Spring 2020

# The Effects of Induced Polycystic Ovary Syndrome in NAG-1 Transgenic Mice

Nicholas Werner
wernern@cwu.edu

# THE EFFECTS OF INDUCED POLYCYSTIC OVARY SYNDROME IN NAG-1 TRANSGENIC MICE

---

A Thesis

Presented to

The Graduate Faculty

Central Washington University

---

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Biological Sciences

---

by

Nicholas James Werner

June 2020

CENTRAL WASHINGTON UNIVERSITY

Graduate Studies

We hereby approve the thesis of

Nicholas James Werner

Candidate for the degree of Master of Science

APPROVED FOR THE GRADUATE FACULTY

_____ _____
Dr. April Binder, Committee Chair

_____ _____
Dr. Lucinda Carnell

_____ _____
Dr. David Darda

_____ _____
Dean of Graduate Studies

ABSTRACT

THE EFFECTS OF INDUCED POLYCYSTIC OVARY

SYNDROME IN NAG-1 TRANSGENIC MICE

by

Nicholas James Werner

June 2020

Polycystic ovary syndrome (PCOS) is the leading cause of infertility among women in the US and the most common endocrine disorder among women. PCOS is characterized by cystic ovaries, hyperandrogenism (heightened levels of male sex hormones), altered menstrual cycles and various metabolic dysfunctions. The metabolic symptoms associated with PCOS are difficult to treat, as they are a result of hormonal imbalances, rather than diet. The human Non-Steroidal Anti-Inflammatory Drug Activated Gene (NAG-1) been shown to prevent diet-induced metabolic disorders and weight gain in mice. We hypothesized that the expression of NAG-1 may also prevent hormonal-induced metabolic disorders. To test this question, we induced PCOS via dihydrotestosterone (DHT) implantation in transgenic mice expressing the human NAG-1 gene. Our findings suggest that NAG-1 mice have similar physiological responses to DHT-treatment as compared to wild-type mice throughout the 90-day study. Specifically no changes in the age of puberty and anal-genital distance (AGD) were observed. NAG-1 mice also display similar ovarian phenotypes, developing fewer corpora lutea, and having disrupted estrus

cycles. NAG-1 mice displayed no significant weight gain between treatment groups throughout the study, and no significant increase in triglyceride levels. Additionally, NAG-1 mice showed no change in white adipocyte morphology after DHT-treatment. However, wild-type mice treated with DHT showed an increased amount of brown adipocytes differentiating to white adipocytes, compared to NAG-1 mice. Our findings indicate that NAG-1 mice respond similarly to DHT-treatment as wild-type mice in ovarian response, but continue to maintain their lean phenotype in the presence of induced PCOS. These findings suggest that expression of NAG-1 may have therapeutic benefits in the prevention of hormonal induced weight gain, and brown adipocyte hypertrophy.

ACKNOWLEDGMENTS

I am incredibly thankful for the members of graduate committee, who have been there to help and support me every step of the way through graduate school. Dr. Binder, Dr. Carnell, and Dr. Darda always made themselves available to help in any way they could throughout the entirety of both this study and the process of writing my thesis, and I can't thank them enough.

I want to thank my committee chair, Dr. Binder, for her constant support and for being such a great mentor over the years. I only wish I could begin to describe the vast amount she has taught me about experimental design, laboratory techniques, and scientific writing during my time at Central Washington University (CWU). I don't know where this thesis would be at without her always being so willing to read over and provide me with such timely and meaningful feedback. I will walk away from my time at CWU with a knowledge of molecular biotechniques that could not have been possible without her guidance. She has always been more than willing to help me in whatever way she can, and the patience and support I have received from her are unmatched.

I also would like to thank Delaney Clem and RaeAnne Tegman for their help in the laboratory. Both worked alongside me preparing, sectioning, staining, and analyzing adipose tissue, and their help is so incredibly appreciated. Their constant enthusiasm and positivity made them amazing to work with, and I could not have asked for a better team. I am also incredibly appreciative of all the help from the other members of the Binder Research Group who spent their weeks helping take care of the mice in the vivarium.

I also want to thank and acknowledge the CWU office of graduate studies for their constant availability and help, the Washington State Distinguished Fellowship in Biology for their generosity in funding my project, and Washington State University for letting me present my research at the Center for Reproductive Biology Retreat in 2019.

Last, I want to thank my parents, Dawn and Greg. They both have always been beyond supportive of me through my college career, and I would not be where I am today without them.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

# CHAPTER I

## INTRODUCTION AND LITERATURE REVIEW

Sexual reproduction is the result of the fusion between the male and female gametes, known as the sperm and the oocyte, respectively. In males, the gametes are produced in the testis through a process known as spermatogenesis, while in the females they are produced in the ovary through oogenesis. Males begin to produce sperm at puberty and continue throughout their life, while females are born with a fixed number of oocytes, produced during fetal development. In order to ensure reproductive success, the ovary must be highly efficient at maintaining the gametes and ensuring their proper growth and release throughout the female's reproductive lifespan. Occasionally, ovarian homeostasis may become impaired, and interrupt this process. One way ovarian homeostasis can become impaired is as the result of increased levels of androgens [1]. This elevated level of androgens is a condition known as hyperandrogenism. Hyperandrogenism in females can lead to infertility, formation of ovarian cysts, and irregularities in estrus or menstrual cycles [2]. In addition to these ovarian phenotypes, hyperandrogenism can also contribute to several types of metabolic disorders. The combination of hyperandrogenism, ovarian dysfunction, and an impaired metabolic phenotype culminate in a disease called polycystic ovary syndrome (PCOS). Common metabolic disorders associated with PCOS include glucose intolerance, type-II diabetes, obesity, and fatty liver disease [3]. Because these metabolic disorders arise as a result of hormonal imbalances, and not diet, they can prove difficult to treat. To understand changes in

ovarian homeostasis, and how they can lead to metabolic disorders, it is important to know the signaling processes that regulate ovarian function.

**The Ovary**

The ovary is the site of oogenesis and the synthesis of reproductive steroid hormone production in the female. It is here that the oocyte will receive nutrients, mature, and be released into the fallopian tube for fertilization beginning at puberty [4]. Due to its vital role in reproduction, the ovary is comprised of many different specialized cells that are responsible for maintaining the homeostasis of certain steroid hormones that ensures the oocytes develop correctly and are released at the appropriate time. Maintenance and production of these hormones occurs in structures within the ovary called follicles.

**The Mechanism of Follicular Development**

The oocyte is stored, undergoes development and maturation in a circular structure known as an ovarian follicle. The follicle is also responsible for producing testosterone, estrogen and progesterone in response to the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH released from the pituitary gland are responsible for ensuring proper growth and development of the oocyte, as well as the proper timing of its release into the fallopian tubes (ovulation) for fertilization. LH is responsible for the ovulation of the oocyte, initiating the synthesis of testosterone, and progesterone that prepares the endometrium for implantation of the fertilized oocyte [5, 6, 7].

The three main cell types of the ovarian follicle are theca cells, granulosa cells, and the oocyte [8]. The oocyte is located in the center of the follicle, where it

will grow and mature in a process called oogenesis. As the oocyte matures, the follicle undergoes transformation in a process called folliculogenesis. The earliest stage of a follicle is known as a primordial follicle and is comprised of an oocyte surrounded by several flat granulosa cells [9]. Folliculogenesis is initiated by the release of FSH from the pituitary gland. FSH binds to its specific G-protein coupled receptor, which stimulates intracellular signaling leading to increased granulosa cell division resulting in an increasingly thick layer of granulosa cells, while the oocyte forms a zona pellucida [9]. At this stage in development, the follicle is referred to as a primary follicle. The follicle then recruits a thin layer of theca cells, and develops into a secondary follicle. Theca cells form the outermost layer of the ovarian follicle, and are primarily responsible for the production of male sex hormones (androgens) [10]. The primary androgen produced is androstenedione, which is a precursor to estradiol [11]. The theca cells express the luteinizing hormone receptor (LHR) and synthesize androgens in response to LH secreted from the pituitary. This leads to increased expression of *Lhr*/LHR on both the granulosa and theca cells as the follicle grows [5]. High levels of vascular endothelial growth factor mRNA have also been observed in theca cells, suggesting they may have a role in vascularization [12].

The release of the mature oocyte, known as ovulation, is triggered by a surge of LH from the pituitary [5]. After ovulation the follicle differentiates into a progesterone producing structure called the corpus luteum, and will regress back into the ovary as scar tissue [13]. The ovary can have multiple stages of folliculogenesis occurring at any given time in order to assure an oocyte is released on a regular basis. As a mature follicle is preparing to ovulate its oocyte, there may

be corpus lutea, as well as immature follicles present in the ovary. In humans, an oocyte will be ovulated approximately once a month, and the entire process of folliculogenesis takes roughly 300 days [14]. In contrast, mice may release multiple oocytes once every four to five days, and a complete round of folliculogenesis takes only 19 days [15]. Although there are differences in the timing of folliculogenesis and ovulation, mice go through folliculogenesis physiologically and biochemically similar to humans making them an ideal model for studying ovarian function.

**Follicular Hormone Signaling**

The hypothalamic-pituitary-gonadal (HPG) axis is responsible for the regulation of development and reproduction in animals and thus plays a critical role in maintaining a healthy ovary and normal reproductive function [16]. The hypothalamus is composed of hypothalamic neurons, and is responsible for the initiation of hormonal signaling cascade involved in the HPG axis. The hypothalamus signals directly to the pituitary gland, which is located at the base of the brain. Signals from the pituitary are released into the bloodstream and travel to the gonads, which in turn can upregulate or downregulate further signaling from the hypothalamus or pituitary [17].

In adolescence, the HPG axis remains inactive, but during puberty the ovaries begin to synthesize and release estrogen in response to LH and FSH secreted by the pituitary, which in turn activates the HPG axis. As the HPG axis is activated, it begins releasing gonadotropin-releasing hormone (GnRH) from the hypothalamus to the pituitary gland [6]. GnRH binds to its G-protein coupled receptor and signals the gonadotrope cells in the anterior pituitary to release FSH and LH (Fig. 1). The HPG

axis remains active until menopause in females, when oocytes are depleted and the ovary stops secreting large amounts of estrogen due to lack of follicular granulosa cells [16].

FSH is responsible for the growth and maturation of immature small antral follicles [7]. As pulses of FSH in the ovary drive the growth of follicles, the granulosa cells begin to produce CYP19A1 (aromatase), which converts androstenedione produced in the theca cells into estradiol. The estradiol from the granulosa cells inhibits the production of both GnRH in the hypothalamus and FSH in the pituitary [18]. This dual inhibition triggers a surge in LH, which in turn triggers the rupturing of the preovulatory follicle, and the oocyte is ovulated into the fallopian tube for potential fertilization. After the oocyte is released, the remaining granulosa cells become luteinized and form a corpus luteum that synthesizes and secretes large amounts of progesterone and a small amount of estrogen [19, 20]. Estrogen initiates and maintains cell proliferation in the endometrium prior to the release of the oocyte, and progesterone prepares the endometrium for possible implantation if fertilization occurs [21]. Progesterone stimulates the endometrium to enter a secretory phase, where the inner lining can serve as an implantation site for a blastocyst [22]. Progesterone also inhibits the hypothalamus from releasing GnRH to the pituitary [23]. If fertilization does not occur, progesterone levels lower to basal levels, the corpus luteum regresses back into the ovary, the release of GnRH will no longer be inhibited, and folliculogenesis can continue [24]. Figure 1 illustrates the pathways through which the HPG axis regulates folliculogenesis.

These cumulative events mark the completion of one round of folliculogenesis in the ovary.

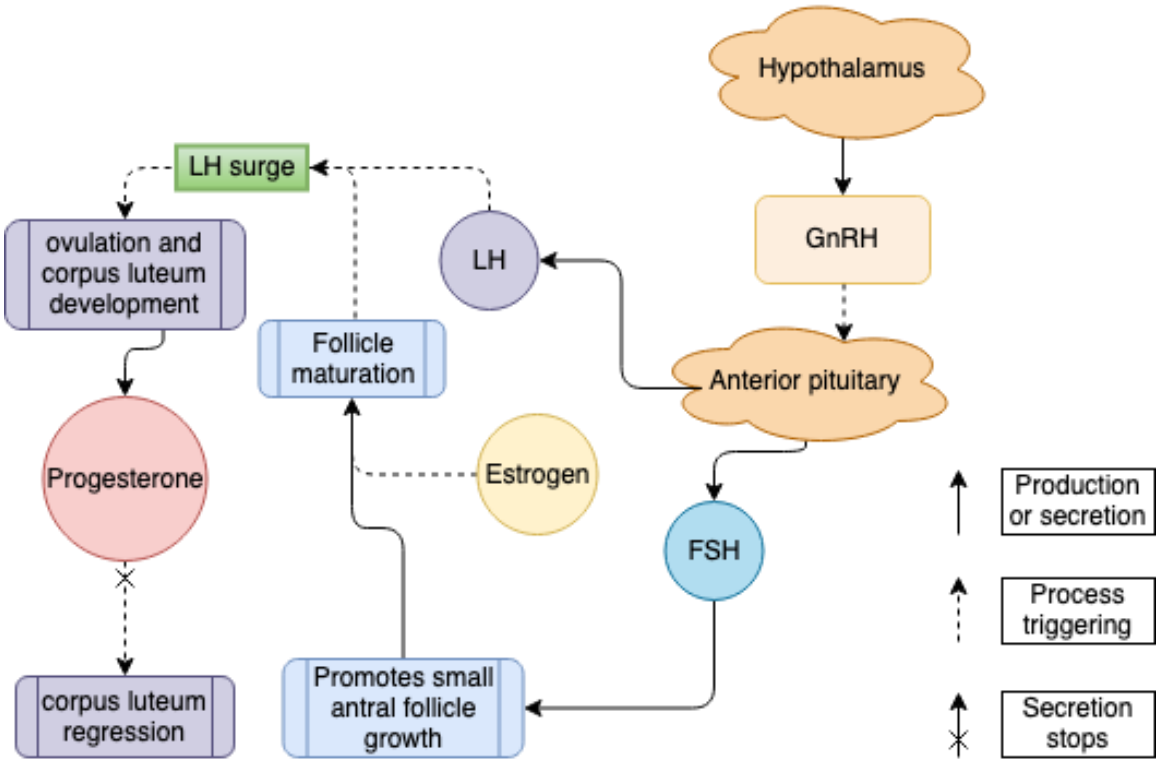


**Figure 1:** Hormonal regulation of folliculogenesis via the HPG axis. The hypothalamus releases GnRH which signals the anterior pituitary to produce LH and FSH. FSH promotes recruitment of early follicles and follicular development. Mature follicles secrete estradiol, which inhibits FSH and GnRH, thus causing a surge in LH. This surge triggers ovulation and the development of the corpus luteum. The corpus luteum produces high levels of progesterone, and will eventually regress into the ovary if oocyte is not fertilized.

## Hyperandrogenism in the Ovary

Hormonal signaling in the ovary is tightly regulated by the HPG axis to assure proper follicular development and ovarian health. The HPG axis is also self-regulating, as it cycles between releasing FSH an LH in response to estrogen and progesterone production as a result of ovulation. As testosterone is the precursor to

estrogen synthesis in the ovary, it has the potential to have highly detrimental effects on ovarian health if dysregulated.

The dysregulation in ovarian androgens begins with the overproduction of LH from the pituitary. High circulating levels of LH cause an upregulated synthesis of testosterone, and dihydroepiandrosterone, another common androgen [25]. These androgens are produced by theca cells in the ovary, and under normal conditions, most are converted to estrogen by the granulosa cells via the enzyme aromatase [13]. This excess of androgens causes the arrest of follicular development, and also impairs the function of aromatase [26]. The impaired aromatase function in turn prevents estrogen production. The arrest of follicular development causes an increase in anti-mullerian hormone, which inhibits follicular recruitment and the formation of primary follicles [26]. When follicles become arrested in development, they can no longer release their oocytes, and may continue to swell until becoming cystic.

