in WT males, but CadKO males were protected from HFD-induced changes in liver weight (Fig 29). Liver weight was not significantly different in HFD-fed females of either genotype (Fig 29).

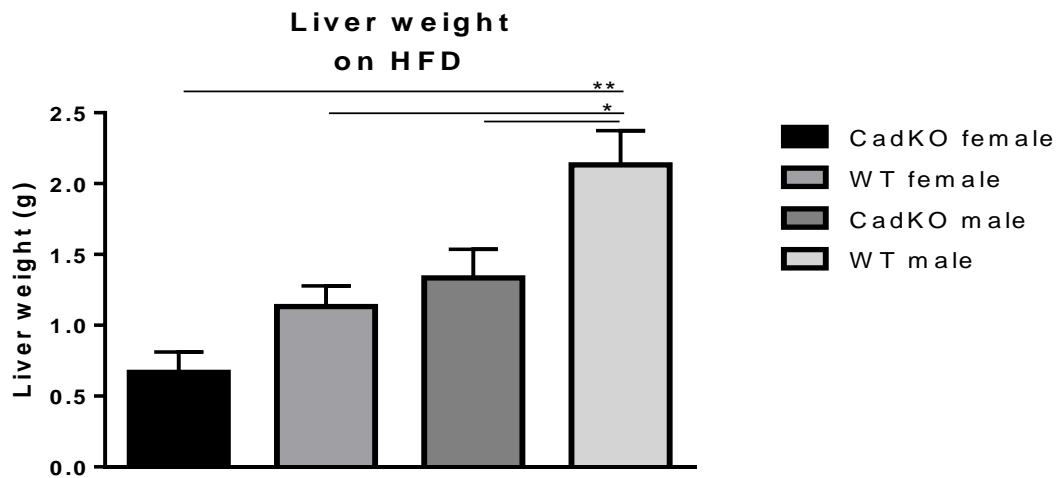


Fig 29. Liver weight after 15 weeks of HFD in male and female of CadKO (n=3) and WT (n=3). *p<0.05, **p<0.01, by one-way ANOVA with Turkey's post hoc comparison. p values of less than 0.05 were considered statistically significant.

H&E staining of the liver was used to investigate hepatic steatosis/NAFLD. No lipid droplets were observed in any NCD groups (Fig 30.a). In contrast, lipid droplet deposition revealed obvious sex differences in HFD, with WT males depositing more lipid droplets

compared to WT females (Fig 30.b, c). Lipid spillover was significantly reduced in HFD-fed CadKO males and females, with CadKO females being indistinguishable similar to NCD groups (Fig 30.a-c).

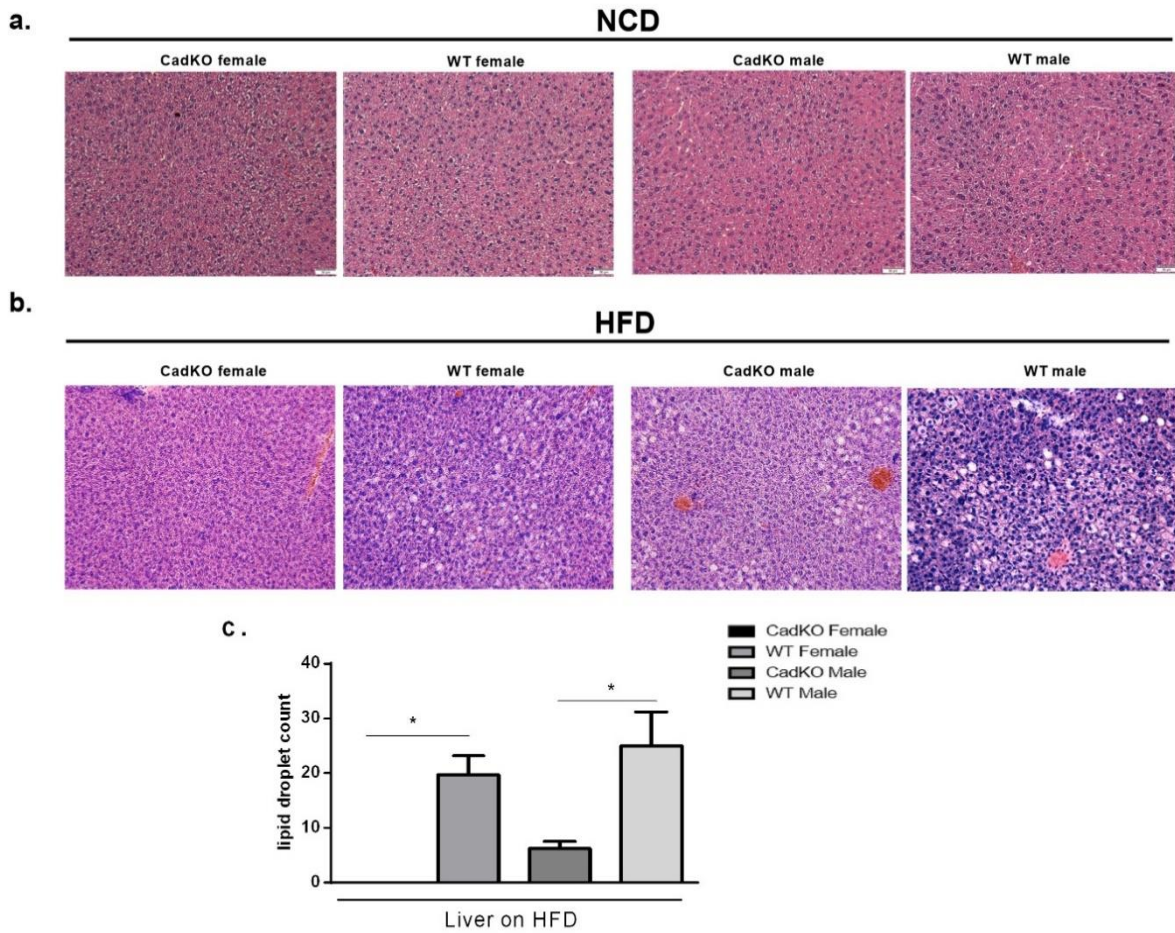


Fig 30. CadKO protects against HFD-induced hepatic steatosis. (a, b) Histological data (H&E staining) obtained in liver of the mice after 15 weeks of NCD (a) and HFD (b) to determine lipid spillover. White circular spot indicates presence of lipid droplets in the staining. n=3-4; Scale bar: 100µm. (c) Lipid droplets quantification from the images obtained from H&E. n=3-4, *p<0.05, by one-way ANOVA with Turkey's post hoc comparison. p values of less than 0.05 were considered statistically significant.

4.4.2 Results Subaim 4.2: Examine different molecular pathways of the liver.

Gene transcripts were explored involving liver steatosis and function. *Ppara* (a gene involved in fatty acid metabolism) was elevated by HFD in males of WT (HFD/NCD=2.1) and CadKO (HFD/NCD=3.2) (Fig 31). However, *Ppara* in female was not changed by diet in WT(HFD/NCD=0.9) but was reduced by HFD in CadKO (HFD/NCD=0.5) (Fig 29). Moreover, transcripts for the fatty acid translocase, CD36, increased slightly on HFD for male WT (HFD/NCD=1.4) and CadKO (HFD/NCD=1.2) (Fig 31). Similar observations were seen in WT females (HFD/NCD=1.3). However, contrasting results were obtained for CadKO female as HFD significantly suppressed CD36 level (HFD/NCD=0.5) (Fig 31). Next, lipogenic gene, *ACC* (*acetyl Coenzyme A carboxylase*) mRNA expression was not altered by HFD in WT male (HFD/NCD=1.1) and CadKO male (HFD/NCD=1.2) (Fig 31). However, in female HFD significantly increased *ACC* expression in WT (HFD/NCD=2.1) compared to CadKO, which was unchanged (HFD/NCD=1.2) (Fig 31). Finally, transcript levels for the rate limiting enzyme of gluconeogenesis and glycogenolysis, *Glucose 6 phosphatase* (G6Pase), was examined. HFD had no effect on G6Pase expression for WT males (HFD/NCD=1.1) but was significantly elevated in CadKO males (HFD/NCD=2.8) (Fig 31). WT=1.1, for female: CadKO=0.9, WT=0.8). G6Pase was not changed by HFD neither WT (HFD/NCD=0.8) nor in CadKO (HFD/NCD=0.9) females (Fig 31).

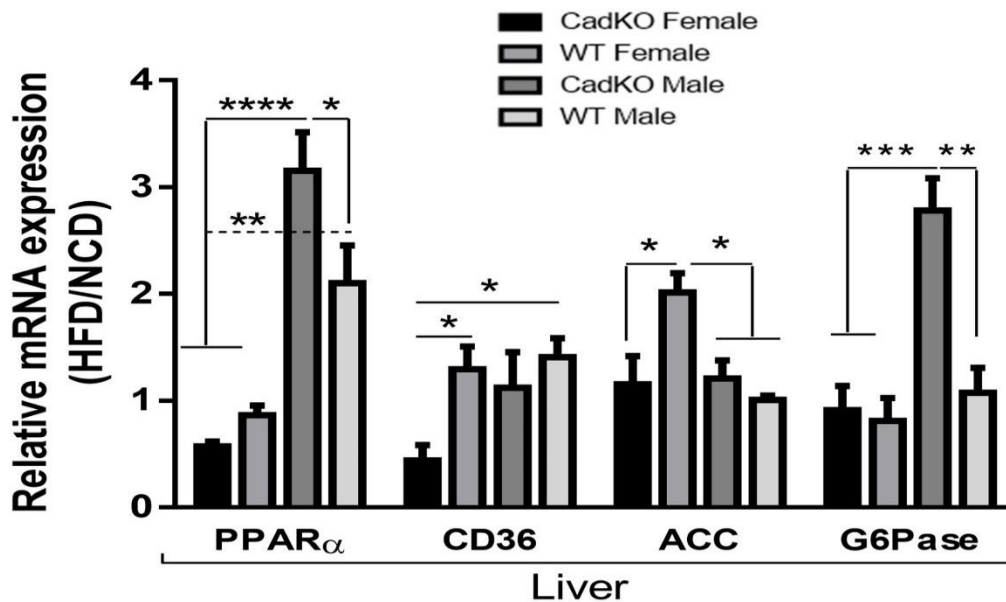


Fig 31. CadKO protects against HFD-induced hepatic steatosis in sexually dimorphic pathway.

