The Role of Ovarian Factors in the Regulation of Metabolism

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THE ROLE OF OVARIAN FACTORS IN THE REGULATION OF METABOLISM

De functie van ovariële factoren in de regulatie van de stofwisseling

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Aan mijn ouders

CONTENTS

Chapter 1

General Introduction

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1.1 INTRODUCTION

The ovary, or female gonad, plays a central role in the production of the female gametes, the oocytes, and is the major organ where sex steroid hormones are produced in females. In humans, the oocytes are produced from a limiting primordial follicle pool, which is established before birth (1). From the primordial follicle pool follicles are continuously recruited to develop through primary and secondary stages to become antral follicles. After puberty, when menstrual cycling has commenced, at each cycle, follicles will be recruited from this growing follicle pool under the influence of Follicle Stimulating Hormone (FSH), and ultimately only one of those follicles will reach the preovulatory stage (dominant follicle) (Figure 1). The remaining follicles that are not rescued by FSH will become atretic (2).

Under the influence of Gonadotropin Releasing Hormone (GnRH) from the hypothalamus, Luteinizing Hormone (LH) and FSH are secreted by the pituitary (3). During a menstrual cycle, which has an average length of 28 days, two phases can be recognized before and after ovulation: the follicular and the luteal phase (4-5). In normal cycling women, FSH levels are highest when the dominant follicle emerges. This leads to the production of estrogens by the granulosa cells of the preovulatory follicle. After estrogen levels peak, the LH surge occurs and ovulation will follow. After ovulation, the follicle remnant without the oocyte forms the corpus luteum, which marks the beginning of the luteal phase. The corpus luteum produces progesterone and estrogens which synergistically stimulate the endometrium. When pregnancy does not occur, progesteron and estrogen levels drop and menses start (Figure 1).

1.2 POLYCYSTIC OVARY SYNDROME

Since the menstrual cycle is the result of the strict interplay of hormones produced by the hypothalamus, pituitary and ovaries, cycle irregularities are an indication of a disbalance and may be an indication of infertility. The most common cause of female infertility is Polycystic Ovary Syndrome (PCOS), which affects about 6-8% of women worldwide (6). Based on the Rotterdam Criteria, PCOS is characterized by two of the following three criteria: clinical or biochemical hyperandrogenism, oligo- or amenorrhea, and polycystic ovaries (PCO) (7). The various combinations of the criteria which emerged due to the introduction of the Rotterdam criteria, introduced three different PCOS phenotypes. (i) The classic phenotype is characterized by hyperandrogenism and oligo-or amenorrhea, with or without PCO morphology. (ii) The ovulatory phenotype is characterized by hyperandrogenism and PCO. Finally, (iii) the normoandrogenic phenotype is characterized by oligo- or amenorrhea and