While excess testosterone may cause follicular arrest, small amounts of testosterone is necessary for maintaining ovarian health. Testosterone has been shown to promote follicular growth in mice, and may act as a regulator of progesterone secretion in granulosa cells [27, 28]. While most androgens will be converted to estrogen and progesterone, androgens alone are necessary for healthy ovarian and follicular development. Testosterone, DHT, and DHEA bind to the androgen receptor, and androgen receptor knockout mice have been shown to have decreased fertility, irregular estrus cycles, and decreased litter sizes [29]. The

mechanisms for these changes are unknown, but it appears that androgens are crucial for ovarian function.

Causes for hyperandrogenism has been attributed to hyperinsulinemia, and overactive adrenal glands [30, 31], but the most prominent theory is that hyperandrogenism in females arises from the ovary [1]. The high levels of circulating androgens in combination with cystic ovaries are two key criteria in diagnosing the endocrine disorder known as polycystic ovary syndrome.

**Polycystic Ovary Syndrome**

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder among women of reproductive age, and is estimated to affect between 6 to 14% of women, depending on which diagnostic criteria is used [32]. The symptoms of PCOS can manifest themselves with a wide range of variation among affected individuals, so to avoid over diagnosis, the Rotterdam Criteria was established. These criteria focus on the most common symptoms of PCOS [4]. Under the Rotterdam Criteria, and individual can be diagnosed with PCOS by displaying 2 of 3 symptoms: presence of ovarian cysts, oligo/anovulation, and/ or hyperandrogenism (clinical or biochemical) [33]. Ovarian cysts are examined via ultrasound [34]. Oligovulation is defined as irregular menstrual cycles, or having fewer than 8 menstrual cycles per year in humans [35]. The most common clinical feature of hyperandrogenism is hirsutism, or the abnormal growth of hair on the face or body [36]. Clinical analysis of serum in blood samples is also performed to determine serum androgen levels.

Ovarian Phenotypes Associated With PCOS

An ovarian cyst is formed when a follicle fails to rupture and ovulate its oocyte, due to the interruption of folliculogenesis. The interruption of folliculogenesis may stem from several causes, but the most common cause is the release of excessive LH, which ultimately can lead to the formation of cysts [37]. The binding of LH to LHR on the theca cells, leads to the synthesis of testosterone, and the resulting surplus of testosterone from LH leads to high levels of estrogen and progesterone conversion in the granulosa cells [25]. The level of estrogen from the ovary causes the secretion of FSH, thus driving the growth of immature follicles [7]. The high levels of FSH also inhibits a process known as follicular atresia, where granulosa cells undergo apoptosis [38]. In healthy individuals, the majority of follicles do not rupture; they instead undergo atresia, and are broken down [39]. In individuals with PCOS, the follicles may not undergo atresia for an extended period of time (or at all) and will continue to grow, giving them a cyst-like appearance. The follicle may eventually undergo atresia, or the oocyte may undergo necrosis [40]. Due to the nature of folliculogenesis multiple immature follicles will be maturing at any given time, so as a cyst regresses, another cyst may take its place [14]. Therefore, it is believed that high levels of LH, testosterone, or a combination of the two are responsible for the formation of ovarian cysts commonly observed in PCOS.

Prevalence of Infertility, and Effects on Mental Health

In an unselected US population, up to 72% of individuals with PCOS reported infertility, in comparison to 16% in those without PCOS [41]. The mental health of

those with PCOS may be impacted as well. Those with PCOS report higher levels of depression and anxiety than unaffected individuals, regardless of age [42]. Symptoms of PCOS such as obesity and hirsutism may be contributing factors to the poor mental wellbeing of affected individuals, and can contribute to the worsening quality of life reported by these individuals [43].

### Metabolic Disorders Associated With PCOS

While ovarian phenotypes and hyperandrogenism provide a partial diagnostic criterion, there exists a myriad of metabolic disorders associated with PCOS, including obesity and type-II diabetes [44]. Women with PCOS are also at a higher risk for development of insulin resistance and non-alcoholic fatty liver disease [45]. It is estimated that PCOS-related obesity affects up to 42% of those with PCOS [46]. Despite being a prevalent feature of the disease, obesity is not universal among those with PCOS, and may not be present in some individuals. The insulin resistance in women with PCOS may be present regardless of if the affected individual is obese, and is estimated to be present in 50-90% of PCOS patients [47, 48].

## PCOS-Associated Metabolic Disorders

Insulin resistance is a common disorder associated with PCOS, and its presence may contribute to the severity of the disease [49]. In healthy individuals, insulin is secreted in response to an increase in blood glucose, and signals for cells in the body to uptake and store glucose, as well as initiating the conversion of

glucose to other metabolite such as DNA, lipids, and proteins [50]. Insulin functions by binding to the insulin receptor, which initiates a signal cascade causing glucose transporter type 4 (GLUT-4) transporters to translocate to the cell membrane, where glucose can then be taken into the cell [50]. When an individual suffers from insulin resistance, their cells have an impaired ability to intake glucose due to a decreased cellular response to insulin. To compensate, the pancreas begins secreting more insulin [51]. Increased insulin levels stimulate the production of Insulin-Like Growth Factor 1 (IGF1), which has been shown to increase the secretion of androgen from theca cells [52].

Other common metabolic symptoms of PCOS that may contribute to glucose intolerance include obesity, dyslipidemia, and hypertension [53]. As glucose intolerance becomes more severe, it can also contribute to the exacerbation of these symptoms [53]. An increase in adipose tissue due to glucose intolerance and weight gain can damage the beta islet cells of the pancreas, lowering insulin production. This decrease in insulin production can lead to the development of type-II diabetes [54]. Serum levels of triglycerides and cholesterol in those with PCOS also tend to be higher [53, 55]. All of these symptoms can be lumped together into another condition known as metabolic syndrome, which is not specific to PCOS, but is characterized by the metabolic symptoms seen in PCOS [55]. The metabolic aspects of PCOS are difficult to treat, as they are a result of the hormonal irregularities associated with the disease, and not due to diet. As diet and lifestyle alterations alone may not be enough to curb the symptoms in affected individuals, researchers are investigating alternative ways of treating these disorders in PCOS.

## Dihydrotestosterone-Induced PCOS

Several rodent models of PCOS currently exist, which mimic many of the symptoms of the PCOS phenotypes observed in humans. These models are generated either by pre- or post-natal introduction of exogenous androgens, or aromatase inhibitors to prevent the conversion of testosterone to estrogen and progesterone [3]. Dihydrotestosterone (DHT) is an androgen functionally similar to testosterone, and can bind to the androgen receptor and regulate androgen dependent gene transcription [56]. DHT differs from testosterone in that it cannot be converted to estrogen or progesterone, which makes it an excellent molecule to induce hyperandrogenism (a key feature of PCOS) in female mice without confounding effects due to estrogen conversion. Treating pre-pubescent mice with 7.5 mg of extended release DHT over 90 days has been shown to induce the major symptoms of PCOS including obesity, impaired glucose tolerance, irregular estrus cycles, and cystic ovaries [2]. DHT- treatment in mice has also been shown to induce adipocyte hypertrophy, dyslipidemia, and hepatic steatosis [3]. Given DHT's ability to reproduce many key human features of PCOS, it has been used to create a mouse model of PCOS that allows for the study of both the ovarian and metabolic components of the disease.

## Transgenic NAG-1 Have a Lean Phenotype

The Non-Steroidal Anti-Inflammatory Drug Activated Gene (NAG-1), also known as macrophage inhibitory cytokine 1(MIC-1) or growth differentiation factor

15 (GDF15), belongs to the transforming growth factor beta family of genes [57]. NAG-1 is present in low levels in many central organs, and can reduce inflammation responses when upregulated [58]. While the function of NAG-1 is unknown, it appears to play a role in body weight and regulation of thermogenesis in mice [59]. Transgenic mice overexpressing the human NAG-1 gene weigh significantly less and have lower levels of white adipose tissue compared to their wild-type littermates [60]. Transgenic NAG-1 mice are more metabolically active, have greater lean body mass, and consume less food than wild-type mice [60]. Transgenic NAG-1 mice also have improved glucose tolerance and insulin production in both males and females, regardless of diet, as well as increased thermogenesis-related gene expression in brown adipose tissue [59]. When a glucose tolerance test is performed after a 12-hour fasting period, NAG-1 mice have been shown to have a much lower spike in initial glucose uptake, and also return to a basal level more quickly than wild-type mice. When the same mice are injected with insulin, the NAG-1 mice also show a larger drop in blood glucose levels, suggesting that expression of NAG-1 improves glucose tolerance [61]. Mice expressing NAG-1 also have an increased amount of brown adipose tissue and a reduced amount of white adipose tissue [61].

Several thermogenesis genes are upregulated in brown adipose tissue in NAG-1 mice, including uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1a), cytochrome c oxidase subunit 8B (COX8B), and peroxisome proliferator-activated receptor alpha (PPARa), which when upregulated, lead to greater heat production from metabolic processes [59]. UCP1 is a proton uncoupler, and decreases the proton gradient in oxidative

phosphorylation [62]. This decrease in the proton gradient results in the mitochondria producing less ATP, and more heat [62]. PGC1a is a regulator of energy metabolism, and controls the growth of mitochondria [63]. COX8B codes for a subunit of the final electron acceptor in oxidative phosphorylation, and its upregulation is linked to higher metabolic activity [64]. PPARa is a transcription factor, and is responsible for regulating lipid metabolism, and fatty acid oxidation in the mitochondria [65]. The increase in heat production from the up regulation of these genes may play a role in the development of the lean phenotype observed in NAG-1 mice and the resistance to diet induced weight gain.

These alterations to metabolism, weight, and thermogenesis regulation occur regardless of whether or not mice are fed a high-fat diet [61]. To date, current studies on NAG-1 and its role in metabolism regulation only focus on diet-induced metabolic dysfunctions, while the metabolic dysfunctions seen in PCOS are a result of hormone dysregulation. This presents a novel opportunity to study the effects of hormonally induced metabolic disorders, specifically those due to PCOS, on transgenic NAG-1 mice.

**Conclusions**

PCOS is a dynamic disease. Based on the name alone, one would surmise that the disease is localized to the ovary, but its symptoms may affect a variety of organs. The primary way PCOS affects the body outside of the ovary and reproductive system is by causing or contributing to many detrimental metabolic disorders. As the metabolic disorders associated with PCOS are a result of hormonal imbalances, they become difficult to treat. The NAG-1 mice provide a unique model to explore

differences in diet versus hormonal induced metabolic disorder. When on a high-fat diet, mice expressing NAG-1 have been shown have a lean phenotype, and also have resistance to the same metabolic disorders seen in PCOS. However, the mechanism behind how NAG-1 can lead to this phenotype and resistance is unknown. As we induce these metabolic disorders through a mouse model of PCOS, we hypothesize that NAG-1 will continue to protect the mice from metabolic disorders, even when they are hormonally induced, due to its efficiency at protecting against the same disorders when diet induced.

REFERENCES

[1] Rosenfield R. The Polycystic Ovary Morphology-Polycystic Ovary Syndrome Spectrum. *J Pediatr Adolesc Gynecol*. 2015;28(6):412-419. doi:10.1016/j.jpag.2014.07.016

[2] van Houten E, Kramer P, McLuskey A, Karels B, Themmen A, Visser J. Reproductive and Metabolic Phenotype of a Mouse Model of PCOS. *Endocrinology*. 2012;153(6):2861-2869. doi:10.1210/en.2011-1754

[3] Caldwell A, Middleton L, Jimenez M et al. Characterization of Reproductive, Metabolic, and Endocrine Features of Polycystic Ovary Syndrome in Female Hyperandrogenic Mouse Models. *Endocrinology*. 2014;155(8):3146-3159. doi:10.1210/en.2014-1196

[4] Legro R, Arslanian S, Ehrmann D et al. Diagnosis and Treatment of Polycystic Ovary Syndrome: An Endocrine Society Clinical Practice Guideline. *The Journal of Clinical Endocrinology & Metabolism*. 2013;98(12):4565-4592. doi:10.1210/jc.2013-2350

[5] Baird D, Swanston I, Mcneilly A. Relationship Between LH, FSH, and Prolactin Concentration and the Secretion of Androgens and Estrogens by the Preovulatory Follicle in the Ewe. *Biol Reprod*. 1981;24(5):1013-1025. doi:10.1095/biolreprod24.5.1013

[6] Lüking Jayes F, Britt J, Esbenshade K. Role of Gonadotropin-Releasing Hormone Pulse Frequency in Differential Regulation of Gonadotropins in the Gilt. *Biol Reprod*. 1997;56(4):1012-1019. doi:10.1095/biolreprod56.4.1012

[7] Vihko K. Gonadotropins and ovarian gonadotropin receptors during the perimenopausal transition period. *Maturitas*. 1996;23:S19-S22. doi:10.1016/0378-5122(96)01006-7

[8] Young J, McNeilly A. Theca: the forgotten cell of the ovarian follicle. *REPRODUCTION*. 2010;140(4):489-504. doi:10.1530/rep-10-0094

[9] Wallace W, Kelsey T. Human ovarian reserve from conception to the menopause. *Nature Precedings*. 2009. doi:10.1038/npre.2009.3179.1

[10] Hillier S, Whitelaw P, Smyth C. Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. *Mol Cell Endocrinol*. 1994;100(1-2):51-54. doi:10.1016/0303-7207(94)90278-x

[11] Saldanha C, Remage-Healey L, Schlinger B. Synaptocrine Signaling: Steroid Synthesis and Action at the Synapse. *Endocr Rev*. 2011;32(4):532-549. doi:10.1210/er.2011-0004

[12] Taylor P, Hillier S, Fraser H. Effects of GnRH antagonist treatment on follicular development and angiogenesis in the primate ovary. *Journal of Endocrinology*. 2004;183(1):1-17. doi:10.1677/joe.1.05685

[13] Garzo V, Dorrington J. Aromatase activity in human granulosa cells during follicular development and the modulation by follicle-stimulating hormone and insulin. *Am J Obstet Gynecol*. 1984;148(5):657-662. doi:10.1016/0002-9378(84)90769-5

[14] Gougeon A. Dynamics of follicular growth in the human: a model from preliminary results. *Human Reproduction*. 1986;1(2):81-87. doi:10.1093/oxfordjournals.humrep.a136365

[15] Hoage T, Cameron I. Folliculogenesis in the ovary of the mature mouse: A radioautographic study. *Anat Rec*. 1976;184(4):699-709. doi:10.1002/ar.1091840409

[16] Baird D, Balen A, Escobar-Morreale H et al. Health and fertility in World Health Organization group 2 anovulatory women. *Hum Reprod Update*. 2012;18(5):586-599. doi:10.1093/humupd/dms019

[17] Downs J, Wise P. The role of the brain in female reproductive aging. *Mol Cell Endocrinol*. 2009;299(1):32-38. doi:10.1016/j.mce.2008.11.012

[18] Dickerson L, Schrader S, Diaz V. *Pharmacotherapy: A Pathophysiologic Approach*. McGraw-Hill Medical; 2008:1313-1328.

[19] Niswender G. Molecular control of luteal secretion of progesterone. *Reproduction*. 2002:333-339. doi:10.1530/rep.0.1230333

[20] Häggström M. Reference ranges for estradiol, progesterone, luteinizing hormone and follicle-stimulating hormone during the menstrual cycle. *WikiJournal of Medicine*. 2014;1(1). doi:10.15347/wjm/2014.001

[21] Hess R, Bunick D, Lee K et al. A role for oestrogens in the male reproductive system. *Nature*. 1997;390(6659):509-512. doi:10.1038/37352

[22] Emera D, Romero R, Wagner G. The evolution of menstruation: A new model for genetic assimilation. *BioEssays*. 2011;34(1):26-35. doi:10.1002/bies.201100099

[23] Hoehn K, Marieb E. *Human Anatomy & Physiology*. San Francisco: Pearson Benjamin Cummings; 2007:1090–1110.