Total RNA was isolated from liver to investigate mRNA levels of PPAR α , CD36, ACC, G6Pase by real-time PCR on male and female CadKO (n=4-6) and WT (n=4-6) mice. Each gene was normalized against the amount of housekeeping gene, Actin. Data are expressed as the ratio of levels in HFD to NCD animals to accentuate changes associated with diet.

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA with Turkey's post hoc comparison. p values of less than 0.05 were considered statistically significant.

4.4.3 Summary

These data indicate CadKO imparts protective effects on HFD-induced hepatic steatosis in both sexes, but in that mechanism underlying protection is sex-specific. These data suggest males in general are more prone to NAFLD and its severity, as indicated by lipid spillover in the H&E staining. CadKO prevents ectopic lipid spillover in the liver for both sexes, with females demonstrating similar liver morphology as lean mice despite 15 weeks of *ad libitum* HFD

diet. Adipose-specific deletion of AhR in female mice may reduce uptake of serum fatty acids into the liver, as indicated by reduced CD36 transcript levels, and prevent an increase in the production of additional fatty acids in response to HFD, as indicated by ACC, thus protecting from lipid accumulation. In contrast, males increase lipid metabolism and mobility to prevent lipid accumulation as indicated by PPAR α . Moreover, the increase in G6Pase transcript levels in CadKO male livers could relate to systemic glucose homeostasis, as no difference was found in fasting and postprandial glucose level in CadKO compared to WT (Fig 19.a, e, g).

4.5 Specific Aim 5: Explore hypothalamic feeding centers to examine systemic effect in energy regulation.

The phenotypic analysis in Aim 2 showed that CadKO females consume fewer calories when presented with HFD compared to their WT counterparts. Adipose tissue plays a crucial role in energy regulation by communicating with various regions of the brain, including the hypothalamus, which is involved in the regulation of energy balance and metabolism (Sandoval et al., 2008). One way the adipose tissue communicates with the hypothalamus is through the release of various signaling molecules, such as leptin. Leptin is produced by the fat cells after a meal to act on the leptin receptor (LepR) of the hypothalamic energy regulatory regions (ARC, VMH), leading to an increase in POMC hormone that decreases food intake and increases energy expenditure (Sandoval et al., 2008). Chronic high-calorie diet typically produces leptin resistance. Chronically elevated leptin levels, resulting from long-term intake of an energy-dense diet lead to a reduction of LepR expression, and thus the brain does not properly recognize the presence of leptin, which defines leptin resistance (Obradovic et al., 2021). This creates an imbalance in energy regulation and potentially contributes to weight gain and obesity. Furthermore, there are fundamental sex differences in body energy regulation, driven primarily

by sex steroid hormones. Estrogen, the primary sex steroid in females released from the ovary, can stimulate the production of POMC through ER α contributing to energy balance (Stincic et al., 2018). Moreover, estrogen signaling has been shown to stimulate the release of leptin from fat cells (Fungfuang et al., 2013). The potential for estrogen to stimulate leptin makes females more vulnerable to the development of leptin resistance. The present study examined how adipose-specific depletion of AhR may alter communication between adipose tissue and the hypothalamic energy regulatory regions to affect feeding behavior on HFD.

Aims 2 and 3 provide an explanation of AhR involvement in adipose tissue and how deficiency of the receptor improves adipose tissue biology in both sexes explaining some of the phenotypic differences observed in HFD. This aim focused on whether the alteration of adipose biology can exert a systemic effect on weight homeostasis via communication with the feeding center of the hypothalamus to affect feeding behavior. Leptin and estrogen signaling, known regulators of the hypothalamic-adipose cross-talk were the focus of this aim.

Hypothesis: Adipose-specific AhR deficiency will exert a systemic effect to improve crosstalk between adipocytes and hypothalamic energy regulatory regions under HFD.

4.5.1 Results Subaim 5.1: Examine leptin signaling in energy regulatory regions of hypothalamus.

Circulating leptin was not different across sex or genotype on NCD. Leptin was elevated by HFD in all groups. However, leptin levels were lower in HFD-fed CadKO females compared to all other HFD groups (Fig 32.a). Overall, these data predict possible protection from HFD-induced leptin resistance in CadKO females. To examine the possibility of leptin resistance, LepR expression in ARC and VMH nucleus of the hypothalamus was investigated. Fig 32.b, illustrates coronal sections of the brain with the third ventricle (3V), Arcuate (ARC), and

Ventromedial hypothalamic (VMH) nucleus identified. The intensity of punctae obtained from immunofluorescence of LepR antibody and quantitated is shown in Fig 32.c. As no real difference in leptin was observed in NCD groups, leptin receptor levels were examined only in HFD groups. CadKO female LepR levels were significantly higher in ARC and VMH nucleus compared to WT female and both male genotypes (Fig 32.c).

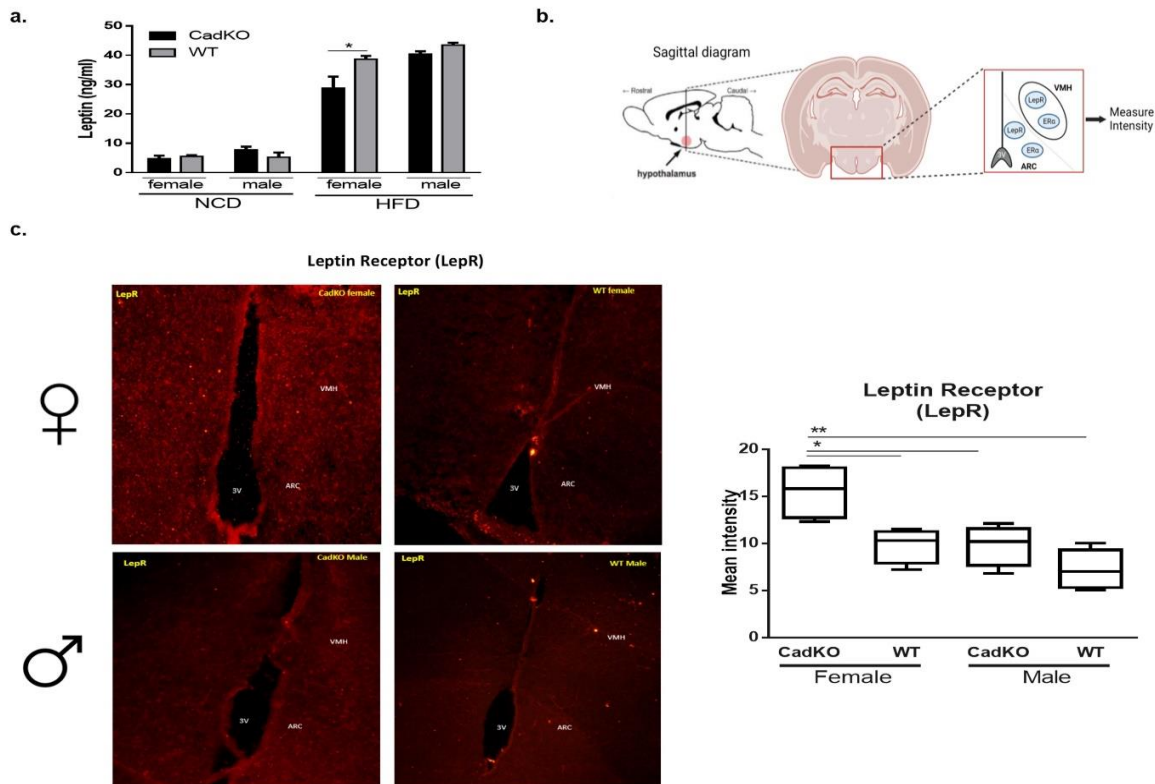


Fig 32. CadKO induces satiety response by promoting leptin signaling in female mice when faced with calorie dense diet. (a) Blood was collected from mice groups at week 15 of NCD or HFD. Serum leptin were measured by ELISA (NCD, n= 5-6; HFD, n=8-10). (b) Diagrammatic illustration of the hypothalamus location in the sagittal section of the brain as well as location of third ventricle (3V), Arcuate (ARC) and ventromedial hypothalamic (VMH) nucleus in a coronal section to measure receptor intensity from immunofluorescence. (c)

Immunofluorescence of Leptin Receptor, LepR (red punctae) expression in arcuate (ARC) and ventromedial hypothalamic (VMH) nucleus located near third ventricle (3V). Scale bar: 50 μ m. Mean intensity measured for LepR in CadKO (n= 4) and WT (n=3) for both the sex, *p<0.05, **p<0.01 by one-way ANOVA. p values of less than 0.05 were considered statistically significant.

To verify, we also examined LepR expression in non-hypothalamic tissue, liver. HFD decreases the mRNA expression of LepR in male WT (HFD/NCD=0.6) and CadKO (HFD/NCD=0.3) drastically (Fig 33). However, HFD had little to no effect in WT female (HFD/NCD=0.9) but was significantly increased in CadKO (HFD/NCD=5.3) (Fig 33).



Fig 33. CadKO female resist lowering of leptin receptor expression in liver on HFD. Total RNA was isolated from liver to investigate mRNA levels of leptin receptor by real-time PCR on male and female of CadKO (n=4-5) and WT (n=4-5) mice. Each gene was normalized against the amount of housekeeping gene, Actin. Data are expressed as the ratio of levels in

HFD to NCD animals to accentuate changes associated with diet. *** $p < 0.001$ by one-way ANOVA with Turkey's post hoc comparison.

4.5.2 Results Subaim 5.2: Examine estrogen signaling in energy regulatory regions of the hypothalamus.

Serum 17β -estradiol and $ER\alpha$ expression in the hypothalamic feeding center was examined to investigate estrogen signaling. Serum 17β -estradiol was not affected by diet, genotype or sex (Fig 34.a). Fig 32.b illustrates sections of the brain including the ARC and VMH showing immunofluorescence secondary to staining with $ER\alpha$ antibody. $ER\alpha$ expression was significantly higher in CadKO females compared to other HFD groups (Fig 34.b).

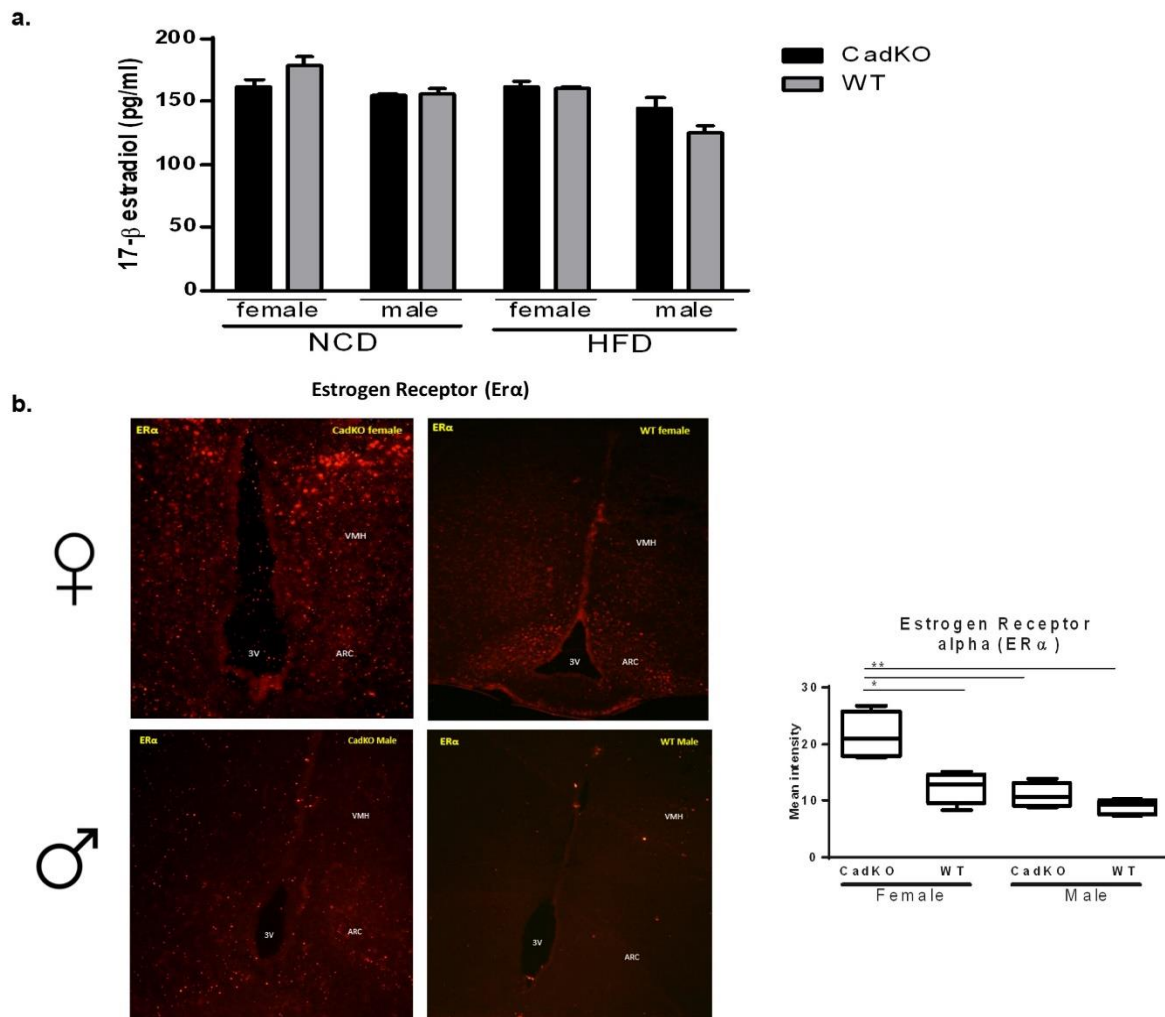


Fig 34. CadKO promotes estrogen signaling in female mice when faced with calorie dense diet. (a) Blood was collected from mice groups at week 15 of NCD or HFD. Serum 17- β estradiol were measured by ELISA (NCD, n= 5-6; HFD, n=8-10). (b) Immunofluorescence of Estrogen Receptor alpha, $ER\alpha$ (red punctae) expression in arcuate (ARC) and ventromedial hypothalamic (VMH) nucleus located near third ventricle (3V). Scale bar: 50 μ m. Mean intensity measured for $ER\alpha$ in CadKO (n= 4) and WT (n=3) for both the sex, * p <0.05,

**p<0.01 by one-way ANOVA. p values of less than 0.05 were considered statistically significant.

4.5.3 Summary

Together with the reduced leptin levels, the increased LepR in CadKO females on HFD is consistent with enhanced leptin sensitivity. Furthermore, elevated ER α in HFD-fed CadKO females suggests enhanced estrogen signaling, which is consistent with improved adipose-hypothalamic crosstalk, and may explain why CadKO females eat less on HFD. Congruent with the phenotypic data, this study reveals CadKO protects female from leptin resistance and improves estrogen signaling to promote better satiety response and may, therefore, account for weight difference among genotypes in females on HFD (Fig 35).

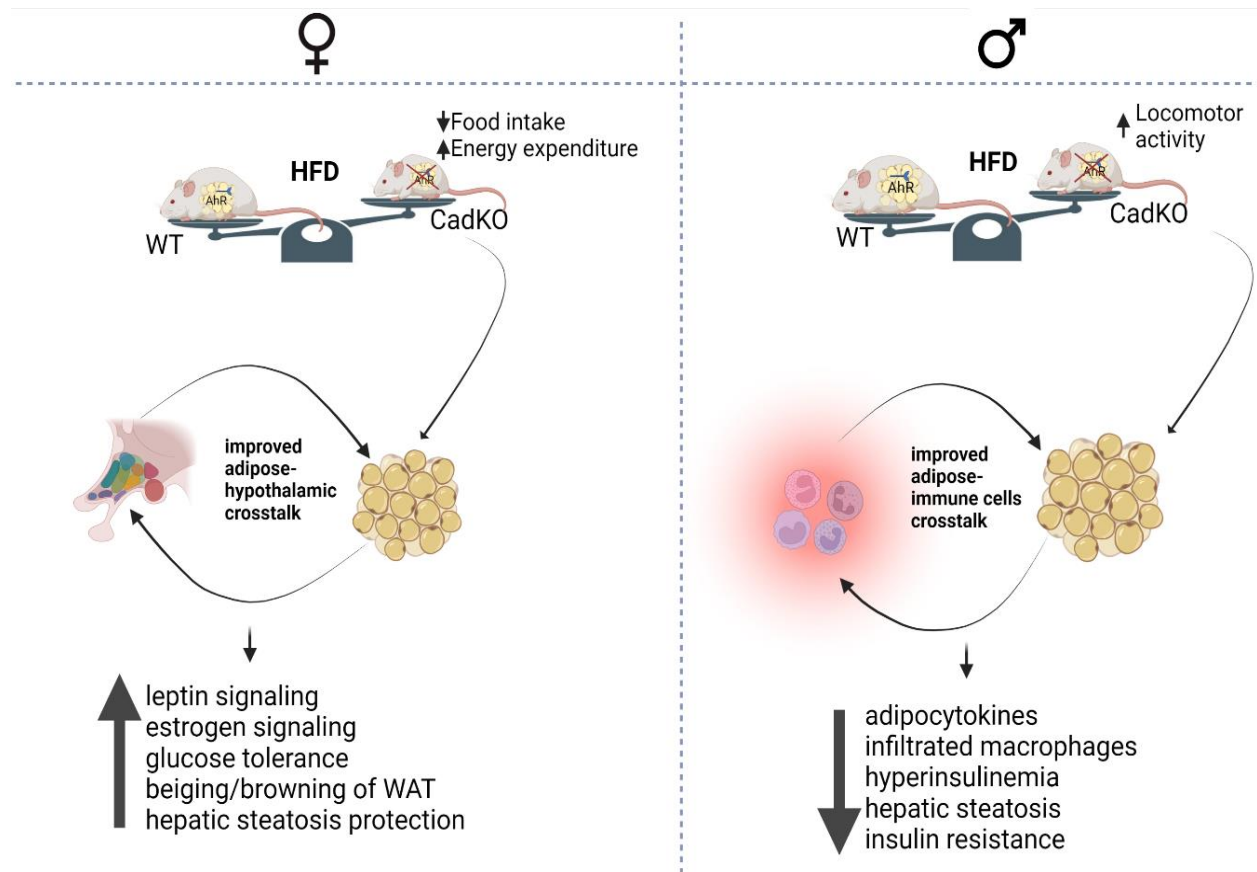


Fig 35. Schematic illustration of sex differences in CadKO mice for protection against HFD-induced metabolic dysfunction. CadKO females gained less weight due to net negative energy balance compared to WT, whereas CadKO males adapted by increased locomotor activity. Both the sexes for CadKO mice have improved adipose biology that helped females to maintain a healthy adipose-hypothalamic network, whereas CadKO males maintained healthy adipose-immune cells crosstalk. Created by Biorender.com.