[24] Patel B, Elguero S, Thakore S, Dahoud W, Bedaiwy M, Mesiano S. Role of nuclear progesterone receptor isoforms in uterine pathophysiology. *Hum Reprod Update*. 2014;21(2):155-173. doi:10.1093/humupd/dmu056

[25] Puppala S. Re: Polycystic ovary syndrome and the differential diagnosis of hyperandrogenism. *The Obstetrician & Gynaecologist*. 2014;16(1):69-69. doi:10.1111/tog.12069_11

[26] Goodarzi M, Dumesic D, Chazenbalk G, Azziz R. Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. *Nature Reviews Endocrinology*. 2011;7(4):219-231. doi:10.1038/nrendo.2010.217

[27] Murray A, Gosden R, Allison V, Spears N. Effect of androgens on the development of mouse follicles growing in vitro. *Reproduction*. 1998;113(1):27-33. doi:10.1530/jrf.0.1130027

[28] Daniel S, Armstrong D. Site of Action of Androgens on Follicle-Stimulating Hormone-Induced Aromatase Activity in Cultured Rat Granulosa Cells. *Endocrinology*. 1984;114(6):1975-1982. doi:10.1210/endo-114-6-1975

[29] Shiina H, Matsumoto T, Sato T et al. Premature ovarian failure in androgen receptor-deficient mice. *Proceedings of the National Academy of Sciences*. 2005;103(1):224-229. doi:10.1073/pnas.0506736102

[30] Rainey W, Carr B, Sasano H, Suzuki T, Mason J. Dissecting human adrenal androgen production. *Trends in Endocrinology & Metabolism*. 2002;13(6):234-239. doi:10.1016/s1043-2760(02)00609-4

[31] Barbieri R, Makris A, Randall R, Daniels G, Kistner R, Ryan K. Insulin Stimulates Androgen Accumulation in Incubations of Ovarian Stroma

Obtained from Women with Hyperandrogenism*. *The Journal of Clinical Endocrinology & Metabolism*. 1986;62(5):904-910. doi:10.1210/jcem-62-5-904

[32] March W, Moore V, Willson K, Phillips D, Norman R, Davies M. The prevalence of polycystic ovary syndrome in a community sample assessed under contrasting diagnostic criteria. *Human Reproduction*. 2009;25(2):544-551. doi:10.1093/humrep/dep399

[33] Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Human Reproduction*. 2004;19(1):41-47. doi:10.1093/humrep/deh098

[34] Balen A, Laven J, Tan S, Dewailly D. Ultrasound assessment of the polycystic ovary: international consensus definitions. *Hum Reprod Update*. 2003;9(6):505-514. doi:10.1093/humupd/dmg044

[35] Hull M. Epidemiology of infertility and polycystic ovarian disease: endocrinological and demographic studies. *Gynecological Endocrinology*. 1987;1(3):235-245. doi:10.3109/09513598709023610

[36] Legro R, Schlaff W, Diamond M et al. Total Testosterone Assays in Women with Polycystic Ovary Syndrome: Precision and Correlation with Hirsutism. *The Journal of Clinical Endocrinology & Metabolism*. 2010;95(12):5305-5313. doi:10.1210/jc.2010-1123

[37] Straus J. Some New Thoughts on the Pathophysiology and Genetics of Polycystic Ovary Syndrome. *Ann N Y Acad Sci*. 2003;997(1):42-48. doi:10.1196/annals.1290.005

[38] Kaipia A, Hsueh A. Regulation Of Ovarian Follicle Atresia. *Annu Rev Physiol*. 1997;59(1):349-363. doi:10.1146/annurev.physiol.59.1.349

[39] Faddy M. Follicle dynamics during ovarian ageing. *Mol Cell Endocrinol*. 2000;163(1-2):43-48. doi:10.1016/s0303-7207(99)00238-5

[40] Van Wezel I, Dharmarajan A, Lavranos T, Rodgers R. Evidence for Alternative Pathways of Granulosa Cell Death in Healthy and Slightly Atretic Bovine Antral Follicles. *Endocrinology*. 1999;140(6):2602-2612. doi:10.1210/endo.140.6.6758

[41] Joham A, Teede H, Ranasinha S, Zoungas S, Boyle J. Prevalence of Infertility and Use of Fertility Treatment in Women with Polycystic Ovary Syndrome: Data from a Large Community-Based Cohort Study. *J Womens Health*. 2015;24(4):299-307. doi:10.1089/jwh.2014.5000

[42] Cinar N, Kizilarslanoglu M, Harmanci A et al. Depression, anxiety and cardiometabolic risk in polycystic ovary syndrome. *Human Reproduction*. 2011;26(12):3339-3345. doi:10.1093/humrep/der338

[43] Eggers S, Kirchegast S. The Polycystic Ovary Syndrome—a medical condition but also an important psychosocial problem. *Collegium Antropologicum*. 2001;25:673–85.

[44] Knudsen N, Laurberg P, Rasmussen L et al. Small Differences in Thyroid Function May Be Important for Body Mass Index and the Occurrence of Obesity in the Population. *The Journal of Clinical Endocrinology & Metabolism*. 2005;90(7):4019-4024. doi:10.1210/jc.2004-2225

[45] Ramezani Binabaj M, Motalebi M, Karimi-Sari H, Rezaee Zavareh M, Alavian S. Are Women With Polycystic Ovarian Syndrome at a High Risk of Non-Alcoholic Fatty Liver Disease? A Meta-Analysis. *Hepat Mon*. 2014;14(11). doi:10.5812/hepatmon.23235

[46] Azziz R, Woods K, Reyna R, Key T, Knochenhauer E, Yildiz B. The Prevalence and Features of the Polycystic Ovary Syndrome in an Unselected Population. *The Journal of Clinical Endocrinology & Metabolism*. 2004;89(6):2745-2749. doi:10.1210/jc.2003-032046

[47] Venkatesan A. Insulin Resistance in Polycystic Ovary Syndrome: Progress and Paradoxes. *Recent Prog Horm Res*. 2001;56(1):295-308. doi:10.1210/rp.56.1.295

[48] Barber T, Franks S. Adipocyte biology in polycystic ovary syndrome. *Mol Cell Endocrinol*. 2013;373(1-2):68-76. doi:10.1016/j.mce.2012.10.010

[49] Dunaif A. Insulin Resistance and the Polycystic Ovary Syndrome: Mechanism and Implications for Pathogenesis. *Endocr Rev*. 1997;18(6):774-800. doi:10.1210/er.18.6.774

[50] Schinner S, Scherbaum W, Bornstein S, Barthel A. Molecular mechanisms of insulin resistance. *Diabetic Medicine*. 2005;22(6):674-682. doi:10.1111/j.1464-5491.2005.01566.x

[51] Shanik M, Xu Y, Skrha J, Dankner R, Zick Y, Roth J. Insulin Resistance and Hyperinsulinemia: Is hyperinsulinemia the cart or the horse?. *Diabetes Care*. 2008;31(Supplement 2):S262-S268. doi:10.2337/dc08-s264

[52] Kristiansen S, Endoh A, Casson P, Buster J, Hornsby P. Induction of steroidogenic enzyme genes by insulin and IGF-I in cultured adult human adrenocortical cells. *Steroids*. 1997;62(2):258-265. doi:10.1016/s0039-128x(96)00223-1

[53] De Leo V, la Marca A, Petraglia F. Insulin-Lowering Agents in the Management of Polycystic Ovary Syndrome. *Endocr Rev*. 2003;24(5):633-667. doi:10.1210/er.2002-0015

[54] Gardner D, Schoback D. *Greenspan's Basic & Clinical Endocrinology*. 9th ed. New York: McGraw-Hill; 2011:Chapter 17: Pancreatic hormones & diabetes mellitus.

[55] Carmina E, Lobo R. Polycystic Ovary Syndrome (PCOS): Arguably the Most Common Endocrinopathy Is Associated with Significant Morbidity in Women. *The Journal of Clinical Endocrinology & Metabolism*. 1999;84(6):1897-1899. doi:10.1210/jcem.84.6.5803

[56] Roy A, Lavrovsky Y, Song C et al. Regulation of Androgen Action. *Vitamins & Hormones*. 1998;55:309-352. doi:10.1016/s0083-6729(08)60938-3

[57] Ago T, Sadoshima J. GDF15, a Cardioprotective TGF-β Superfamily Protein. *Circ Res*. 2006;98(3):294-297. doi:10.1161/01.res.0000207919.83894.9d

[58] Kim J, Kosak J, Kim J et al. NAG-1/GDF15 Transgenic Mouse Has Less White Adipose Tissue and a Reduced Inflammatory Response. *Mediators Inflamm*. 2013;2013:1-10. doi:10.1155/2013/641851

[59] Chrysovergis K, Wang X, Kosak J et al. NAG-1/GDF-15 prevents obesity by increasing thermogenesis, lipolysis and oxidative metabolism. *Int J Obes*. 2014;38(12):1555-1564. doi:10.1038/ijo.2014.27

[60] Tsai V, Macia L, Johnen H et al. TGF-b Superfamily Cytokine MIC-1/GDF15 Is a Physiological Appetite and Body Weight Regulator. *PLoS ONE*. 2013;8(2):e55174. doi:10.1371/journal.pone.0055174

[61] Macia L, Tsai V, Nguyen A et al. Macrophage Inhibitory Cytokine 1 (MIC-1/GDF15) Decreases Food Intake, Body Weight and Improves Glucose Tolerance in Mice on Normal & Obesogenic Diets. *PLoS ONE*. 2012;7(4):e34868. doi:10.1371/journal.pone.0034868

[62] Crichton P, Lee Y, Kunji E. The molecular features of uncoupling protein 1 support a conventional mitochondrial carrier-like mechanism. *Biochimie*. 2017;134:35-50. doi:10.1016/j.biochi.2016.12.016

[63] Valero T. Editorial: Mitochondrial Biogenesis: Pharmacological Approaches. *Curr Pharm Des*. 2014;21(999):1-1. doi:10.2174/1381612819999140307101132

[64] Calhoun M, Thomas J, Gennis R. The cytochrome oxidase superfamily of redox-driven proton pumps. *Trends Biochem Sci*. 1994;19(8):325-330. doi:10.1016/0968-0004(94)90071-x

[65] Kersten S. Integrated physiology and systems biology of PPARα. *Mol Metab*. 2014;3(4):354-371. doi:10.1016/j.molmet.2014.02.002

# CHAPTER II

# METABOLIC AND OVARIAN PHENOTYPES OF DIHYDROTESTOSTERONE TREATMENT IN NAG-1 TRANSGENIC MICE

**JOURNAL-READY ARTICLE**

**FOR SUBMISSION TO:**

***ENDOCRINOLOGY***

# METABOLIC AND OVARIAN PHENOTYPES OF DHT INDUCED PCOS IN NAG-1 TRANSGENIC MICE

Nicholas Werner[1], Delaney Clem[1], and April K. Binder[1, 2]

[1]Department of Biological Sciences, Central Washington University, Ellensburg, WA

[2]Center for Reproductive Biology, Washington State University, Pullman, WA

**Abstract**

Polycystic ovary syndrome (PCOS), a leading cause of infertility in women, is a dynamic disease characterized by cystic ovaries, hyperandrogenism, altered menstrual cycles and metabolic dysfunction. As the metabolic disorders present in PCOS are caused as a result of hormonal dysfunction, they can be difficult to treat. Understanding the underlying cellular mechanisms and genes involved in metabolic disorders may lead to novel therapeutic approaches. The human Non-Steroidal Anti-Inflammatory Drug Activated Gene (NAG-1) also known as GDF15 has been shown to prevent diet-induced metabolic disorders when overexpressed in transgenic mice. NAG-1 transgenic mice have a lean phenotype, and resistance to development of metabolic disorders when on a high-fat diet. We hypothesized that NAG-1 mice would maintain this lean phenotype in response DHT-induced PCOS. Our findings indicate that transgenic NAG-1 mice treated with DHT have similar ovarian responses to wild-type mice but enter puberty earlier than placebo-treated littermates. NAG-1 DHT-treated mice also display similar ovarian phenotypes to wild-type DHT-treated mice with disrupted estrus cycles and reduced numbers of corpus lutea in the ovary. These results suggest NAG-1 mice have a similar ovarian response to DHT as wild-type mice. We next examined changes in metabolism in DHT-treated NAG-1 mice. NAG-1 mice displayed no significant weight gain between treatment groups and no significant increase in triglyceride levels. NAG-1 mice showed no change in white adipocyte morphology or differentiation from brown adipocytes into white adipocytes after DHT-treatment in contrast to wild-type mice treated with DHT. Our findings indicate that NAG-1 mice have similar ovarian

responses to DHT-treatment as wild-type mice, but continue to maintain their lean phenotype over wild-type mice in the presence of DHT-induced hyperandrogenism. Our results suggest that the overexpression of NAG-1 may have therapeutic potential in preventing some hormonal induced metabolic disorders.

**Introduction**

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder among women worldwide affecting 8-14% of the population, and is characterized by symptoms such as ovarian cysts, hyperandrogenism, and development of metabolic disorders [1, 2]. The presence of two out of three of the aforementioned symptoms are required under the Rotterdam diagnostic criteria for the diagnosis [3]. While not all individuals with PCOS may exhibit metabolic disorders, roughly 90% of the affected population has some level of metabolic disruption [4, 5]. The metabolic symptoms can include obesity, type-II diabetes, and fatty liver disease [6]. These metabolic symptoms are believed to be a result of hyperandrogenism associated with PCOS, and therefore can be challenging to treat.

Evidence suggests these metabolic symptoms are due to altered levels of androgen hormones [7]. Ovarian theca cells produce androgens that are converted to estrogen by aromatase (*Cyp19a1*) expressed in ovarian granulosa cells [8]. In women with PCOS, there appears to be an excess of androgen production, coupled with a lack of conversion of androgens to estrogen due to reduced expression of aromatase [9]. This condition known as hyperandrogenism can have widespread effects on the body including development of both reproductive and metabolic phenotypes. The reproductive phenotypes may include ovarian cysts, irregular

estrus or menstrual cycles, and infertility [10]. The metabolic aspects of the disease include obesity, glucose intolerance, type-II diabetes, and fatty liver disease [3]. These phenotypes caused by hyperandrogenism also serve as key diagnostic criteria for PCOS.

There are several mouse models for studying metabolic disorders, which include inducing metabolic disorders via being fed on a high-fat diet, or the use of transgenic or knockout mice that display altered metabolic responses. Transgenic mice that overexpress the human Non-Steroidal Anti-Inflammatory Drug Activated Gene (NAG-1) have been shown to be resistant to development of diet-induced metabolic disorders [11, 12, 13]. These transgenic mice when fed a high-fat diet do not become obese or suffer any insulin resistance, are more metabolically active than wild-type littermates, and have reduced levels of white adipose tissue [11]. It is hypothesized that the transgenic mice's lean phenotype is due to a significant up-regulation of genes involved in thermogenesis [11]. Implanting mice with a subcutaneous Dihydrotestosterone (DHT) pellet results in the mice displaying ovarian, hormonal, and metabolic phenotypes similar to those seen in humans with PCOS [14]. In this study we examined the metabolic response of NAG-1 mice in a PCOS model induced via DHT implantation.

Transgenic NAG-1 and wild-type mice were treated with placebo or DHT pellets for 90 days. NAG-1 mice were found to be resistant to weight gain and both white and brown adipocyte hypertrophy. In contrast, NAG-1 mice exhibited similar ovarian phenotypes to wild-type mice when exposed to DHT. This study suggests that NAG-1 mice are resistant to hormonal-induced weight gain, brown adipocyte

hypertrophy, and have some resistance to white adipocyte hypertrophy. NAG-1 overexpression also appears to have no impact on the ovarian phenotype of DHT-induced PCOS.