CHAPTER 5

DISCUSSION

5.1 Specific Aim 1: Establish inducible adipose specific AhR knockout mouse models (CadKO) using Cre-loxp system and characterize them.

AhR is an established regulator of energy metabolism. However, tissue-specific functions for AhR in regulating metabolism remain unclear. To date, most of the studies have used AhR^{-/-}, AhR^{+/-}, a low-affinity AhR mice strain (C57BL/6.D2) or AhR inhibitors (such as α -naphthoflavone, CH-223191) to explore AhR's role in metabolic function. However, the tissue-specific function of AhR is important to explore, as it can provide insight into the specific functions and mechanisms in different tissues that can facilitate in developing targeted therapies and reducing side effects.

Some efforts have been made to explore tissue specific role of AhR using Cre-loxp, either by conditional knock out (CKO) or inducible CKO (iCKO). Both of these gene manipulations permanently knock-out AhR from certain cell types, but at different times. CKO deletes genes throughout development, achieved by introducing a *Cre recombinase* gene, driven by tissue-specific promoters, into the cell, which specifically targets and removes the *loxP-flanked* gene of interest. Alternatively, iCKO deletes the gene of interest by introducing an external chemical inducer (such as tamoxifen, tetracycline), at a specified time, to activate *Cre recombinase* for *loxP-flanked* gene deletion. Inducible knockouts are particularly useful for studying the role of a gene in adult animals, by reducing developmental effects of gene deletion.

Studies on CKO and iCKO of AhR have produced in conflicting results, which may be due to the timing of AhR deletion. CKO from both adipose and liver augmented the metabolic dysfunction in response to HFD (Baker et al., 2015; Wada et al., 2016). In contrast, iCKO from

adult liver (Girer et al., 2019; Girer et al., 2016) ameliorates the disease condition, and mimics at least part of the phenotype demonstrated in AhRKO, low affinity AhR (C57BL/6.D2) or AhR inhibition mice (Xu et al., 2015).

The present study used tamoxifen-inducible adipose-specific AhR depletion to explore metabolic regulation under HFD in a time dependent manner. For tamoxifen dependent *Cre recombinase* (*Cre^{ER} recombinase*) tamoxifen activates *Cre* to generate both time and tissue specific mouse mutants. Tamoxifen usually has low affinity towards ER, but the metabolite of tamoxifen 4-hydroxytamoxifen (4-OHT) and *N*-desmethyl-4-hydroxytamoxifen has 30–100 times more affinity (Ahmad et al., 2010; Desta et al., 2004). It has been reported after 5 consecutive days of gavage at 100 mg/kg/day, only 1% remained in WAT 10 days after the last gavage (Ye et al., 2015). Hence, it is unlikely that tamoxifen can have a long-term effect in our study as we provided 7-days wash out time after 2 consecutive days of 150mg/kg/day (IP) tamoxifen. Our data validated and established that the tamoxifen inducible adiponectin-Cre has compromised AhR functionality in mature adipose tissues.

5.2 Specific Aim 2: Phenotypic investigation of energy and glucose homeostasis in different AhR genotypes after subjecting mice to different diet regimen.

Phenotypic studies on examining body energy homeostasis suggest AhR is an important regulator of adult adipose tissue in response to HFD that mediates systemic regulation of body weight in a sexually dimorphic manner. Direct comparison of WT males to females indicates males gained more body mass (117% increase) than the females (100% increase) after 12 weeks of HFD. However, considering weight gain on NCD, where males gained around 15% body weight and females only 10%, the relative increase for HFD/NCD for males was 7.8 and for

females was around 10. Thus, inclusion of the body mass gain from control diet allows a relative measure of body mass, which we found to be more informative than absolute measures.

Ample numbers of papers demonstrate that AhR inhibition (Moyer et al., 2016; Moyer et al., 2017), reduced AhR affinity (C56BL/6.D2) (Kerley-Hamilton et al., 2012; Moyer et al., 2017), whole body knock out (Xu et al., 2015), and iCKO liver specific knock out (Girer et al., 2019) protects against weight gain from HFD in male mice. The few studies that examined females revealed more protection from HFD compared to male (Girer et al., 2019; Moyer et al., 2017). The current study showed that adipose-specific AhR-deficiency protects both male and female mice from gaining weight on HFD, with a greater effect in females.

A previous study (Baker et al., 2015) on tissue-specific AhR deletion from adipocytes unexpectedly found an increase in body weight in males on HFD. Although our result failed to demonstrate robust protection from HFD-induced weight gain in males, it certainly was not exacerbated. The differences could reflect the timing of AhR depletion, as the current study allowed for adipose tissue to develop normally before depletion with tamoxifen treatment in adults. However, adipose-specific AhR depletion using platelet-derived growth factor receptor alpha (*Pdgfra*)-Cre mice, where AhR is depleted beginning at the preadipocytes phase demonstrated reduced weight gain in male mice, similar to our study (Gourronc et al., 2020). Although the reasons behind the differences with Baker *et al.* are not clear, deletion of AhR from adipose tissue from the time of fertilization can change its development, whereas depletion in preadipocytes or mature adipocytes affects only adult adipose tissue biology.

Conditional AhR deletion from hepatocytes in males (Wada et al., 2016) had no effect on HFD-induced weight gain. Inducible liver specific knockout (Girer et al., 2019), where AhR is depleted from only the adult hepatocytes, showed protection from HFD in males, and a bigger

effect in females, similar to our study. Based on these studies one can deduce that differences in the mice lines can easily be due to the timing of AhR depletion. Deleting AhR before tissues are matured can cause developmental effects that can contribute to systemic differences observed when depletion occurs after animals are mature. Moreover, congenic (Baker et al., 2015) and transgenic (Xu et al., 2015) AhR deficiency protects from HFD-induced obesity, suggesting AhR-mediated regulation of metabolism may involve combined effects in various cell types. Due to very limited available data, the story is not clear for females. Hence, more cell-specific sex-based studies are warranted to define sex differences for AhR's involvement in energy balance.

This study revealed that AhR-deficiency in females led to reduced calorie intake and an increased metabolic rate that contributed to protection from HFD-induced weight gain. In general, females have an increased susceptibility to overfeeding and are more sensitive to macronutrient changes (Cornier et al., 2004). Compared to males, WT females tend to reduce appetite when challenged with a dense calorie diet, due to more central leptin sensitivity (Kautzky-Willer et al., 2016). AhR effects on calorie intake in males are equivocal according to current literature. While a majority of studies showed no change in consumed calories (Baker et al., 2015; Kerley-Hamilton et al., 2012; Xu et al., 2015), one study did demonstrate reduced consumption of kilocalories in low-affinity AhR or AhR depleted mouse strains, when fed HFD (West et al., 1992). AhR involvement in the regulation of calorie intake in females is also ambiguous, but mainly due to a lack of available data. One study that used an AhR antagonist to inhibit AhR demonstrated protection from weight gain/obesity in both males and females under western diet (35% kcal carbohydrates, 45% kcal fat), but contrary to the present study the protection was not associated with reduced calorie intake (Moyer et al., 2017). Unfortunately, that study did not investigate adipose tissues, which would have helped in comparing with the

present study. Nevertheless, changes in body weight and fat mass can develop with very small mismatches of food intake and energy expenditure, especially over prolonged periods of time (Preitner et al., 2009).

Whole body energy expenditure (EE) is determined by adaptive thermogenesis, basal metabolism and physical activity. Females have lower 24-h EE, basal metabolic rate and sleeping metabolic rate in general, and obese females have lower EE compared to males (Ferraro et al., 1992). Global AhR deficient male mice (AhRKO) maintain a lean phenotype on HFD due to an increase in EE (Xu et al., 2015). In this study, CadKO males did not show increased EE on HFD compared to WT. However, both CadKO and AhRKO females did increase EE under HFD conditions compared to WT. Similar observations have also been reported in inducible liver-specific AhR knock-out in females (Girer et al., 2019). Collectively, the available data highlight AhR's possible involvement in EE and demonstrate that AhR deficiency in mature adipose tissue has sex-specific effects on EE.

Increased locomotor activity may also contribute to reduced weight gain under HFD conditions. However, because weight differences at the time of the experiment may influence locomotion, it is not clear that AhR deficiency is the direct cause of changes in locomotor activity, particularly in females. In general, males are more physically active compared to females, and testosterone plays a big role in this difference (Jardí et al., 2018). CadKO males showed increased activity, which might assist in their protection from weight gain on HFD compared to WT (Fig 35). One caveat to this study is the male sex steroid (testosterone) level and its signaling were not investigated, which can be explored in the future.

Investigation of glucose homeostasis suggests AhR deficiency specifically from mature adipose tissue improves fasting glucose and glucose tolerance in females, similar to AhR global

knockout. In contrast, males require AhR deficiency beyond adipose tissue for significant improvement in glucose homeostasis. Similar to the present study, our previous study in male AhRKO (Xu et al., 2015) demonstrated significant improvement in glucose tolerance. Even mice that express only a single AhR allele (*AhR*^{+/-}), which may in some ways be more similar to the mice with the low-affinity allele, are resistant to the harmful effects of HFD-induced glucose burden (Jaeger et al., 2017; Xu et al., 2015). AhR ablation specifically from adipose tissue did not provide a robust defense against glucose intolerance in males, suggesting that AhR depletion must occur in other organs (liver), or multiple organs to achieve protection in males. A previous study by Baker et al (Baker et al., 2015) using tissue-specific AhR deletion from adipocytes (from fertilization) unexpectedly found increased body weight and impaired glucose homeostasis in HFD-fed males. It wasn't clear from their study the exact reasoning behind that, and based on the available literature, that study is an outlier. This study failed to demonstrate robust protection from weight gain and glucose homeostasis in males, but neither was exacerbated. Moreover, tissue-specific AhR depletion using (*Pdgfra*)-Cre to deplete AhR beginning at the preadipocyte phase in males produced effects on glucose tolerance was also comparable to the current study; neither form of adipose-specific depletion was effective to protect male mice from HFD-induced glucose insensitivity. These data suggest AhR knockout in other tissues besides adipose is important for overt improvement in glucose homeostasis in males (Gourronc et al., 2020). However, congenic (Kerley-Hamilton et al., 2012) and transgenic (Xu et al., 2015) AhR deficiency does protect from glucose insensitivity in males on HFD, suggesting AhR can regulate glucose homeostasis in males, but that this effect is not solely mediated by adipose tissue. Due to very limited data, the story is not clear for female. The current study does show protected glucose sensitivity in AhRKO and CadKO females, indicating that adipose AhR may

play a bigger role in mediating systemic glucose sensitivity in females, compared to males.

These data provide evidence that adipose tissue function, and its contribution to global metabolism, may differ between the sexes.

The two major processes responsible for the maintenance of normal glucose homeostasis are insulin sensitivity and insulin secretion. However, insulin secretion is likely most important, as the body with insulin resistance can maintain normal glucose level by pumping more insulin. However, this hyperinsulinemia eventually leads to burnout of the pancreatic beta cells and is considered a hallmark of insulin resistance. Hyperinsulinemia in WT males on HFD suggests the possibility of developing insulin resistance with pancreatic β -cell compensation to maintain glucose levels similar to CadKO male. Similarly, Xu et al found AhR^{-/-} and AhR^{+/-} male mice fed HFD were protected from hyperinsulinemia (Xu et al., 2015). Based on the current study, it is clear AhR depletion plays a significant role in the maintenance of normal insulin levels under HFD. Moreover, plenty of reports also suggest AhR signaling contributes to insulin sensitivity and insulin regulation (e.g. (Cranmer et al., 2000; Henriksen et al., 1997; Zhao et al., 2020)). Overall, the phenotypic data presented in this study clearly demonstrate that AhR depletion from adipose tissue has sex-specific effects on systemic metabolism in face of HFD.

5.3 Specific Aim 3: Examine molecular changes in adipose tissue in CadKO after HFD feeding.

The phenotypic data presented in this study clearly demonstrate that AhR depletion from adipose tissue has sex-specific effects, suggesting that adipose function may be different between males and females. Effects of adipose-specific AhR depletion on metabolic health are partly mediated through the preservation of adipose tissue function. Morphological and transcript level analysis suggests maintenance of small adipocytes in CadKO females on HFD promotes healthy adipose function with active adipogenesis, and lipolysis, which was not seen in males. Small

adipocytes can be able to store and release fat more efficiently. The ability to recruit new mature adipocytes in the face of increased energy abundance can be critical to combat metabolic disease. It follows that decreased ability to recruit and differentiate new adipocytes can lead to hypertrophic obesity due to the inappropriate expansion of available adipocytes. When adipocytes become too large, their function deteriorates. They lose the ability to remove lipids from the blood. On the other hand, expansion of adipocyte numbers can result in adiposity with the maintenance of metabolic function, also known as a metabolically healthy, obese state. Therefore, factors that influence adipogenesis are critical in the maintenance of systemic metabolism. Fat mobility can also help maintain the size of adipocytes, by promoting the release of stored fat from the cells. When the body needs to use energy, it can break down stored fat and release it into the bloodstream through the process of lipolysis. This can help keep the fat cells from becoming too enlarged, which can occur in obesity and subsequently leads to a metabolically unhealthy obese state, and the development of the metabolic syndrome.

Adipogenesis is controlled by a complex network of transcription factors, but PPAR γ is considered the master regulator (Wafer et al., 2017). Similarly, lipolysis is a multi-step process controlled by several enzymes, but HSL, which catalyzes the hydrolysis of stored fat (triglycerides) into FFA and glycerol is considered key to this process. AhR activation, which may occur in response to certain components of the HFD in mature adipocytes, will inhibit adipogenesis and lipolysis in mature adipocytes through PPAR γ and HSL pathways, respectively (Kerley-Hamilton et al., 2012; Khazaal et al., 2023; Shimba et al., 2003; Shimba et al., 1998). The PPAR family of nuclear receptors are well-studied targets of AhR activation. Upon ligand binding, e.g. end product of HFD, AhR translocate inside the nucleus and functions as the substrate receptor in E3 ubiquitin ligase for recruiting and degradation of PPAR γ (Dou et al.,

2019). AhR overexpression reduces PPAR γ stability and suppresses adipocyte differentiation, and AhR knockdown stimulates adipocyte differentiation in 3T3-L1 cells. Like AhR, PPAR γ can also interact with many nuclear receptors, such as the androgen receptor (Buchanan et al., 2007; Jin et al., 2012), estrogen receptor (Burns et al., 2014; Yan et al., 2007), glucocorticoid receptor (Chodankar et al., 2014), and PPAR α (Georgiakaki et al., 2006). Their functions are likely sex-specific, but differences in mechanism between the sexes remain to be determined. Such studies could facilitate explaining the sex differences in adipogenesis observed in the current study as described below, but for now, the exact mechanism is not clear.

CadKO females respond to HFD by increasing PPAR γ , which promotes adipogenesis and may explain the maintenance of smaller adipocytes in VAT. AhR regulates cell proliferation and differentiation of mesenchymal stem cells and preadipocytes within adipose tissue. AhR protein is downregulated when preadipocytes differentiate into mature adipocytes (Shimba et al., 2003; Shimba et al., 1998). Study on preadipocytes specific, Pdgfr α -Cre Ahr-floxed (Ahr^{fl/fl}), AhR knockout mice demonstrated protection from HFD-induced obesity. The Pdgfr α -Cre Ahr^{fl/fl} knockout mice were also protected from increased adiposity, and enlargement of adipocyte size while on the HFD compared to control mice (Gourronc et al., 2020). Unexpectedly results from CKO AhR-specific knockout from adipocytes by Baker et al (Baker et al., 2015) revealed conflicting results compared to preadipocytes specific (Gourronc et al., 2020) and iCKO from mature adipocytes (present study) as they reported exacerbated HFD-induced obesity. One way to explain this discrepancy is that AhR knockout in adipocytes after they are matured or in preadipocytes might be more effective in communicating between preadipocytes and mature adipocytes regarding the process of adipogenesis. Delay of knockout of AhR until the adipogenesis program is fully activated (when adiponectin is expressed during

the mature phase) may result in a completely different phenotype in responses to AhR ligands. These contrasting effects may also suggest differences in cell-autonomous and non-autonomous actions of the AhR in adipocytes can vary depending upon the timing of AhR knockout and may be important for determining the outcome.

Upregulation of HSL in females indicates enhanced lipolysis that increases the ability to provide FFA for energy during fasting and exercise, which is important for systemic energy homeostasis. A previous study from the Tischkau lab found activation of AhR by BNF inhibits the expression of lipolysis genes in differentiated 3T3-L1 adipocyte cultures and suppresses lipolysis (Khazaal et al., 2023). These results are synergistic with the current results in CadKO females, but not with males, and the reason behind that is not clear. It could be due to the intrinsic sex differences, as the current result demonstrated females, in general, have higher HSL levels compared to males. It could also be that AhR depletion does not always give exact opposite results compared to AhR activation (Julliard et al., 2014; Murray et al., 2014; Ojo & Tischkau, 2021). Many studies demonstrate complexity around AhR activation and inhibition that are not straightforward. Further studies are necessary to investigate the specifics of the relationship between AhR and lipolysis.

The rate-limiting lipogenic gene, *Srebp1c*, was significantly higher in CadKO male VAT. *Srebp1c* is activated by insulin, primarily in WAT and liver, when the levels of glucose/FFA are high; it activates the transcription of genes responsible for lipogenesis, including enzymes involved in fatty acid synthesis (acetyl-CoA carboxylase, fatty acid synthase) and enzymes involved in the synthesis of triglycerides. Low *Srebp1c* levels in VAT of HFD-fed WT males may reflect the appearance of an inflammatory and insulin-resistant state. Sex differences were apparent in the expression of this transcript, which was not surprising, as males are known to

increase *de novo* lipogenesis in VAT when presented with excess calories, which may also explain why VAT mass was similar in CadKO and WT males. In the study by Baker et al (Baker et al., 2015) using CKO AhR deletion from adipocytes, adiposity and adipocyte size were unexpectedly increased in males on HFD. The authors speculated the reason could be due to enhanced triglyceride synthesis/*de novo* lipogenesis. Without AhR, there is potential to upregulate *de novo* lipogenesis, as lipogenesis is inhibited by AhR activation (Liu et al., 2021). In the present study, CadKO males were protected from HFD-induced weight gain, but it is possible that *de novo* lipogenesis was increased, which produces increased adiposity. More *de novo* lipogenesis under HFD can be beneficial for reducing ectopic lipid spillover in adjacent organs.