## Materials and Methods

### DHT-Treated Mice

Male transgenic C57BL/6 mice expressing the human NAG-1 gene were obtained from Dr. Tom Eling at the National Institute of Environmental Health Sciences and bred at Central Washington University. The transgenic line was maintained by breeding male NAG-1 mice to wild-type C57BL/6 females. Mice were fed Mazuri Rat and Mouse Diet *ad libitum* and kept on a 12-hour light/dark cycle. At weaning mice were genotyped as previously described [15], and 31 female mice divided into groups based on genotype (16 wild-type and 15 NAG-1). Mice were then randomly placed into groups to receive either placebo (wild-type n = 8, NAG-1 n = 8) or DHT (wild-type n = 8, NAG-1 n = 7) implants. Between 23-25 days of age, mice received a subcutaneous beeswax-based pellet containing either 7.5 mg of sesame oil or 7.5 mg of DHT (5a-Androstan-17B-ol-3-one, A8380, Sigma-Aldrich Corp., St. Louis, MO). Pellets were made using a modified protocol published by Leonie, E., et al. [14]. Modifications made to the protocol include the use of beeswax (243221, Sigma-Aldrich Corp., St. Louis, MO) as a medium for the pellets, and the use of 7.5 mg of DHT in place of 2.5 mg. At 90 days post-implantation, the distance between the anus and genitalia were recorded using a digital caliper. The protocols for all procedures performed on mice was approved by the Central Washington

University Institutional Animal Care and Use Committee (Protocol #A101801 and #A101602).

Body Weights and Glucose Tolerance

Animals were monitored daily and body weights were measured weekly for the duration of the study.

At 111 days of age (90 days post-implantation) mice had their fasting blood glucose measured. Mice were fasted 12 hours, and glucose levels were measured using a glucometer and glucose test strips (ATC Medical, Germantown, TN). Afterwards, mice were injected intraperitoneally with 2000 mg/kg of dextrose (Sigma-Aldrich Corp., St. Louis, MO). Blood glucose measurements were taken from tail snips at 20, 40, 60, 120, and 180 minutes post injection.

Tissue Collection

At the end of the study, mice were euthanized via $CO_2$ inhalation followed by cervical dislocation as a secondary method. Immediately post-euthanasia, the final weight of the mice was determined and cardiac puncture was performed to collect serum samples. Ovaries, white adipose tissue, and brown adipose tissue were collected and part of the sample was prepared for histological examination using standard procedures while the remainder was stored at -80° C for gene expression analysis.

Measuring Reproductive Development

Vaginal openings were monitored in each treatment group as an assessment of the onset of puberty. Mice from each group were visually inspected once daily and the date of vaginal opening was recorded. Ten weeks post-implantation we performed estrus cycle monitoring for 14 consecutive days. Each morning, saline solution was used to wash the vagina of each mouse individually. The washes from each mouse were fixed on glass slides and stained using hematoxylin and eosin (Sigma-Aldrich Corp., St. Louis, MO), dehydrated and sealed with a glass coverslip. Estrus cycles were assessed from the slides based on inspection via light microscopy of cell count and presence of nucleated cells [16]. Slides were analyzed separately by two individuals and categorized as being predominantly proestrus, estrus, metestrus, or diestrus. Proestrus slides were defined as having a mixture of nucleated and anucleated cells, estrus slides as having no nucleated cells, metestrus slides as having a mixture of nucleated and anucleated cells, as well as leukocytes, and diestrus slides as having extremely few cells of any kind in comparison to the other types.

Histology

Tissues were fixed in 10% formalin immediately after collection, and stored until further use at -4° C. Tissues were then dehydrated through a series of ethanol washes, and cleared with Citrisolv (Sigma-Aldrich Corp., St. Louis, MO) and embedded in blocks of paraffin wax. Tissues were sectioned in 8 uM slices, stained following standard H&E protocols and coverslipped. Images were taken using an

EVOS XL Microscope. One section of each type of adipose tissue was selected and used for analysis for each mouse. The area of white adipocytes was measured using the Watershed Algorithm package on ImageJ. Using the smallest measured white adipocyte as reference (90 $um^2$), the brown adipose tissue was analyzed using the Watershed Algorithm for any adipocytes that passed this threshold, which were classified as enlarged adipocytes. To examine ovarian follicles, ovaries were sectioned and every $10^{th}$ section (totaling 5 sections per mouse) was used for analysis. Only follicles with oocytes present were counted and categorized into five stages of development: small preantral (1-2 layers of granulosa cells), large preantral (3-5 layers of granulosa cells), small antral (5+ layers of granulosa cells), large antral (formation of an antrum), and corpora lutea (large mass of luteinized cells).

Measuring lipid levels

ELISA reagents were prepared in accordance to the protocols provided in the Aviva Systems triglyceride (OKEH02597) and cholesterol (OKEH02598) kits (Aviva Systems Biology Corporation, San Diego, CA). Triglyceride and Cholesterol ELISAs were performed using 5 µl of serum diluted in 45 µl of sample diluent. Serum and standards were ran in duplicate in both triglyceride and cholesterol kits, and was absorbance readings were taken at 450 nm. A standard curve was generated from standards ran in each kit, and triglyceride and cholesterol concentrations were quantified by comparing readings against the standard curve as described by the manufacturer.

Statistical Analysis

Comparisons of treatment groups were made using Graph Pad Prism Version 8 (GraphPad Software, Inc.). Vaginal openings and anal genital distances were analyzed via a one-way ANOVA, with a significance set at a p value of $p<0.05$. Body weights, estrus cycles, and glucose tolerance tests were analyzed using a two-way ANOVA, with a significance set at a p value of $p<0.05$. Adipocyte sizes were analyzed between groups using a one-way ANOVA with a significance set at a p value of $p<0.05$. ELISA data and follicle counts were analyzed between groups using a Student's t-test, and with a significance set at a p value of $p<0.05$.

## Results

DHT-treatment mimics the physiological effects of PCOS

A key feature of PCOS is hyperandrogenism, and a mouse model of PCOS can be created through pre-pubescent introduction of androgens [14]. In order to induce PCOS, mice were implanted with dihydrotestosterone (DHT) pellets at 23-25 days of age, and monitored for 90-days. Two non-invasive methods of analyzing whether or not the DHT is effecting the physiology of the mice is by monitoring vaginal opening after implantation to determine the age of puberty, and measuring anal-genital distance (AGD) after 90 days. Exposure to hormones, such as DHT, can cause female mice to enter puberty at an earlier age than placebo groups [17, 18]. We found that treatment of DHT, regardless of genotype, caused mice to enter puberty significantly earlier than the placebo group. DHT-treated mice entered

puberty early at 26 days of age on average, and placebo mice entered puberty at 36 days (Fig.1, $p < 0.05$). AGD was also observed to be significantly longer in the DHT group having an average AGD of 6.7 mm compared to the placebo group, which has an average AGD of 4.6 mm (Fig.1, $p < 0.05$).

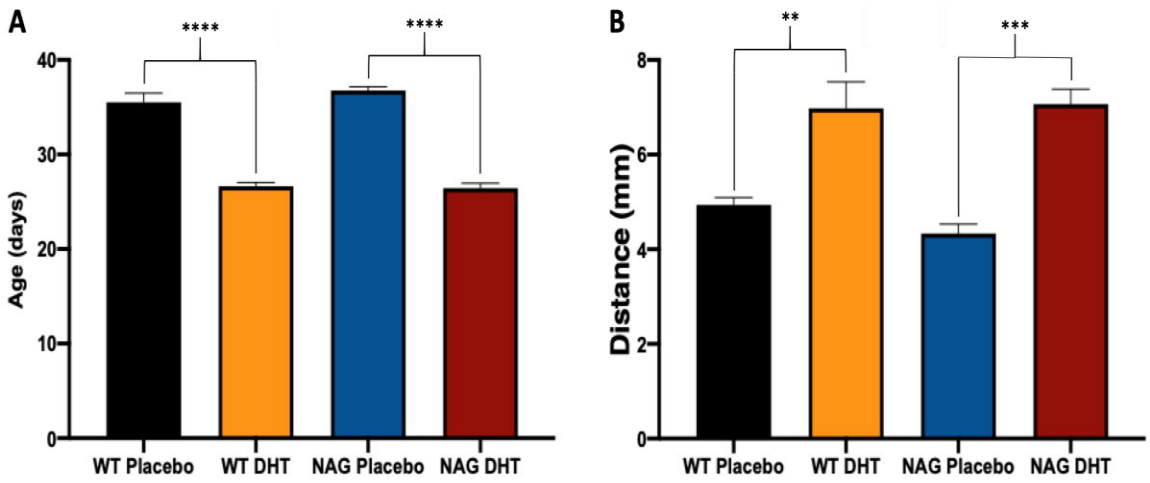


**Figure 1: Effects of DHT on Secondary Sex Characteristics.** A) The age of puberty was determined by observing the age when vaginal opening occurred. B) Anal genital distance was measured at 90 days post-implantation using a digital caliper. Data shown is average ± SEM (n = 8 for both placebo groups, n = 7 for both DHT groups). Data was analyzed using Tukey's multiple comparison tests between DHT and Placebo groups (*$p < 0.05$).

On average, mice have a four-day estrus cycle, and completing one cycle typically correlates with oocyte release [19]. Therefore, we chose to monitor estrus cycles as an indirect measure of fertility. Estrus cycles were monitored for a total of 14 consecutive days at 6 weeks post-implantation via daily vaginal smears to determine which stage of the estrus cycle they were in (Fig. 2). Mice in the placebo groups on average completed more estrus cycles in a 14-day period than mice in the DHT groups, regardless of genotype (Fig. 3, $p < 0.05$).

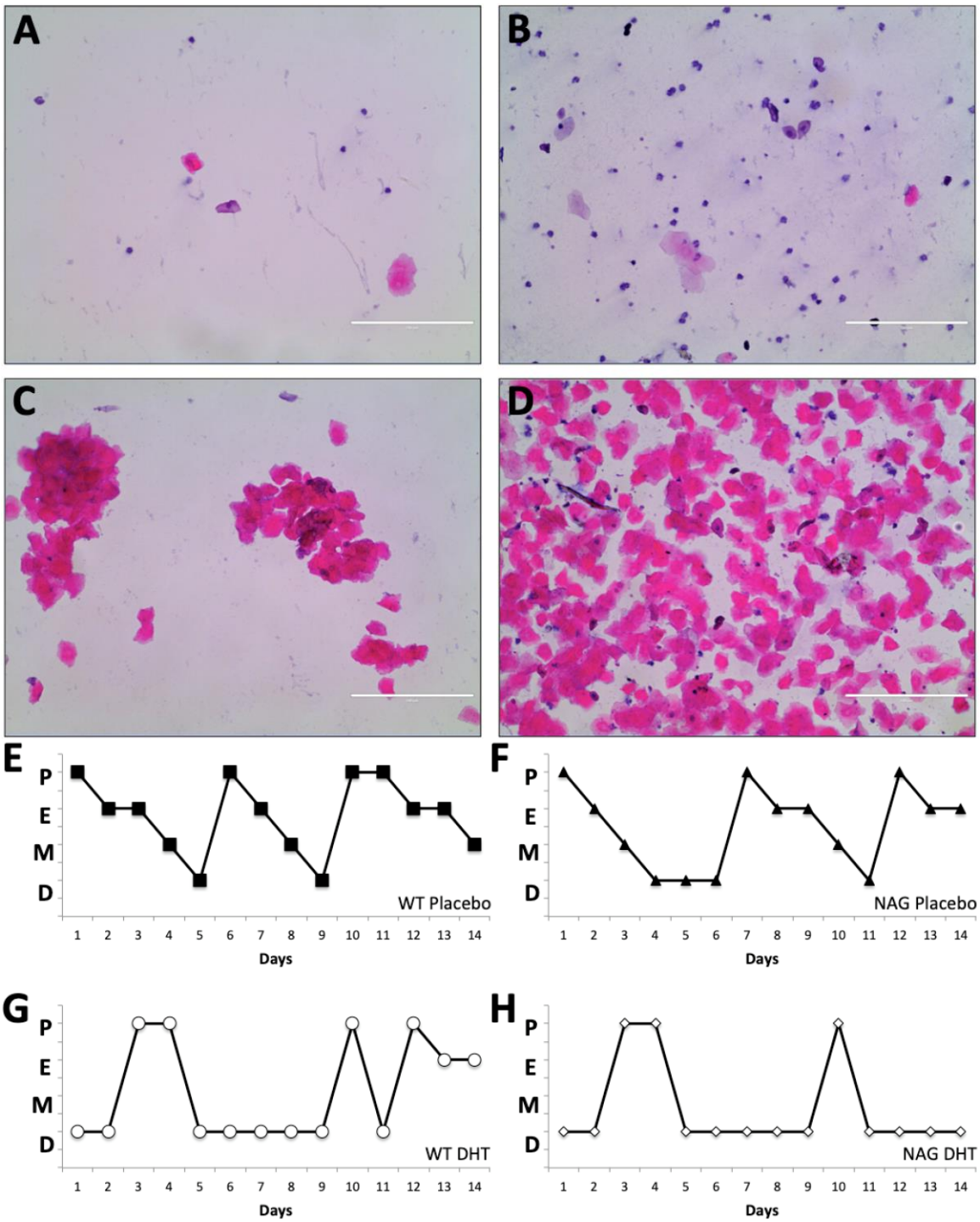


**Figure 2: DHT-Treated Mice Have Disrupted Estrus Cycles.** A-D) Examples of vaginal washes from in diestrus (A), proestrus (B), estrus (C), and metestrus (D) phases of the estrus cycle. E-H) Estrous cycle pattern in representative mice from wild-type placebo (E), NAG-1 placebo (F), wild-type DHT (G), and NAG-1 DHT (H) groups. (P, Proestrus; E, Estrus; M, Metestrus; D, Diestrus). Images were taken at 200x, and scale bars represent 200 μm.

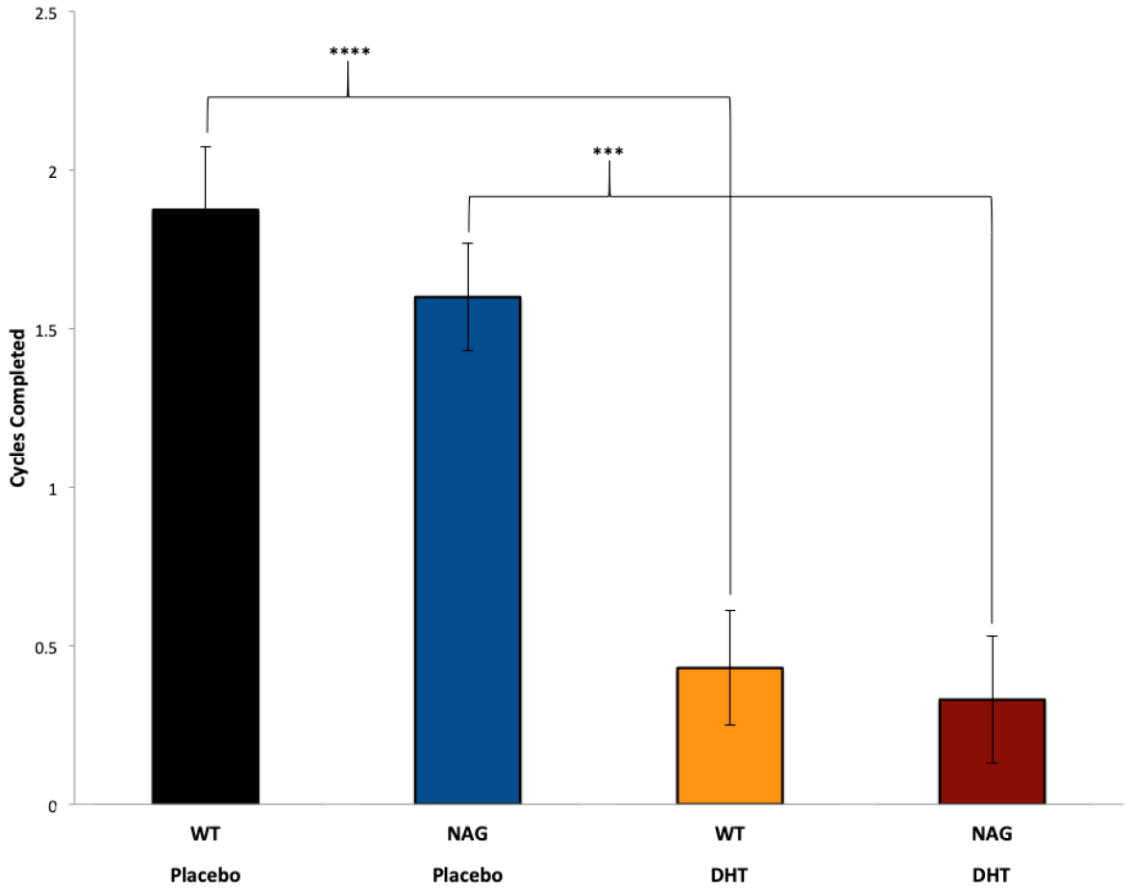


**Figure 3: Effects of DHT on Estrus Cycles.** Number of cycles completed over 14 days. Percentage of mice to complete one estrus cycle is reported as average (n = 8 for both placebo groups, n = 7 for both DHT groups). Tukey's multiple comparison tests were conducted for the comparison of estrus cycles between all groups (*$p < 0.05$).