Although the exact reason for the sex differences remains unclear, we did explore the possibility of AhR interactions with the female sex steroid receptor, ER α . AhR inhibits the signaling pathways of ER α . The precise mechanism for this interaction is not clear and can be multifactorial. Activated AhR recruits ER α away from estrogen-responsive elements in target genes, increases proteasomal degradation of ER α , and increases the synthesis of an unknown inhibitory protein (Matthews & Gustafsson, 2006). In females, ER α inhibits WAT development, amount, as well its size, suggesting ER α also regulates triglyceride accumulation (Heine et al., 2000; Pedersen et al., 1992). In this study, deleting AhR from adipose tissue increased the expression of ER α in female VAT, which may provide a mechanism for the observed protection against adiposity. Thus, an increase in ER α favors the anti-obesity phenotype in CadKO females on HFD.

In males, AhR depletion in adipose tissue may improve crosstalk with local and peripheral immune cells (Fig 35). Inflammatory cytokines secreted from adipose tissue play a

critical role in such crosstalk. Agonists of AhR, such as obesogenic POPs, coplanar PCB, and TCDD, increase inflammation of murine and human adipocytes, and selective ablation of AhR in adipose tissue abolishes the negative effects on adipose tissue inflammation (Arsenescu et al., 2008; Baker, Karounos, et al., 2013; Kim et al., 2012; Nishiumi et al., 2010). Moreover, AhR activation by obesogens and other metabolites present in HFD, such as Kyn, also negatively affects adipose function through the AhR/Stat3/IL6 pathway (Huang et al., 2022), which can be prevented by the removal of AhR. Altogether, the present study suggests CadKO provides a mechanism to improve adipose biology in males on HFD through reduced secretion of proinflammatory adipocytokines (TNF α , IL1 β , IL6), thus less inflammation, macrophage infiltration, and lower serum insulin (Fig 35).

Xu et al. 2015 (Xu et al., 2015), demonstrated robust improvement of AhRKO males in glucose homeostasis, which was not observed in our present study on CadKO males. Both adipocytokines and adipokines (such as adiponectin) regulate adipose tissue inflammation and contribute to glucose homeostasis. HFD-fed AhRKO males showed reduced adipocytokine, combined with increased adiponectin secretion and improved leptin signaling. This might contribute to the preservation of healthy fasting and postprandial glucose levels. Studies on CKO and iCKO of AhR from adipocytes, however, revealed similar data on adipose tissue inflammation. Depletion via CKO in male mice demonstrates that deficiency of adipocyte AhR protects adipose tissue from inflammation and infiltration of macrophages on HFD (Baker et al., 2015). Interestingly, the same study also revealed exacerbated weight gain on HFD in male CKO mice, highlighting, similar to the present study, that AhR depletion from adipose tissue is more effective in protecting from adipose tissue inflammation than in weight homeostasis. In females, CadKO prevents weight gain that facilitates lean phenotype, with highly functional adipocytes

and hence maintenance of healthy coordination with other metabolic organs. Healthy collaboration of metabolic tissues (e.g. liver, muscle, WAT) regulates processes such as gluconeogenesis, lipolysis, insulin-dependent glucose uptake that helps to maintain normal blood glucose. Failure of coordination among tissues can be a major contributor to hyperglycemia (Petersen & Shulman, 2018). Hence, the conservation of lean adipocytes in CadKO females could be the primary reason for improved fasting and postprandial glucose levels.

Direct regulation of Fgf21 may provide an important link between AhR and metabolic function in adipose tissue (Girer et al., 2019). Fgf21 promotes EE and improves lipid as well as glucose metabolism, and thus has the potential to protect against metabolic diseases (Girer et al., 2020; Iglesias et al., 2012). This hormone is mainly produced by the liver and can act on adipocytes to promote thermogenesis, insulin sensitivity, lipid and glucose homeostasis (Fisher et al., 2012; Hondares et al., 2010; Kharitononkov et al., 2005). Fgf21 stimulation by physical activity, cold exposure, and/or a calorie-dense diet (Chartoumpekis et al., 2011; Cuevas-Ramos et al., 2010; Cuevas-Ramos et al., 2012; Giralt et al., 2015; Hondares et al., 2011) can directly induce thermogenic gene expression in both BAT and WAT (beiging) (Straub & Wolfrum, 2015). Whether AhR promotes or suppresses Fgf21 gene expression remains ambiguous, with data to support each claim. For instance, Girer et al. 2016, found AhR activation downregulates Fgf21 expression, and iCKO of AhR in the liver stimulates hepatic Fgf21 expression (Girer et al., 2016). However, Cheng et al. 2014, demonstrated that AhR activation with TCDD elevates Fgf21 expression (Cheng et al., 2014). The duration of AhR activation might be important. Acute activation of AhR may increase Fgf21, whereas Fgf21 resistance may develop with chronic AhR activation. AhR liver-specific depletion increases Fgf21 production by the liver and promotes beiging (WAT) and thermogenesis (BAT) (Girer et al., 2019). In the current study, CadKO did

not affect Fgf21 transcript levels in liver and thus failed to stimulate BAT thermogenesis. However, Fgf21 can also be secreted from WAT to act in an autocrine/paracrine fashion to stimulate beiging, and a key modulator for this process is PPAR γ (Cuevas-Ramos et al., 2019; Emanuelli et al., 2014; Hondares et al., 2011; Hondares et al., 2010). HFD-fed CadKO females demonstrated increases in Fgf21, PPAR γ , and UCP1, suggesting that increased thermogenesis may contribute to their heightened adipose function and protection from metabolic dysfunction. These pathways were unaffected in males.

Beiging of WAT and subsequent increases in EE that protect from HFD-induced obesity may also occur secondary to leptin and insulin synergistic signaling in hypothalamic POMC neurons (Dodd et al., 2015). Leptin can elicit acute effects on food intake and EE by promoting BAT activity. Chronic, postprandial elevated leptin and insulin coordinate to act on POMC neurons to promote signals that induce WAT beiging, which may be a major contributor to diet-induced changes in thermogenesis (Dodd et al., 2015; Rothwell & Stock, 1979). Increased expression of UCP1 in VAT of HFD-fed CadKO females suggests increased beiging. CadKO females were the only group to demonstrate protection from insulin resistance (improved fasting, postprandial glucose and insulin levels) and leptin resistance (low serum leptin levels) on HFD. One caveat of the current study is we examined beiging in epididymal visceral adipose tissue found, where beiging occurs less frequently. Examination of other more susceptible adipose depots (such as inguinal WAT) may reveal a larger effect in the CadKO mice.

Interestingly for males, both whole-body AhR knockout (Xu et al., 2015) and CKO of AhR from preadipocytes (Gourronc et al., 2020) increase EE mediated by heightened BAT respiration. This is conflicting with iCKO (present study) and CKO (Baker et al., 2015) studies on adipose-specific AhR deletion. The exact reason behind this is not clear. One reason can

easily be that AhR regulation in preadipocytes of BAT might be more responsive towards maturation and differentiation into functional thermogenic-responsive brown adipocytes, that facilitate HFD-induced norepinephrine-mediated induction of UCP1.

In conclusion, the current study highlights the critical role of AhR in mature adipocytes for controlling energy homeostasis in response to a calorie-dense diet. Deletion of AhR from adipose tissue provides a good working system to examine the effects of HFD-induced obesity and diabetes in a sexually dimorphic way. Improved adipose biology is likely the cause for the amelioration of weight gain and insulin resistance on HFD for both sexes, although the specifics of adipose physiology are changed in a sex-specific manner.

5.4 Specific Aim 4: Explore hepatic steatosis (NAFLD) to examine systemic effect CadKO after HFD feeding.

The current study demonstrates that males are more susceptible to HFD-induced fatty liver, which leads to non-alcoholic fatty liver disease (NAFLD), compared to females. In humans, the prevalence, and severity of NAFLD are lower in females than males, until menopause (Lonardo et al., 2019). After menopause, the rate of NAFLD increases in women, suggesting that estrogen is protective. Adipose tissue communication with the liver is a significant contributor to changes in hepatocyte biology leading to NAFLD. For example, enlarged metabolically unhealthy VAT (found in male), leads to systemic insulin resistance, hyperinsulinemia, and inflammation initiated by inflammatory adipocytokine release. This facilitates energy influx in the liver, making the tissue more vulnerable to metabolic stress (Fried et al., 2015). Furthermore, too much insulin in the blood induces the production of fat in the liver, aggravating the disease condition (Fujii et al., 2020), which parallels our data on WT males. Adiponectin, another key anti-inflammatory adipokine, which is higher in females, plays

a protective role against NAFLD (Gatselis et al., 2014). Sex differences in both adiponectin levels and the appearance of fatty liver were also apparent in the current study.

Studies on different types of AhR mouse models were also consistent with our study. Deficiency or modulation of AhR via whole-body knockout (Xu et al., 2015), hemizygous depletion (Jaeger et al., 2017; Xu et al., 2015), low-affinity mouse strain (Moyer et al., 2017), pharmacological inhibition (Moyer et al., 2017), and CKO from preadipocytes (Gourronc et al., 2020), all demonstrated protection from hepatic steatosis in HFD-fed male mice. Studies on females are limited, although one study showed fatty liver can be prevented to a higher degree by inhibition of AhR (using α -naphthoflavone) under HFD compared to males (Moyer et al., 2017).