After an oocyte is ovulated, a structure called a corpus luteum forms from the ruptured follicle. The observation of a corpus lutea in the ovary signifies that the mouse has ovulated and can be an indirect measure of normal ovarian health [20]. DHT-treatment in C57BL/6 mice has been shown to have negative impacts on folliculogenesis, including a lack of small preantral follicle maturation, and the formation of ovarian cysts [14]. Ovaries were collected from mice at 90 days post-implantation and the numbers of follicles at different stages of folliculogenesis were counted. Only follicles with an oocyte present were counted, and the stage of

folliculogenesis was determined based on the thickness of the cell layer surrounding the oocyte. On average, mice in the placebo groups have significantly larger number of small preantral follicles and corpus lutea than mice in the DHT group, regardless of genotype (Fig. 4 E & F, $p < 0.0001$). NAG-1 DHT-treated mice were also observed to have a significantly larger number of small antral and large antral follicles than the NAG-1 placebo group (Fig. 4 F, $p < 0.0001$).

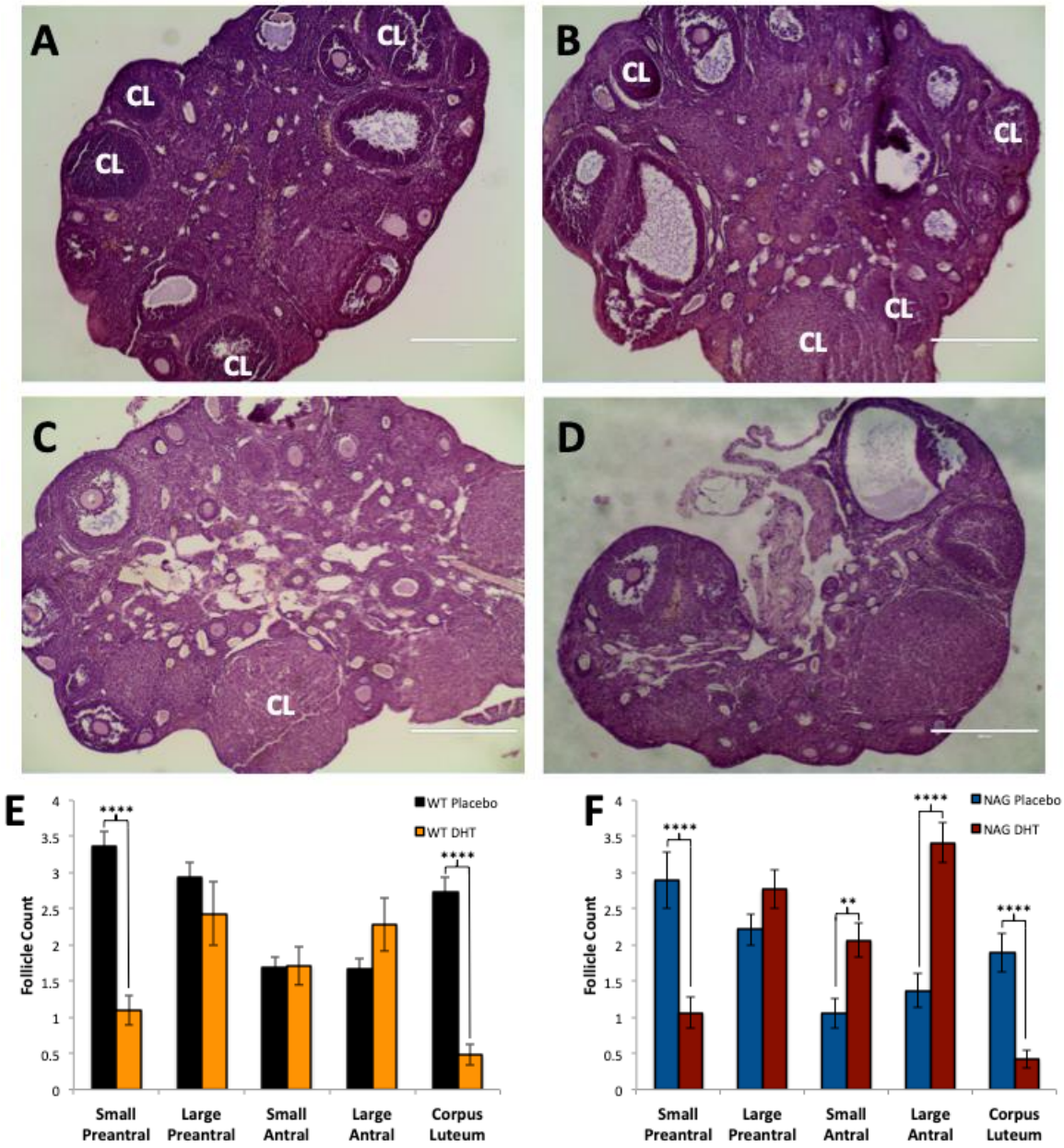


**Figure 4: Effects of DHT on Ovarian Follicles.** A representative ovarian section at from mice belonging to the wild-type placebo (A), NAG placebo (B), wild-type DHT (C), and NAG DHT (D) groups. "CL" indicates presence of corpus lutea. E) Follicle counts of wild-type placebo (n = 7) and DHT (n = 6) groups. F) Follicle counts of NAG placebo (n = 5) and NAG DHT (n = 5) groups. Data shown is average ± SEM. Two tailed t-tests were conducted to compare follicle counts between groups (*$p < 0.05$). Images were taken at 200x, and scale bars represent 200 µm.

NAG-1 Mice Are Resistant to DHT-Induced Fat Storage

C57BL/6 mice exposed to DHT-treatment have been shown to display increased weight gain over the course of the 90-day treatment [21, 22]. In order to examine whether NAG mice exhibited significant weight gain after DHT exposure, we recorded body weights weekly for 90 days. NAG mice weighed significantly less than wild-type mice, regardless of being treated with placebo or DHT pellets (Fig. 5 A & B, $p < 0.05$). In weeks two through nine, wild-type placebo mice weighed less than the wild-type DHT group (Fig. 5 C, $p < 0.05$). There was no significant difference among the weights of the NAG placebo and DHT-treated mice aside after the first week post-implantation (Fig. 5 D, $p < 0.05$). Regardless of treatment, wild-type mice weighed more than the NAG mice, and the wild-type-DHT mice weighed more than the wild-type placebo group. The findings that wild-type mice weigh more than NAG-1 mice and wild-type mice treated with DHT weight more than the wild-type placebo group are consistent with previously published studies [11, 23].

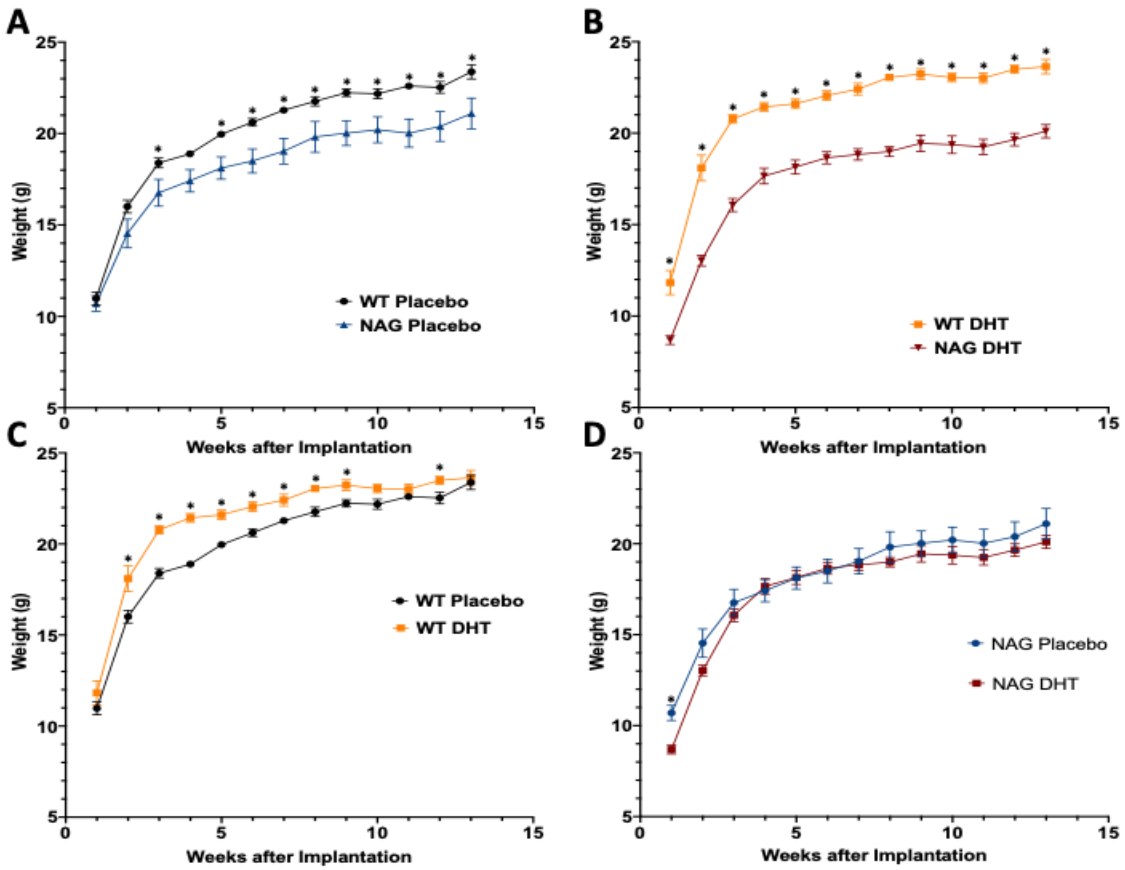


**Figure 5: Effects of DHT and Genotype on Body Weight.** Weights were recorded each week over 90 days. Figures A and B show comparisons between genotype, and figures C and D show comparisons between treatments. Data shown is average ± SEM (n = 8 for both placebo groups, n = 7 for both DHT groups). Tukey's multiple comparison tests were conducted for the comparison of weight between all groups (*$p < 0.05$).

White adipose tissue stores fat in the form of lipids. Each white adipocyte is comprised of one large lipid droplet accounting for most of the cell's area. When mice gain weight, they are not necessarily gaining more white adipocytes, but rather the lipid droplets stored within the adipocytes grow larger [24]. We compared the sizes of white adipocytes between genotypes and treatment groups to analyze potential effects DHT on fat storage. Adipocytes were classified using the Adipocytes Tools plugin on ImageJ into four different sizes. Small adipocytes were between 0 and 500 $um^2$, medium adipocytes were between 500 and 1000 $um^2$, large

adipocytes were between 1000 and 1500 $um^2$, and extra large were greater than 1500 $um^2$. Representative sections from each treatment groups are shown in Figure 6. We observed NAG-1 mice trending towards having a greater number of small adipocytes, regardless of treatment, and we also observed the NAG-1 placebo mice having significantly fewer large and extra-large adipocytes than the wild-type placebo group (Fig. 7) ($p < 0.05$). Our results indicate that DHT does not have a significant impact on adipocyte hypertrophy, regardless of genotype. However, white adipocyte hypertrophy is significant between wild-type and NAG-1 placebo groups when white adipocytes are larger than 1000 $um^2$.

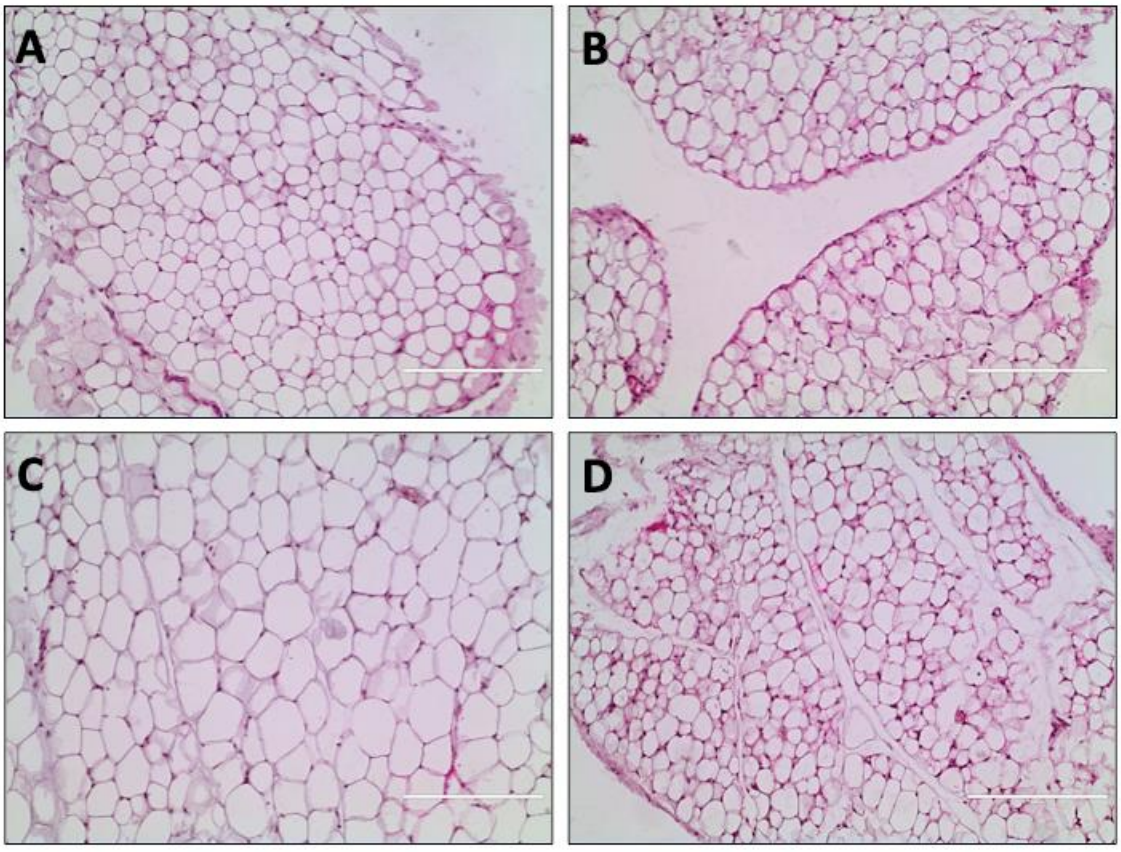


**Figure 6: White Adipocyte Histology After DHT-Treatment.** Representative H&E stained images of white adipocytes taken from mice belonging to the wild-type placebo (A), NAG Placebo (B), wild-type DHT (C), and NAG DHT (D) groups. Data is representative of n = 8 for both placebo groups, and n = 7 for both DHT groups. Images were taken at 200x magnification, and scale bars represent 200 μm.