However, one contrasting study using liver-specific CKO revealed increased hepatic steatosis in males due to a significant increase in *de novo* lipogenesis (Wada et al., 2016). AhR depletion was reported to increase lipogenesis (Alexander et al., 1998), which was also observed in male VAT in the current study. Based on these studies, AhR depletion from adipose tissue leading to increased triglyceride synthesis may be beneficial for hepatic steatosis protection, as the excess FFA from the HFD can be stored more in its designated storage organ, i.e., adipose tissue, rather in the liver. Moreover, the timing of AhR deletion may also be important, as no studies have explored the effects of liver-specific depletion only in adult hepatocytes.

Molecular analyses in the present study demonstrated CadKO females and males have a unique way to acquire protection that doesn't overlap. In the case of females, the protection starts from less intake of food on HFD, which accounts for fewer serum triglycerides in the blood. Molecular changes due to adipose-specific AhR deletion also add to the protection. CD36 is a known target gene for AhR (Lee et al., 2010) that promotes serum FFA translocation inside the liver and was reduced in HFD-fed CadKO females. Upregulation of the hepatic lipogenic gene,

ACC, was observed in HFD-fed WT females, but not in CadKO females, promoting protection from steatosis. Furthermore, increased Fgf21 levels in CadKO female adipose tissue (shown in Chapter 6) may provide signaling to the liver to assist in fighting against hepatic steatosis. Additionally, increased adipogenesis capacity in female CadKO WAT (shown in Chapter 6) may allow the deposition of additional lipids in fat and less lipid spillover.

Protection in males after AhR-specific depletion from adipose tissue was more apparent at the molecular level of liver tissue. PPAR α , important for mitochondrial fatty acid β -oxidation in the liver, was upregulated in CadKO males. This result is different from the effect in AhRKO and AhR^{+/-} males, in which mice showed reduced expression in the liver on HFD (Jaeger et al., 2017; Xu et al., 2015). As AhR is still available in the liver of CadKO male mice, increased PPAR α may be an adaptive response to attenuate fat accumulation in the liver (Lee et al., 2001).

The liver also plays a vital role in glucose homeostasis. Gluconeogenesis (production of glucose from non-carbohydrate substrates) and glycogenolysis (release of glucose by breaking down glycogen) are two important processes in the liver to restore normal blood glucose when required, such as at the time of fasting. G6pase is a rate-limiting enzyme for both processes. AhR activation by obesogens can suppress gluconeogenesis and glycogenolysis, and suppression was attenuated in the low-affinity AhR mouse strain, C57BL/6.D2₂ (Zhang et al., 2015). C57BL/6.D2 mice were less susceptible to the negative metabolic impacts of HFD compared to C57BL/6 mice with a high-affinity AhR allele. Similarly, AhRKO males showed unaltered G6pase on HFD in the liver (Korecka et al., 2016). However, the present study demonstrated an increase in G6Pase transcript levels in CadKO male livers. The reason for the difference can solely be due to the fundamental difference between the two genotypes, which is the presence of AhR in the liver of CadKO and the absence of adipose, which can alter the crosstalk between the two organs. Such

alteration can be manifested in systemic glucose homeostasis, as no difference was found in fasting and postprandial glucose level in CadKO compared to WT (Fig 19.a, e, g), whereas AhRKO demonstrated improvement (Xu et al., 2015).

Overall, these data suggest protective effects of CadKO on HFD-induced hepatic steatosis in a sexually dichotomous manner. This may be due to not only AhR involvement but also intrinsic sex differences between males and females.

5.5 Specific Aim 5: Examine energy regulation in the hypothalamic feeding centers.

Sex differences in calorie intake under HFD prompted our exploration of hypothalamic centers associated with feeding. CadKO females consumed fewer calories on HFD compared to WT. Increased ER α levels in hypothalamic regions of CadKO female explain for the sex differences, but how AhR deletion from adipose tissue accomplishes this remains unclear.

It is possible that this is a consequence of adipose-hypothalamus cross-talk, and is a response to signals coming from healthy, lean adipocytes in CadKO females (Fig 35). Leptin, derived from adipocytes, is a vital hormone for connecting the bridge between adipose tissue and the hypothalamus via afferent signaling. Typically, adult females tend to have both increased leptin levels and sensitivity compared to males (Clegg et al., 2003; Rosenbaum et al., 1996; Saad et al., 1997). Evolutionarily, sex-specific survival strategies in preparation for food scarcity may underlie sex differences. Males prepare by increasing fat stores via consuming more food, whereas females reduce fat store loss by reducing EE (Shi et al., 2007). High levels of leptin in females help meet the survival strategies, but the chronic elevation of these hormones increases the risk of developing resistance. Sex hormones can also alter leptin levels. Androgen reduces leptin, whereas estrogen increases leptin concentrations (Luukkaa et al., 1998; Unluhizarci et al.,

2021). Healthy adipose-hypothalamic crosstalk, as well as better leptin signaling, may provide an important mechanism to protect CadKO females from HFD-induced weight gain.

The exact reason for the reduced weight gain in CadKO males is not understood well. The arcuate nucleus of the hypothalamus contains neurons that are sensitive to insulin to promote POMC neuron activity. Insulin signaling in the ARC may play an important role in regulating the effects observed in CadKO males. As CadKO males are more protected from systemic insulin resistance on HFD compared to WT (Fig 20.a, 23.b, 24), that can contribute to the weight difference observed. Moreover, investigation of leptin signaling after the HFD regimen demonstrated that, similar to WT males, CadKO males became leptin resistant, but differences in leptin signaling prior to initiation of HFD feeding were not explored. Even a small difference in leptin signaling especially over prolonged periods might contribute to a small mismatch in food intake and EE, which can affect overall weight difference (Preitner et al., 2009), which cannot be ruled out by the present study.

Studies that explore AhR-specific regulation of the hypothalamic feeding center are very limited, and no study has been conducted in females. Thus, the current study may pave a path for investigating AhR-specific role in hypothalamic appetite regulation that also includes sex differences.

Table 1. Serum measurements from NCD or HFD fed mice.

Serum Protein	NCD				HFD			
	CadKO Male	WT Male	CadKO Female	WT Female	CadKO Male	WT Male	CadKO Female	WT Female
Leptin (ng/ml)	7.76±2.64 ^{####}	5.27±3.88 ^{####}	4.77±2.57 ^{####}	5.56±1.01 ^{####}	40.40±2.76 ^{SS}	43.47±2.40	28.81±11.19	38.67±3.19
Insulin (ng/ml)	0.34±0.22 [#]	0.81±0.3 ^{####}	0.46±0.34	0.63±0.34	1.59±0.46 ^S	6.46±5.14 ^{SSSS}	0.49±0.344	0.88±0.54
Adiponectin (mg/ml)	48.52±10.38	35.85±6.25 ^{S#}	75.75±18.9	71.52±31 [#]	71.42±9.64	73.9±7.96 [#]	78.15±24.22	106.85±26.6
17β-estradiol (pg/ml)	155.10±1.99	155.73±12.4 ^{S#}	161.39±13.4	178.8±16.63	144.76±24.92	125.74±13 ^{SSS}	161.48±11.5	160.13±6.57
Triglycerides (mg/dl)	n/a	n/a	n/a	n/a	195.88±26 ^{SSS}	181.74±6.23	155.33±17.78	173.20±5.68

Abbreviations: CadKO: Conditional adipose Knock Out; WT: Wild Type; HFD: high-fat diet; NCD: normal chow diet. Blood was collected from mice groups at week 15 of NCD or HFD. Serum leptin, insulin, adiponectin, 17β-estradiol and triglycerides were measured by ELISA or commercial kits (NCD, n= 5-6; HFD, n=8-10). *P<0.05, **p<0.001, ***p<0.0001; #P<0.05, ##p<0.001, ###p<0.0001, ####p<0.00001: compared with diet but same sex; ^Sp<0.05, ^{SS}p<0.001, ^{SSS}p<0.0001, ^{SSSS}p<0.00001: compared with sex but same diet, by 2-way ANOVA with Turkey's post hoc comparison.

Table 2. List of Antibodies.

Antibody	Company	Catalog#	Source	Dilution
Leptin receptor	Thermo Fisher Scientific	PA1-053	Rabbit polyclonal	1:100
Estrogen Receptor α	Abcam	ab32063	Rabbit monoclonal	1:100
Uncoupling protein 1	Invitrogen	PA5-120958	Rabbit polyclonal	1:250
IL-6	Santa Cruz biotechnology	10E5	Mouse monoclonal	1:200
phospho-Stat3	Cell signaling	9145	Rabbit monoclonal	1:2000
B-actin	Sigma-Aldrich	A1978	Mouse monoclonal	1:4000
Anti-Mouse	LI-COR	926-68070	Goat	1:10,000
Anti-Rabbit	LI-COR	926-32211	Goat	1:10,000
Anti-mouse IgG2a (γ2a) (Alexa FlourTM 488)	Thermos Fisher Scientific	A21131	Goat	1:1000
Anti-rabbit IgG (H+L) (Alexa FlourTM 488)	Thermos Fisher Scientific	A11008	Goat	1:1000
Anti-rabbit IgG (H+L) (Alexa Fluor[®] 594)	Thermos Fisher Scientific	A11012	Goat	1:1000

Table 3. List of PCR Primers.