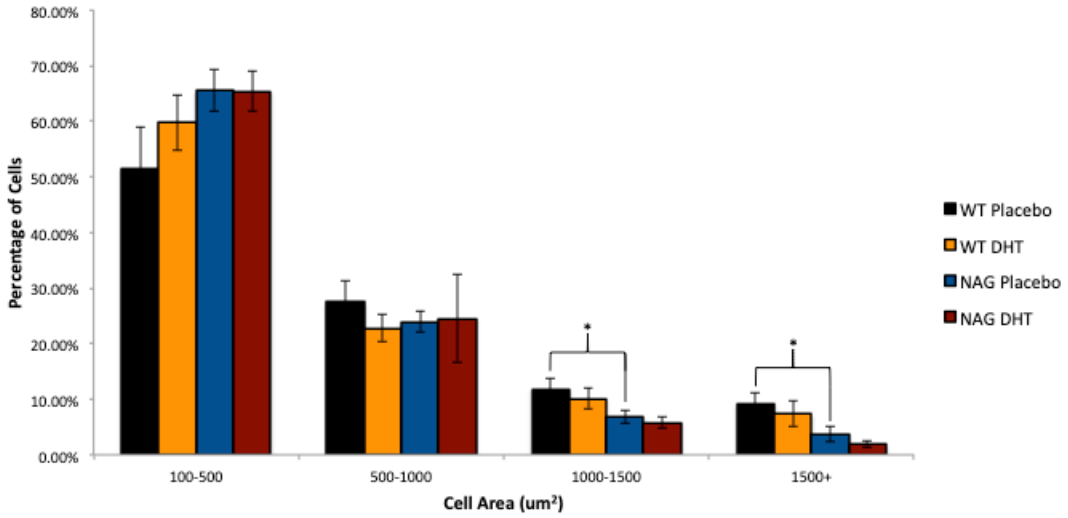


**Figure 7: Percentages of Cell Sizes of White Adipocytes.** Cell sizes from white adipose tissue collected from mice 90 days post-implantation. Data is shown as average ± SEM (n = 8 for both placebo groups, n = 7 for both DHT groups). Tukey's multiple comparison tests were conducted for the comparison of cell sizes between all groups (*$p < 0.05$).

Hepatic steatosis is an extreme phenotype that can be present in individuals with PCOS. Hepatic steatosis occurs when fat deposits accumulate in the liver, which can ultimately impact the ability of the liver to function normally [9, 25]. Hepatic steatosis may also develop into cirrhosis or liver cancer [25]. Liver samples were analyzed across treatment groups for the presence of adipocytes, and no visible adipocytes or differences in liver morphology were observed across treatment or genotype (Fig. 8). To confirm a lack of steatosis, a representative slide from each liver sample was run on ImageJ using Watershed Algorithm package, and no white adipocytes were detected.

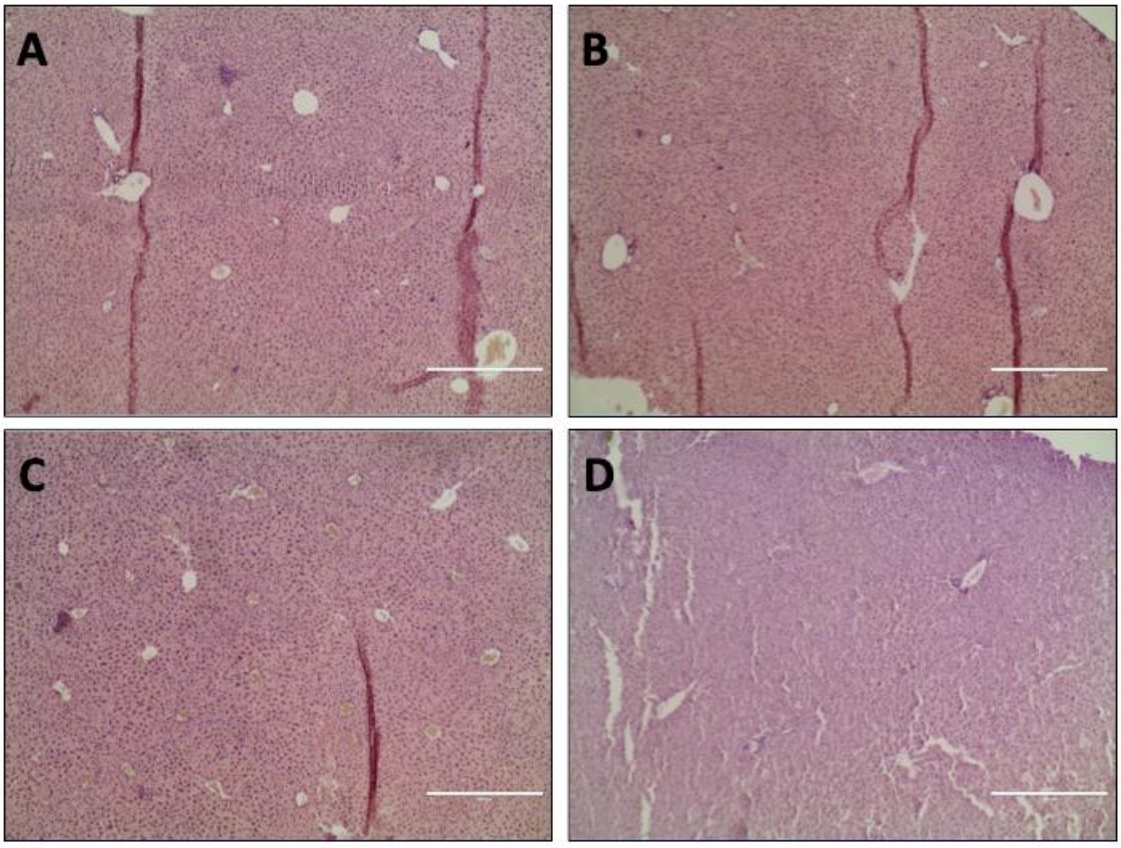


**Figure 8: Liver Histology After DHT-Treatment.** Representative images of liver taken from mice belonging to the wild-type placebo (A), NAG Placebo (B), wild-type DHT (C), and NAG DHT (D) groups. Images were taken at 200x magnification, and scale bars represent 200 μm.

Differences in Glucose Tolerance due to DHT-Treatment are Insignificant

Glucose intolerance is common in PCOS patients and is one method to diagnose type-II diabetes [26]. To test for the presence of glucose intolerance, we performed an intraperitoneal glucose tolerance test 13 weeks post-implantation. No statistical significance was observed in the wild-type mice between placebo and DHT-treated groups, but the DHT-treated group did have higher fasting glucose than the placebo group, and did not return to a basal level in 180 minutes (Fig. 9 A). There was no statistical significance between the NAG-1 mice placebo and DHT-treated groups. Both groups had the same fasting glucose levels and returned to

similar basal levels by 180 minutes. Although not statistically significant, the NAG-1 DHT group had a notably smaller spike in glucose at the 20- and 40-minute mark post-injection (Fig. 9 B) compared to the placebo treated NAG-1 mice.

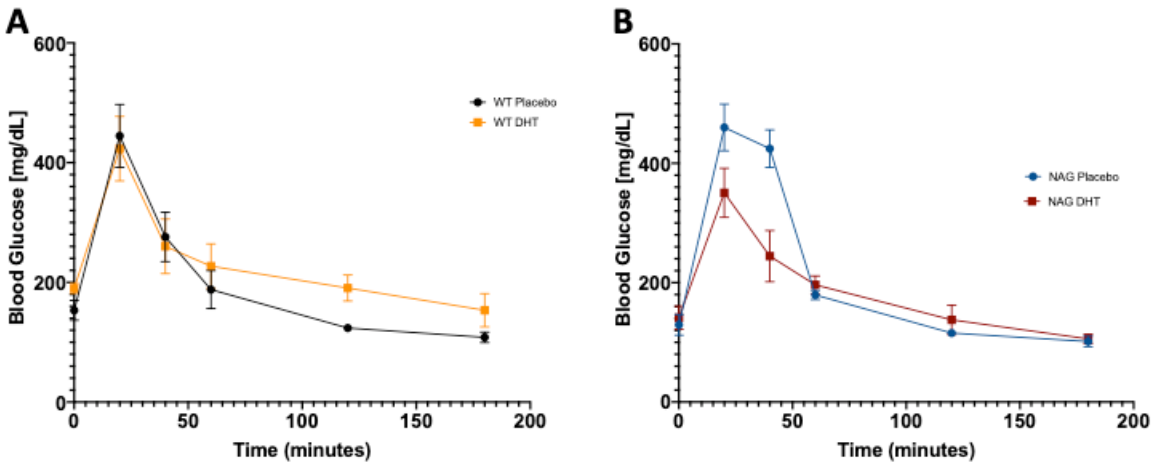


**Figure 9: Glucose Tolerance Tests.** Mice were fasted overnight and IPGTT was performed 90 days post-implantation. Data shown is average ± SEM at each time point (n = 5 for wild-type and NAG placebo groups, n = 5 for wild-type DHT group, and n = 6 for NAG DHT group). Data was analyzed using multiple t-tests and no significance was found.

Brown Adipose Morphology

Brown adipose tissue is highly metabolically active and thermogenic [11]. Its abundance decreases as mice age, but is still present in small amounts in adults [27, 28]. Brown adipocytes are much smaller and compact than white adipocytes, and are comprised of several small lipid droplets rather than one large droplet. The brown coloration of the adipocytes is due to the abundance of mitochondria [29]. When brown adipose tissue was collected from mice at 90 days post-implantation, we noticed no atypical morphological features in wild-type placebo, NAG-1 placebo, and NAG-1 DHT groups (Fig. 10 A, B, & D). In the wild-type DHT group, we observed a large amount of brown adipocytes that appeared to be storing more lipids, based

on the increase in size of lipid droplets over any of the other groups (Fig. 10 C). These brown adipocytes also appear to have begun to take on the appearance of white adipocytes in certain regions. This brown adipocyte hypertrophy occurred in the wild-type DHT-treatment group but was not observed in the transgenic NAG-1 DHT-treatment group. To further assess whether the brown adipocytes seen in the wild-type DHT group were significantly larger than brown adipocytes in the wild-type control group, we analyzed all cells to see if they measured at or above our smallest white adipocyte (< 90 $um^2$) sampled in Figure 6. This area was deemed appropriate as a threshold, as the area of brown adipocytes rarely exceeds 25 $um^2$ [30]. We observed that the wild-type DHT group had a significantly greater number of enlarged brown adipocytes compared to the other groups, which showed no difference between one another (Fig. 11) ($p < 0.01$).

DHT Has No Effect on NAG Mice Triglyceride Levels

High triglyceride and cholesterol levels serum levels are not direct symptoms of PCOS, but can arise as a consequence of metabolic symptoms associated with PCOS such as glucose intolerance and type-II diabetes [31]. Cardiovascular disease is estimated to be twice as common in women with PCOS than in the general population, and high levels of triglycerides and cholesterol can be precursors to cardiovascular disease [32]. We examined serum levels of triglycerides and cholesterol to see if the DHT implantation was having an effect on circulating levels of either. Serum was collected from mice 90 days post-implantation, and ELISAs were performed in duplicate for both triglyceride and cholesterol levels.

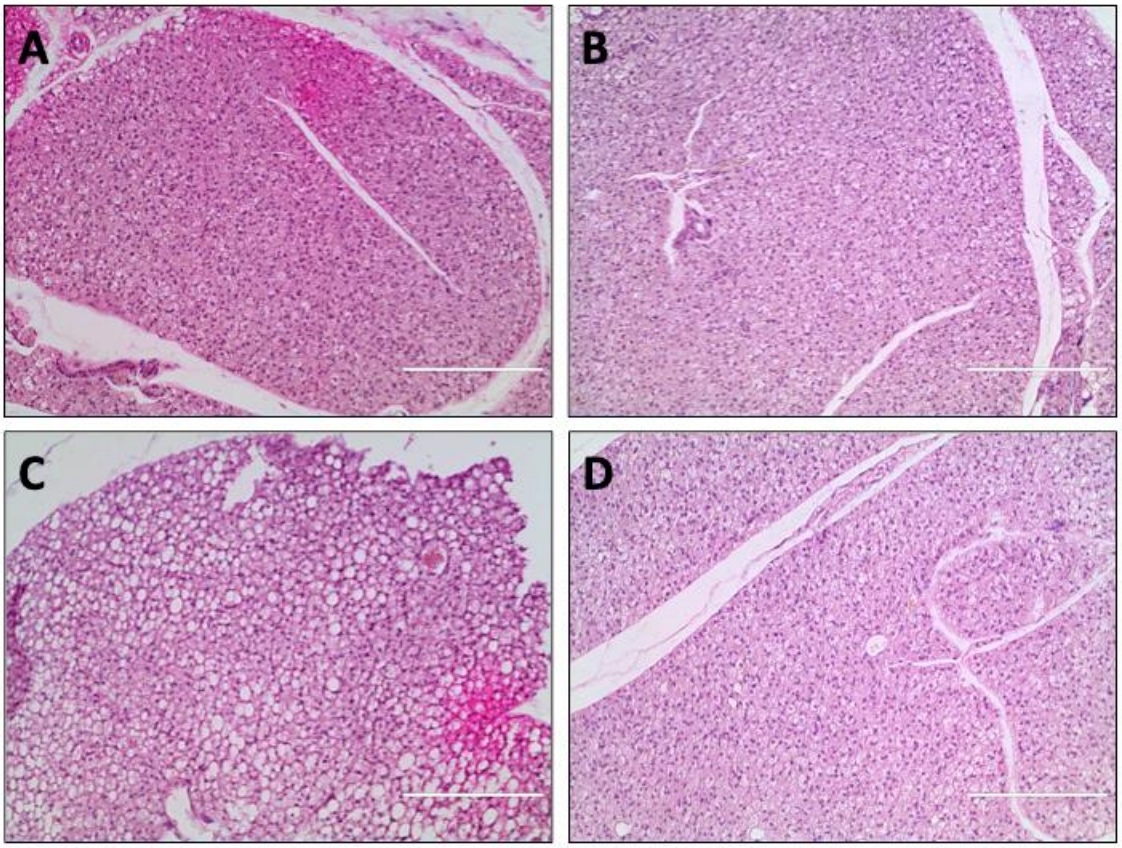


**Figure 10: Brown Adipocyte Histology After DHT-Treatment.** Representative images of brown adipocytes taken from mice belonging to the wild-type placebo (A), NAG Placebo (B), wild-type DHT (C), and NAG DHT (D) groups. Data shown is representative of n = 8 for both placebo groups, and n = 7 for both DHT groups. Images were taken at 200x magnification, and scale bars represent 200 μm.

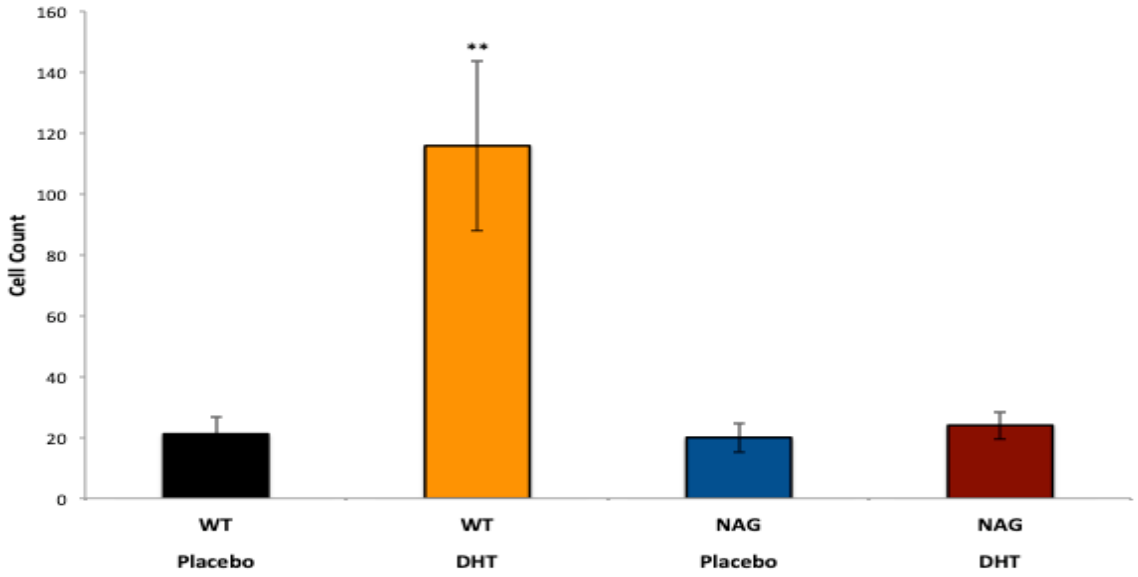


**Figure 11: Enlarged Adipocytes Within Brown Adipose Tissue.** Number of enlarged adipocytes within brown adipocytes collected from mice 90 days post-implantation. Enlarged adipocytes were defined as having an area >90 $um^2$. Data shown is average ± SEM (n = 8 for both placebo groups, n = 7 for both DHT groups). Student's t-tests were conducted for the comparison of cell sizes between all groups (***p < 0.01*).