Gene	Forward Primer	Reverse Primer
Fgf21	GTGTCAAAGCCTCTAGGTTTCTT	GGTACACATTGTAACCGTCCTC
UCP1	GCTTTGCCTCACTCAGGATTGG	CCAATGAACACTGCCACACCTC
Leptin receptor	CTTTCCTGTGGACAGAACCAGC	AGCACTGAGTGACTCCACAGCA
Ppar γ	TCTGGGAGATTCTCTGTGA	GGTGGCCAGAATGGCATCT
ER α	TGCACCATTGACAAGAACCGGA	AGCACCCATTTCATTTGGCCT
Adiponectin	TCCAATGTACCCATTTGCT	TCCTTCTGAAGAGGCTCAC
CD36	GGAAGTGTGGGCTCATTGC	CATGAGAATGCCTCCAACAC
G6PC	AGGTCGTGGCTGGAGTCTTGTC	GTAGCAGGTAGAATCCAAGCGC
Ppara α	ACTGGTAGTCTGCAAAACAAA	AGAGCCCCATCTGTCTCTC
ACC	GCCTCCGTCAGCTCAGATAC	ATGTGAAAGGCCAAACCATC
Srebp1c	CCATCGACTACATCCGTTCTT	ACTTCGTAGGGTCAGGTTCTC
HSL	GGCTCACAGTTACCATCTCACC	GAGTACCTTGCTGTCTGTCC
TNF α	CCACCAGCTCTTCTGTCTAC	AGGGTCTGGGCCATAGAACT
IL1 β	AGATGAAGGGCTGCTTCCAAA	GGAAGGTCCACGGGAAAGAC
Cyp1A1	GTGCATCGGAGAGACCATTG	GGTAGGAGTCATATCCACCTT
Cyp1B1	ACATCCCAAGAATACGGTC	TAGACAGTTCCTCACCGATG
B ₂ M	CAGGCGTATGTATCAGTCTTC	AAGTATACTCACGCCACCA
B-actin	CACCCGCGAGCACAGCTTCTT	TTTGACATGCCGAGCCGTT

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

6.1 Summary

This study highlights the role of AhR in mature adipocytes for controlling energy homeostasis in response to a calorie-dense diet. Deletion of AhR from adipose tissue provides a good working system to examine the effects of HFD-induced obesity and diabetes in a sexually dimorphic way. Improved adipose biology in CadKO mice is likely the cause for the amelioration of weight gain and insulin resistance on HFD for both sexes, although the specifics of adipose physiology changes in a sex-specific manner. In females, maintenance of small, healthy adipocytes on HFD facilitates crosstalk with feeding centers in the hypothalamus to maintain communication similar to lean animals. In males, local effects within adipose tissue and interactions with the immune system may be more important.

Regulation of insulin and leptin may be key to understanding the sex-specific interactions with the brain and/or immune systems. Certainly, hypothalamic changes manifest after AhR depletion from adipose tissue is important in females. The suppressive effect of AhR on ER α may be the link to sex-dependent changes in both adipose depots and the hypothalamus, as CadKO females show elevated ER α under HFD conditions. Other proteins/hormones that also interact with AhR, such as Fgf21, the PPAR family, HSL, CD36, and TNF α also modulate lipid and energy metabolism pertinent to protection from diet-induced metabolic dysfunction and sex differences. This study demonstrates that AhR is an important regulator of adult adipose tissue responses to HFD. AhR contributes to the regulation of body weight and glucose homeostasis in a sexually dimorphic manner. Importantly, this study reveals the significance of studying both sexes and highlights the potential need for developing therapeutics based on sex. Sex is a

fundamental factor in the incidence of obesity and diabetes. Since females are prone to obesity etiology and males to diabetes, understanding sex-specific pathophysiological changes may lead to new therapeutic targets. AhR and its downstream signaling pathway provide an interesting candidate, particularly in females.

6.2 Future Directions

The results from the present study provide important information regarding sex-specific effects of AhR function in adipose tissue and systemic impacts in the presence of dietary stress. The study also raises some important questions that can be addressed in the future. Inducible AhR-specific deletion from mature adipose tissue prior to special diet reveals whether the model can be preventive of HFD diet-induced metabolic dysfunction. Future studies can alter the time of AhR deletion by generating CadKO (administering tamoxifen) subsequent to the diet regimen, examining the model's potential as curative. Although it's hard to hypothesize what will be the outcome of such a scenario, a previous study has shown that AhR antagonism can reverse diet-induced obesity in male mice (Moyer et al., 2016). The present study demonstrated that maintenance of a lean adipose phenotype allowed CadKO female mice to maintain healthy metabolic function in the face of HFD. Whether CadKO can reverse the enlarging of adipocytes, and consequently pathophysiology, resulting from HFD is the confounding question for females. Adipocyte size can be reversible and decreasing the load of triglycerides due to less intake of HFD can be one way to do it. Certainly, the CadKO females showed the ability to reduce food intake on HFD, so this may be possible. Another mechanism could be by increasing the number of adipocytes to withstand the load of excess triglycerides. It is important to note that while it is possible to shrink fat cells, the number of fat cells in the body remains relatively constant throughout adulthood. Whether AhR depletion in already compromised adipose tissue could turn

on adipogenesis, which would be required to increase the number of active adipocytes, remains an open question. As CadKO females have the capability to regulate feeding behavior by improving leptin and estrogen signaling in the feeding center of the brain, CadKO may be curative in females. Apart from calorie restriction, another way to shrink adipocytes is through physical activity. In the current study, we demonstrated that an increase in locomotor activity can be one plausible reason for CadKO males resisting weight gain on HFD. However, at the end of the HFD regimen, CadKO males appeared to be catching up to the WT, thereby failing to maintain the same protection, despite the increase in locomotor activity, which calls into question the ability for AhR knockout to be curative in males.

One caveat of this study is a lack of mechanistic, molecular data to explain differences in locomotor activity. Future studies can focus on this avenue by studying organs such as muscles and body sympathetic tone. Moreover, our study also demonstrated delayed progression in insulin resistance in CadKO males. Hence, whether CadKO can be curative in a system that is already insulin resistant can be interesting to investigate, especially in males. A different approach to determine if AhR knockout could be curative when performed in already obese animals would be to graft healthy AhR-depleted adipose tissue into obese WT mice. Adipose tissue can be collected from mature, (couple of month old) lean mice of AhR-depleted and WT mice, and injected into HFD-induced obese WT mice. A preliminary study in the Tischkau lab demonstrated short-term improvement in glucose sensitivity in obese males and females after the transplant of healthy AhR-depleted adipose tissue (Tischkau lab, unpublished results). One caveat of this approach is the injected fat may not be long-lasting and may require multiple treatments to maintain the desired results.

The present study mainly focused on exploring VAT due to its major contribution on metabolic dysfunction. We cannot negate the fact SAT also plays a critical role and needs to be explored in future studies. A tissue specific role for different types of adipose tissue is hard to design, due to lack of specific promoter distinguishing the tissues in order to target via Cre-LoxP. Tissue specific grafting of VAT and SAT from lean CadKO to obese WT mice can be performed to tackle the issue.

Improved estrogen signaling mediated by ER α provides an additional avenue for controlled weight and glucose homeostasis by providing anti-obesity and anti-inflammatory effects in CadKO females. In the future, a double conditional knock-out (DCKO) of ER α and AhR can be constructed in adipose tissue to explore interactions of these two nuclear receptors. We anticipate to observe weight gain differences on HFD for both sexes on DCKO due to changes in leptin signaling and the anti-adiposity effects of estrogen in adipose tissue, although it is difficult to predict whether the effects will be as robust as observed in the current study. AhR can also modulate the activity of androgen receptor (AR) and can have an effect on fat synthesis, storage, and energy expenditure. Understanding exact mechanisms of how AR regulates these pathways, and how it might interact with AhR to treat or prevent obesity are not understood well. Future studies can identify the changes in AR signaling in adipose tissue and its subsequent impact systemically can be examined in CadKO mice.

Extensive studies in liver and adipose tissue have explored the role of AhR in energy homeostasis. However, very limited research has been conducted in the specific regions of the brain. For example, no lab to this date has explored the possible outcome of hypothalamic AhR loss in energy balance. Knowing both the sexes express high amounts of AhR in hypothalamus, investigating its sex-specific role will also be interesting to look at (Juricek & Coumoul, 2018;

Petersen et al., 2006). Alternatively, higher energy expenditure in the CadKO female can also be due to alteration of the sympathetic nervous system (SNS), which was not explored in this investigation.

Finally, the gut-brain axis is another critical avenue that has not been investigated in the current study. The communication system between the gut and the brain is involved in the regulation of various physiological processes including digestion, metabolism, and energy balance. AhR is widely expressed in the large and small intestine. AhR has been shown to be involved in the regulation of energy metabolism in the gut, in part by modulating the composition of the gut microbiome (Natividad et al., 2018). Certain by-products of bacterial metabolism in the gut microbiome are AhR ligands. Furthermore, the adipose-gut-brain axis is a complex system that plays a role in the regulation of energy balance. The gut and the brain communicate through a variety of signaling pathways, including the enteric nervous system, the vagus nerve, and hormones, to regulate energy intake, storage, and expenditure. A specific role of AhR in the gut-brain axis, as well as any alteration in the adipose-gut-brain axis due to CadKO can be investigated in the future. Tissue-specific roles for AhR will guide a more extensive understanding of AhR regulation of energy metabolism for both the sexes and underscores the potential revealing many more novel mechanisms, including those that can lead to innovative therapies in the fight against obesity and its downstream pathologies (Abate et al., 1996).

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