On average, NAG-1 mice have lower serum triglyceride levels than wild-type mice, regardless of treatment group (Fig. 12 A) (.81 nmol/ml vs. 14.7 nmol/ml, *p < 0.05*). Both groups of DHT-treated mice trended towards having higher cholesterol levels than the placebo groups, but no significance was observed.

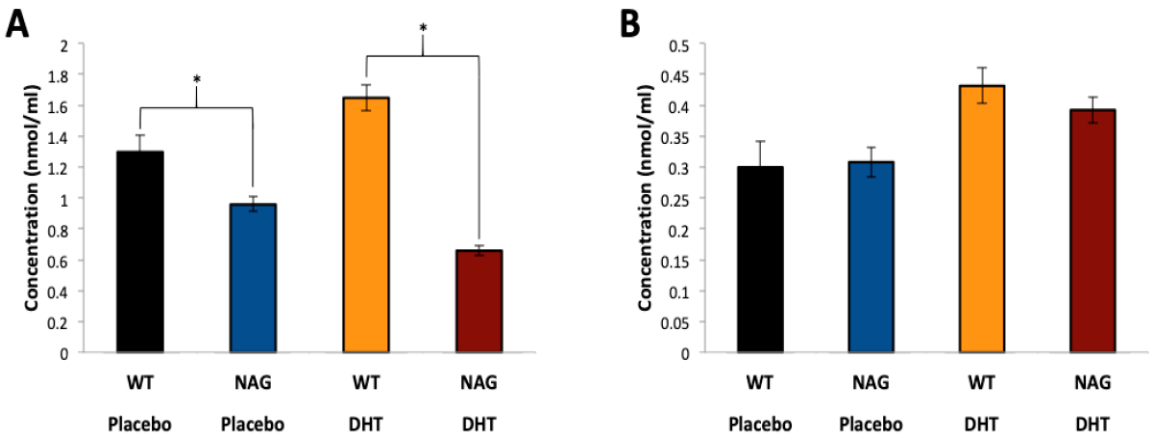


**Figure 12: Serum Triglyceride and Cholesterol Levels in DHT -Treated Mice.** A) Triglyceride ELISA results from serum samples collected from mice 90 days post-implantation. B) Cholesterol ELISA results from serum samples collected from mice 90 days post-implantation. Data shown is average ± SEM (n = 8 for both placebo groups, n = 7 for both DHT groups). Tukey's multiple comparison tests were conducted for the comparison of triglyceride and cholesterols levels between all groups (*$p < 0.05$).

## Discussion

This study was designed to examine if female transgenic NAG-1 mice were resistant to hormonally induced metabolic disorders observed in PCOS, including changes in body weight, adipocyte morphology, glucose intolerance, and serum triglyceride and cholesterol levels. As a first step we determined that prolonged DHT-treatment could induce the same physiological effects of PCOS in NAG-1 mice as previously observed in wild-type mice. To test this, we measured age at which mice entered puberty, AGD, estrus cycles, and ovarian follicle counts.

Our data indicate that NAG-1 mice show no difference in response to DHT-treatment when compared to wild-type mice treated with DHT in regards to changes in the onset of puberty and AGD [17, 18]. Female mice exposed to DHT entered puberty earlier and have increased AGD at 90 days post-implantation than either placebo group, regardless of genotype. The responses to DHT seen in this study align with previous research [14]. In addition, NAG-1 mice also display similar

ovarian physiology to wild-type mice when treated with DHT. Both NAG-1 and wild-type DHT groups had disrupted estrus cycles across a two-week period, and also completed significantly fewer cycles in comparison to the placebo groups. Both the wild-type and NAG-1 treatment groups have decreased numbers of corpus lutea present, which suggests anovulation [33]. Our results indicate that NAG-1 transgenic mice have a similar ovarian response to excess DHT as wild-type mice, which is similar to physiological changes reported in PCOS [14, 21, 22]. Given that the ovarian and reproductive phenotypes associated with PCOS are some of its primary components, demonstrating that our DHT-induced mouse model of PCOS still produces key reproductive phenotypes in mice was a critical first step in confirming that PCOS is inducible in NAG-1 mice [14, 21, 22].

Body weight has been shown to increase in wild-type mice treated with DHT, but no studies have been done on DHT-treatment in NAG-1 mice [14, 21]. NAG-1 mice appear to be resistant to hormonal-induced weight gain. We observed a significant difference in weights across genotype, regardless of treatment, as reported previously in NAG-1 mice [12]. There was significant difference in weights between the wild-type groups in weeks 2-9, and again at week 12, which is similar to previous research that has shown that DHT-induced PCOS leads to significance in weight gain several weeks post implantation, but can taper off and lose significance around week 7 [22]. Another study in which significant weight gain is observed through the entirety of the study used mice fed a high-fat diet, whereas the mice in this study were not, which may account for the loss of significance at weeks 10 and

11 [34]. NAG-1 mice only had differing weights in the first week, which suggests that the NAG-1 transgene may prevent hormonal induced weight gain in mice.

As the severity of weight gain increases in both humans and mice, white adipocytes increase in size as well [35]. In our study we observed that wild-type mice trended towards having lower amounts of small (100-500 $um^2$) adipocytes, as well as increased numbers of large (1000-$1500^2$) and extra large ($1500^2$+) adipocytes. As adipocyte size has been shown to increase as a result of weight gain, it was hypothesized that there would be differences in cell sizes between the wild-type treatment groups, but the trends were not significant [36, 37]. The lack of significance in adipocyte size may be due to adipocytes being collected at 90 days post implantation, when differences in weight gain began to lose significance between wild-type groups several weeks before tissue collection. The NAG-1 placebo group had significantly less adipocytes with areas larger than 1000 $\mu m^2$ than the wild-type placebo group, supporting that NAG-1 mice have leaner phenotypes than wild-type mice. Together, the lack of weight gain observed between NAG-1 treatment groups and the lack of alteration in white adipocyte morphology is suggests that NAG-1 transgenic mice are resistant to DHT-induced weight gain, and white adipocyte hypertrophy. Although we did not see a change in white adipocyte size in the NAG-1 treatment groups, we cannot make a definitive conclusion as to whether NAG-1 mice are resistant to excess lipid storage, as we also observed no significant difference between the wild-type treatment groups. Additionally, no white adipocytes were seen in liver samples in both genotypes and treatment groups when examining liver morphology for the presence of steatosis.

The lack of significant white adipocyte hypertrophy and steatosis may be indicative that DHT-treatment alone is not adequate to induce profound changes in adipocyte morphology, and the use of a high-fat diet may be necessary as well for the study of these phenotypes [34].

NAG-1 mice display no morphological increases in adipocyte size in brown adipose tissue when treated with DHT in contrast to wild-type mice, which had a 5-fold increase in hypertrophic brown adipocytes (Fig. 11). We observed a significantly higher number of unilocular adipocytes in the wild-type DHT group in comparison to all other groups, which suggests that there is some conversion from brown adipocytes to white adipocytes in response to DHT treatment, and possibly loss of the high metabolic activity associated with brown adipocytes [38]. We observed no difference in adipocyte morphology in the NAG-1 groups, which suggests that DHT-treatment does not cause this observable differentiation in NAG-1 mice. If brown adipocytes are differentiating into white adipocytes in wild-type mice, this may be a contributing factor to the observed weight gain, and the lack of adipocyte hypertrophy seen in NAG-1 mice may contribute to their ability to resist hormonal-induced metabolic disorders. More research needs to be done to confirm these hypotheses, but we hypothesize that analyzing gene expression of thermogenesis-related genes (i.e. *UCP1*, *PGC1a*, *Cox8B*, and *Dio2*) in brown adipose tissue could provide more insight, as upregulation of these genes can indicate an increased level of thermogenesis [11].

Excess DHT has been shown to cause glucose intolerance in mice, however we observed no significant difference in glucose tolerance between treatment

groups [22]. Although DHT-treated wild-type mice did not have significant changes in glucose tolerance, the results are consistent with previous research on insulin resistance and glucose intolerance in PCOS rodent models [39]. The NAG-1 placebo mice had a notably higher initial spike in blood glucose in response to the injection as opposed to the NAG-1 DHT group, which may suggest a level of improved glucose uptake in DHT-treated NAG-1 mice. The improved glucose tolerance in NAG-1 mice is consistent with previous research [23], but the further improvement when mice are treated with DHT is novel. The lack of significance in the wild-type groups in this study may be attributed to the mice not being fed a high-fat diet in comparison to similar studies [23, 34]. Additionally, the small sample size may also contribute to the lack of significance, as other studies have between 9-12 mice [14, 21].

The combination of results regarding brown adipocyte hypertrophy and glucose tolerance indicate that DHT may be having a more significant impact on brown adipocyte function than on glucose intolerance, as there seems to be a correlation between the degree of brown adipocyte hypertrophy and weight gain in wild-type mice. This may provide insight into brown adipose tissue's role in regulating weight gain, and its link to NAG-1. Our results suggest that as NAG-1 is expressed in mice, brown adipose tissue retains its normal morphology under hyperandrogenic conditions, and may also remain highly thermogenic.

Additionally, NAG-1 mice had lower triglyceride levels than wild-type mice, regardless of treatment groups, which is consistent with previous research on transgenic mice [11, 40]. A lack of elevation in serum cholesterol and triglyceride levels has been reported in DHT-treated wild-type mice, but elevated levels of

cholesterol and triglycerides have been reported in studies using high-fat diets [21, 34, 39]. As NAG-1 mice maintain lower triglyceride levels, even when hyperandrogenism is induced, expression of NAG-1 may play a role in reducing triglycerides, but appears to have no impact on serum cholesterol levels. Although increased concentrations of both triglycerides and cholesterol can predispose an individual to cardiovascular disease, a reduction in triglycerides alone can lower the risk of cardiovascular disease [41].

NAG-1 mice have been shown to display upregulation of thermogenesis related genes, and so the lack of weight gain induced by DHT-treatment may be attributed to an elevated level of thermogenesis. It appears that brown adipose-mediated thermogenesis may not be impaired, as indicated by the lack of morphological changes in brown adipose tissue in the NAG-1 DHT-treatment group. Previous research has shown distinct difference in white adipocyte morphology in DHT-treated mice, we conclude that the lack of significance in adipocyte size is due to adipocytes being collected at a point in the study where the weights between treatment groups had eclipsed one another. The lack of significance in weights between weeks 10-12 and at week 14 in the wild-type placebo and DHT groups may be attributed to the mice not being fed on a high-fat diet as described in previous studies [23, 34]. The lack of significance in glucose tolerance between wild-type groups in this study compared to similar studies may also be a result of not being fed on high-fat diet [23, 34]. The phenotypes observed in NAG-1 mice in this study were consistent with previous research, and we are able to conclude that NAG-1

mice are able to maintain a lean phenotype, even when hyperandrogenism is induced via DHT.

Women with PCOS have chronic low-level inflammation in addition to the common ovarian and metabolic phenotypes discussed in this paper [42, 43]. As serum levels of inflammatory cytokines, such as Tumor Necrosis Factor-alpha (*TNF-a*), interleukin-18 (*IL-18*), and interleukin-6 increase (*IL-6*), inflammatory cytokine levels also increase in adipose tissue [44, 45]. Increased inflammation is also linked to insulin resistance, which is estimated to be present in 50-70% of women with PCOS [46, 47]. Obese patients with PCOS have also been shown to have elevated levels of the cytokine TNF-*a* compared to non-PCOS patients [48]. Elevated IL-18 levels have been linked to higher incidences of cardiovascular disease [49]. Together, this data suggest that inflammation plays a key role in the development of metabolic disorders in women with PCOS. NAG-1 is secreted in direct response to these cytokines, and while the mechanism is unclear, it appears to play a role in the regulation of inflammatory responses through downstream activation of transforming growth factor-beta [50]. NAG-1 is secreted as a disulfide-linked dimer in response to cytokine release, and binds to the GFRAL receptor where it promotes weight loss and lowers pro-inflammatory cytokine levels [50, 51]. While inflammatory responses were not explored in this paper, we propose a model that in addition to the increased levels of thermogenesis in NAG-1 transgenic mice, the anti-inflammatory and anti-insulin-resistance properties of the gene may also play a role in preventing the metabolic disorders associated with PCOS [33]. Figure 13

describes a possible mechanism for how the overexpression of NAG-1 may reduce inflammation and prevent hormonal induced weight gain.

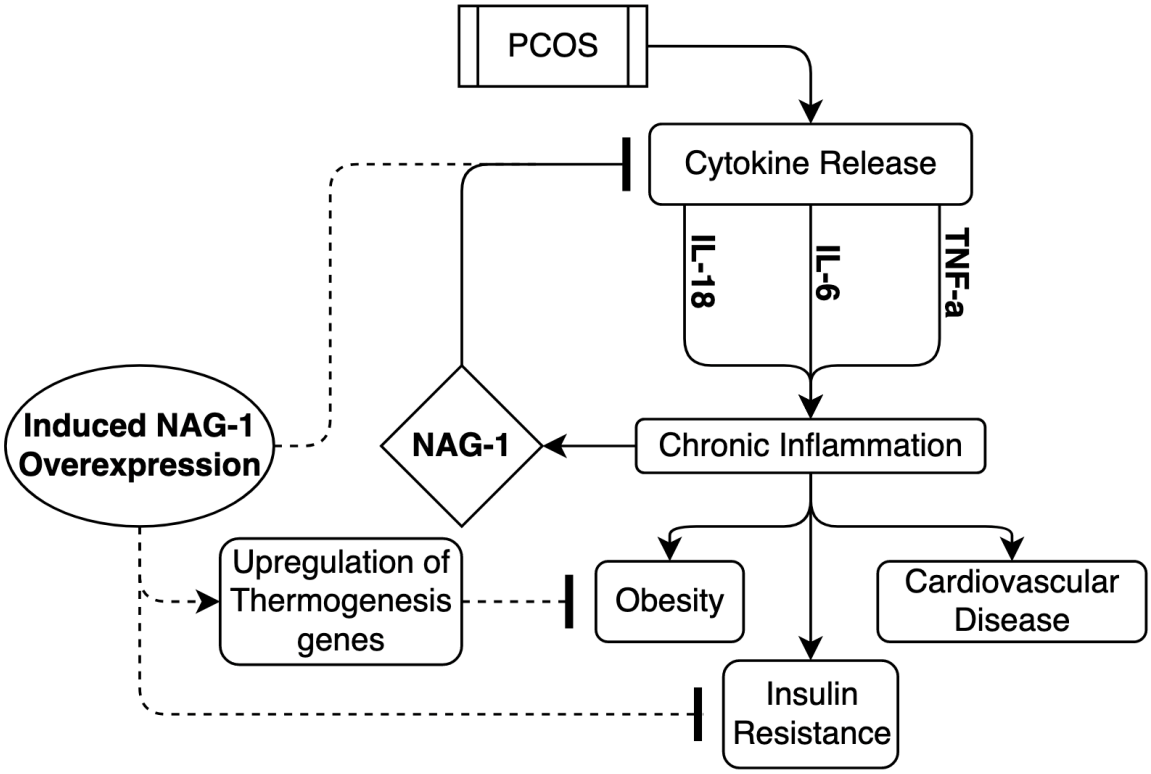


**Figure 13: NAG-1 and Inflammation.** NAG-1 is secreted in response to an initial release of cytokines, and functions to mediate the release of more cytokines. When the expression of NAG-1 is initiated, it further inhibits the release of cytokines and thus reduces the chronic inflammation in PCOS. In addition, the expression of NAG-1 also aids in preventing insulin resistance, and may also cause downstream upregulation of thermogenesis related genes that prevent weight gain and obesity.

In conclusion, this study explores the ovarian and metabolic phenotypes associated with DHT-induced PCOS in NAG-1 mice. We have shown that transgenic NAG-1 mice are resistant to hormonal induced weight gain, white and brown adipocyte hypertrophy. In addition, NAG-1 mice also exhibit similar ovarian phenotypes to wild-type mice when exposed to DHT (Table 1 summarizes the features of PCOS observed between wild-type and NAG-1 mice). More research needs to be performed on the effects of NAG-1 overexpression in response to DHT-

treatment when mice are fed a high-fat diet, and also in regards to the chronic inflammation associated with PCOS, but our results indicate that overexpression of NAG-1 may be promising in preventing hormonal induced metabolic disorders.

**Table 1:** Summary of metabolic and ovarian phenotypes between genotypes observed in a DHT-induced mouse model of PCOS

| | **Treatment Groups** | |
|---|---|---|
| **Phenotypes of DHT-induced PCOS in wild-type Mice** | **Wild-type DHT** | **NAG DHT** |
| Early onset of puberty | ✔ | ✔ |
| Increased anal-genital distance | ✔ | ✔ |
| Weight gain | ✔ | ✔ |
| Irregular estrus cycles | ✔ | ✔ |
| Glucose intolerance | ✔ | ✔ |
| Decreased corpus lutea count | ✔ | ✔ |
| White adipocyte hypertrophy | ✔ | ✔ |
| Brown adipocyte hypertrophy | ✔ | ✔ |
| Increased triglyceride levels | ✔ | * |
| Increased cholesterol levels | ✔ | ✔ |
| Hepatic steatosis | ✔ | ✔ |

✔, present; ✗, not present; *, maintained lower than wild-type

**Acknowledgments**

We thank Raeanne Tegman for her assistance in histological preparation, and Amanda Qvigstad for her assistance in developing DHT pellets.

Address all correspondence and requests for reprints to: April Binder, Ph.D. and associate professor of biology, Department of Biology, Central Washington University, 400 E University Way MS7537, Ellensburg, WA 98926. E-mail: BinderA@cwu.edu

This study was supported by grants from the Washington State Distinguished Fellowship in Biology.

Disclosure Statement: The authors have nothing to disclose.

## References


[1] Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Human Reproduction*. 2004;19(1):41-47. doi:10.1093/humrep/deh098

[2] Knudsen N, Laurberg P, Rasmussen L et al. Small Differences in Thyroid Function May Be Important for Body Mass Index and the Occurrence of Obesity in the Population. *The Journal of Clinical Endocrinology & Metabolism*. 2005;90(7):4019-4024. doi:10.1210/jc.2004-2225

[3] Legro R, Arslanian S, Ehrmann D et al. Diagnosis and Treatment of Polycystic Ovary Syndrome: An Endocrine Society Clinical Practice Guideline. *The Journal of Clinical Endocrinology & Metabolism*. 2013;98(12):4565-4592. doi:10.1210/jc.2013-2350

[4] Venkatesan A. Insulin Resistance in Polycystic Ovary Syndrome: Progress and Paradoxes. *Recent Prog Horm Res*. 2001;56(1):295-308. doi:10.1210/rp.56.1.295

[5] Barber T, Franks S. Adipocyte biology in polycystic ovary syndrome. *Mol Cell Endocrinol*. 2013;373(1-2):68-76. doi:10.1016/j.mce.2012.10.010

[6] Ramezani Binabaj M, Motalebi M, Karimi-Sari H, Rezaee Zavareh M, Alavian S. Are Women With Polycystic Ovarian Syndrome at a High Risk of Non-Alcoholic Fatty Liver Disease? A Meta-Analysis. *Hepat Mon*. 2014;14(11). doi:10.5812/hepatmon.23235

[7] Wild R, Carmina E, Diamanti-Kandarakis E et al. Assessment of Cardiovascular Risk and Prevention of Cardiovascular Disease in Women

with the Polycystic Ovary Syndrome: A Consensus Statement by the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society. *The Journal of Clinical Endocrinology & Metabolism*. 2010;95(5):2038-2049. doi:10.1210/jc.2009-2724

[8] Dickerson L, Shrader S, Diaz V. *Pharmacotherapy: A Pathophysiologic Approach*. McGraw-Hill Medical; 2008:1313-1328.

[9] Puppala S. Re: Polycystic ovary syndrome and the differential diagnosis of hyperandrogenism. *The Obstetrician & Gynaecologist*. 2014;16(1):69-69. doi:10.1111/tog.12069_11

[10] Hull M. Epidemiology of infertility and polycystic ovarian disease: endocrinological and demographic studies. *Gynecological Endocrinology*. 1987;1(3):235-245. doi:10.3109/09513598709023610

[11] Chrysovergis K, Wang X, Kosak J et al. NAG-1/GDF-15 prevents obesity by increasing thermogenesis, lipolysis and oxidative metabolism. *Int J Obes*. 2014;38(12):1555-1564. doi:10.1038/ijo.2014.27

[12] Tsai V, Macia L, Johnen H et al. TGF-b Superfamily Cytokine MIC-1/GDF15 Is a Physiological Appetite and Body Weight Regulator. *PLoS ONE*. 2013;8(2):e55174. doi:10.1371/journal.pone.0055174

[13] Rainey W, Carr B, Sasano H, Suzuki T, Mason J. Dissecting human adrenal androgen production. *Trends in Endocrinology & Metabolism*. 2002;13(6):234-239. doi:10.1016/s1043-2760(02)00609-4

[14] Leonie E, van Houten E, Kramer P, McLuskey A, Karels B, Themmen A, Visser J. Reproductive and Metabolic Phenotype of a Mouse Model of PCOS. *Endocrinology*. 2012;153(6):2861-2869. doi:10.1210/en.2011-1754

[15] Baek S, Okazaki R, Lee S et al. Nonsteroidal Anti-Inflammatory Drug-Activated Gene-1 Over Expression in Transgenic Mice Suppresses Intestinal Neoplasia. *Gastroenterology*. 2006;131(5):1553-1560. doi:10.1053/j.gastro.2006.09.015

[16] McLean A, Valenzuela N, Fai S, Bennett S. Performing Vaginal Lavage, Crystal Violet Staining, and Vaginal Cytological Evaluation for Mouse Estrous Cycle Staging Identification. *Journal of Visualized Experiments*. 2012;(67). doi:10.3791/4389

[17] Dean A, Smith L, Macpherson S, Sharpe R. The effect of dihydrotestosterone exposure during or prior to the masculinization programming window on reproductive development in male and female rats. *Int J Androl*. 2012;35(3):330-339. doi:10.1111/j.1365-2605.2011.01236.x

[18] Ojeda S, Urbancki H. *Physiology Of Reproduction*. New York: Raven Press; 1994:363-409.

[19] Byers S, Wiles M, Dunn S, Taft R. Mouse Estrous Cycle Identification Tool and Images. *PLoS ONE*. 2012;7(4):e35538. doi:10.1371/journal.pone.0035538

[20] Hoage T, Cameron I. Folliculogenesis in the ovary of the mature mouse: A radioautographic study. *Anat Rec*. 1976;184(4):699-709. doi:10.1002/ar.1091840409

[21] Caldwell A, Middleton L, Jimenez M et al. Characterization of Reproductive, Metabolic, and Endocrine Features of Polycystic Ovary Syndrome in Female Hyperandrogenic Mouse Models. *Endocrinology*. 2014;155(8):3146-3159. doi:10.1210/en.2014-1196

[22] Hurliman A, Keller Brown J, Maille N, Mandala M, Casson P, Osol G. Hyperandrogenism and Insulin Resistance, Not Changes in Body Weight, Mediate the Development of Endothelial Dysfunction in a Female Rat Model of Polycystic Ovary Syndrome (PCOS). *Endocrinology*. 2015;156(11):4071-4080. doi:10.1210/en.2015-1159

[23] Macia L, Tsai V, Nguyen A et al. Macrophage Inhibitory Cytokine 1 (MIC-1/GDF15) Decreases Food Intake, Body Weight and Improves Glucose Tolerance in Mice on Normal & Obesogenic Diets. *PLoS ONE*. 2012;7(4):e34868. doi:10.1371/journal.pone.0034868

[24] Fang L, Guo F, Zhou L, Stahl R, Grams J. The cell size and distribution of adipocytes from subcutaneous and visceral fat is associated with type 2 diabetes mellitus in humans. *Adipocyte*. 2015;4(4):273-279. doi:10.1080/21623945.2015.1034920

[25] Lieber C. Pathogenesis of Hepatic Steatosis. *Gastroenterology*. 1963;45(6):760-764. doi:10.1016/s0016-5085(19)34810-3

[26] De Leo V, la Marca A, Petraglia F. Insulin-Lowering Agents in the Management of Polycystic Ovary Syndrome. *Endocr Rev*. 2003;24(5):633-667. doi:10.1210/er.2002-0015

[27] Virtanen K, Lidell M, Orava J et al. Functional Brown Adipose Tissue in Healthy Adults. *New England Journal of Medicine*. 2009;360(15):1518-1525. doi:10.1056/nejmoa0808949

[28] Cypess A, Lehman S, Williams G et al. Identification and Importance of Brown Adipose Tissue in Adult Humans. *Obstet Gynecol Surv*. 2009;64(8):519-520. doi:10.1097/ogx.0b013e3181ac8aa2

[29] Enerbäck S. The Origins of Brown Adipose Tissue. *New England Journal of Medicine*. 2009;360(19):2021-2023. doi:10.1056/nejmcibr0809610

[30] Vernon R, Flint D. *Encyclopedia Of Food Sciences And Nutrition*. 2nd ed.; 2003:23-29.

[31] Wild R, Bartholomew M. The influence of body weight on lipoprotein lipids in patients with polycystic ovary syndrome. *Am J Obstet Gynecol*. 1988;159(2):423-427. doi:10.1016/s0002-9378(88)80099-1

[32] de Groot P, Dekkers O, Romijn J, Dieben S, Helmerhorst F. PCOS, coronary heart disease, stroke and the influence of obesity: a systematic review and meta-analysis. *Hum Reprod Update*. 2011;17(4):495-500. doi:10.1093/humupd/dmr001

[33] Lunn S, Fraser H.M., Mason H.D. Structure of the corpus luteum in the ovulatory polycystic ovary. *Human Reproduction*. 2002;17(1):111-117. doi:10.1093/humrep/17.1.111

[34] Ressler I, Grayson B, Ulrich-Lai Y, Seeley R. Increased fat consumption exacerbates metabolic and behavioral effects of polycystic ovary syndrome in

a rodent model. *Fertil Steril*. 2013;100(3):S355. doi:10.1016/j.fertnstert.2013.07.835

[35] Kauffman A, Thackray V, Ryan G et al. A Novel Letrozole Model Recapitulates Both the Reproductive and Metabolic Phenotypes of Polycystic Ovary Syndrome in Female Mice1. *Biol Reprod*. 2015;93(3). doi:10.1095/biolreprod.115.131631

[36] Dunaif A, Segal K, Shelley D, Green G, Dobrjansky A, Licholai T. Evidence for distinctive and intrinsic defects in insulin action in polycystic ovary syndrome. *Diabetes*. 1992;41(10):1257-1266. doi:10.2337/diabetes.41.10.1257

[37] Manneråas-Holm L, Leonhardt H, Kullberg J et al. Adipose Tissue Has Aberrant Morphology and Function in PCOS: Enlarged Adipocytes and Low Serum Adiponectin, but not Circulating Sex Steroids, Are Strongly Associated with Insulin Resistance. *Endocrinology*. 2011;152(1):332-332. doi:10.1210/endo.152.1.9990

[38] Rosenwald M, Perdikari A, Rülicke T, Wolfrum C. Bi-directional interconversion of brite and white adipocytes. *Nat Cell Biol*. 2013;15(6):659-667. doi:10.1038/ncb2740

[39] Manneråas L, Cajander S, Holmäng A et al. A New Rat Model Exhibiting Both Ovarian and Metabolic Characteristics of Polycystic Ovary Syndrome. *Endocrinology*. 2007;148(8):3781-3791. doi:10.1210/en.2007-0168

[40] Xiong Y, Walker K, Min X et al. Long-acting MIC-1/GDF15 molecules to treat obesity: Evidence from mice to monkeys. *Sci Transl Med*. 2017;9(412):eaan8732. doi:10.1126/scitranslmed.aan8732

[41] Berglund L, Brunzell J, Goldberg A et al. Evaluation and Treatment of Hypertriglyceridemia: An Endocrine Society Clinical Practice Guideline. *The Journal of Clinical Endocrinology & Metabolism*. 2012;97(9):2969-2989. doi:10.1210/jc.2011-3213

[42] Kelly C, Lyall H, Petrie J, Gould G, Connell J, Sattar N. Low Grade Chronic Inflammation in Women with Polycystic Ovarian Syndrome. *The Journal of Clinical Endocrinology & Metabolism*. 2001;86(6):2453-2455. doi:10.1210/jcem.86.6.7580

[43] González F, Considine R, Abdelhadi O, Acton A. Inflammation triggered by saturated fat ingestion is linked to insulin resistance and hyperandrogenism in PCOS. *The Journal of Clinical Endocrinology & Metabolism*. 2020. doi:10.1210/clinem/dgaa108

[44] ClÉment K, Viguerie N, Poitou C et al. Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. *The FASEB Journal*. 2004;18(14):1657-1669. doi:10.1096/fj.04-2204com

[45] Blankenberg S, Tiret L, Bickel C et al. Interleukin-18 Is a Strong Predictor of Cardiovascular Death in Stable and Unstable Angina. *Circulation*. 2002;106(1):24-30. doi:10.1161/01.cir.0000020546.30940.92

[46] Bloomgarden Z. Inflammation and Insulin Resistance. *Diabetes Care*. 2003;26(5):1619-1623. doi:10.2337/diacare.26.5.1619

[47] Legro R, Castracane V, Kauffman R. Detecting Insulin Resistance in Polycystic Ovary Syndrome: Purposes and Pitfalls. *Obstet Gynecol Surv*. 2004;59(2):141-154. doi:10.1097/01.ogx.0000109523.25076.e2

[48] Gonzalez F, Thusu K, Abdel-Rahman E, Prabhala A, Tomani M, Dandona P. Elevated serum levels of tumor necrosis factor alpha in normal-weight women with polycystic ovary syndrome. *Metabolism*. 1999;48(4):437-441. doi:10.1016/s0026-0495(99)90100-2

[49] Bootcov M, Bauskin A, Valenzuela S et al. MIC-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-b superfamily. *Proceedings of the National Academy of Sciences*. 1997;94(21):11514-11519. doi:10.1073/pnas.94.21.11514

[50] Mullican S, Lin-Schmidt X, Chin C et al. GFRAL is the receptor for GDF15 and the ligand promotes weight loss in mice and nonhuman primates. *Nat Med*. 2017;23(10):1150-1157. doi:10.1038/nm.4392

[51] Mullican S, Rangwala S. Uniting GDF15 and GFRAL: Therapeutic Opportunities in Obesity and Beyond. *Trends in Endocrinology & Metabolism*. 2018;29(8):560-570. doi:10.1016/j.tem.2018.05.002