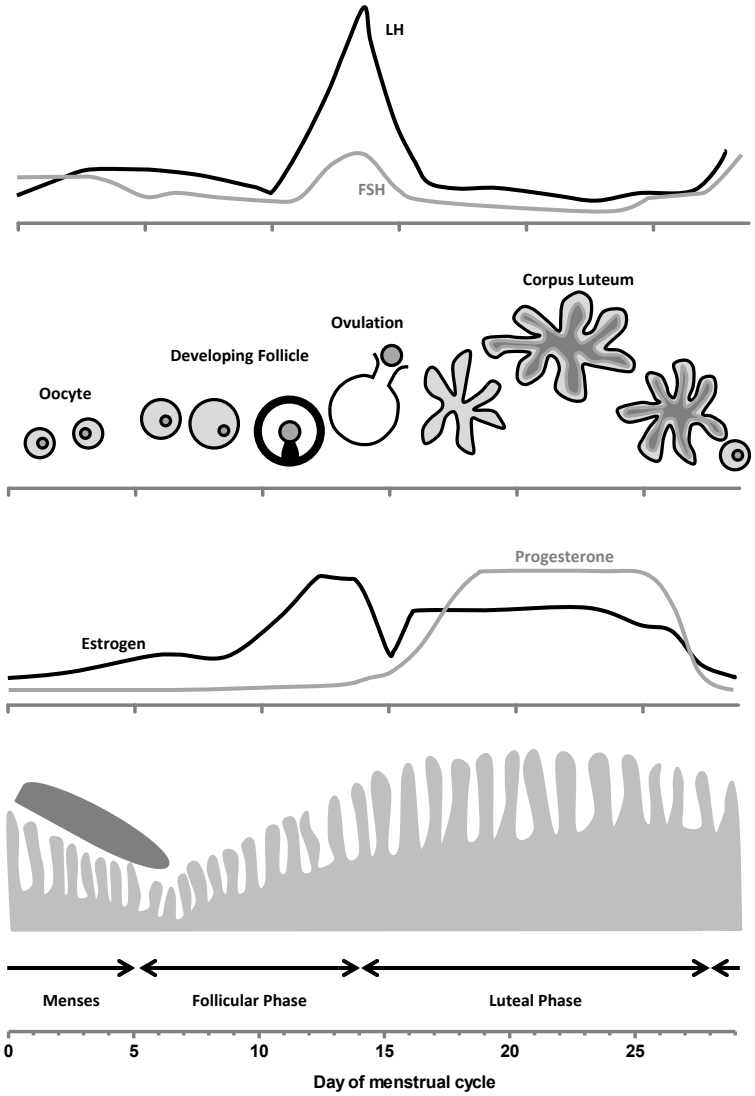

FIGURE 1. Menstrual cycle. Hormonal, ovarian and endometrial stages throughout the menstrual cycle.

PCO (8). Although the etiology of PCOS is unknown, the failure in dominant follicle selection leading to an accumulation of small antral follicles suggests that FSH sensitivity is altered in PCOS ovaries. This is further discussed in §1.2.1.

Treatment of women with PCOS focuses on infertility and the metabolic characteristics which are often present, such as obesity and insulin resistance (discussed in §1.2.2). For the treatment of infertility usually pharmacological ovulation induction is used. Clomiphene citrate is the most commonly used agent. It is a selective

estrogen receptor modulator and inhibits the binding of estradiol in the pituitary and hypothalamus by competitive receptor antagonism, thereby stimulating the release of FSH and LH, which lead to stimulation of follicular growth, eventually leading to ovulation (9). Metformin is used as an oral antihyperglycaemic reagent in the treatment of diabetes. However, in PCOS metformin has been suggested to improve fertility by lowering insulin levels and thereby restoring the hormonal milieu in the ovary and thus restoring ovulation (10). In addition, treatment of insulin resistance and obesity also often indirectly leads to the treatment of infertility.

There are multiple methods to treat obesity within PCOS: lifestyle changes, pharmaceutical interventions and bariatric surgery. Lifestyle changes usually encompass exercise and calorie restriction, which lead to changes in body composition, improvement of insulin sensitivity and improvement of hyperandrogenism (11). Regarding pharmaceutical interventions, metformin (see above) is most often used. Besides lowering the insulin levels, it has also been associated with weight loss (12). Especially the use of metformin in combination with a hypocaloric diet leads to weight reduction, improvement of the ovulatory function and fertility rates (13). Finally bariatric surgery, which is increasingly used, has proven to result in very positive effects on the metabolic, hyperandrogenic and reproductive manifestations and can even lead to complete resolution of PCOS (14). However, this procedure remains drastic. Although positive effects are reached with these interventions, treatment is palliative rather than curative and focuses on symptomatic approaches. Nevertheless palliative treatment may become increasingly important since over the years it has become evident that PCOS is associated with long-term health consequences beyond the reproductive system. Around 7.5% of the affected women will develop type 2 diabetes (15). Furthermore, women with PCOS have more extensive cardiovascular diseases (16). Also, it has been assumed that PCOS predisposes to endometrial cancer (17).

1.2.1 *The reproductive phenotype in PCOS*

Approximately 85% of PCOS patients have clinically evident menstrual dysfunction (18), although occasional ovulations have been reported (19). Interestingly, the greater the menstrual irregularity is, the more severe the metabolic alterations are, such as BMI and fasting insulin (20). Besides menstrual dysfunction there are other reproductive manifestations, including hyperandrogenism, referring to high levels of circulating endogenous androgens, including testosterone, dehydroepiandrosterone (DHEA), dehydroepiandrosteronesulfate (DHEAS) and androstenedione. Similar to the number of ovulations and its correlation to phenotype severity, there is also a correlation between hyperandrogenism and the metabolic phenotype severity. Normoandrogenic PCOS patients have milder metabolic phenotypes (21).

The characteristic morphological feature of ovaries in anovulatory PCOS women is an accumulation of small antral follicles and the polycystic appearance of the ovaries as a result of the arrest in follicle growth (22). This suggest an intrinsic abnormal ovarian environment which contributes to the follicular arrest (22), as exemplified by the higher FSH threshold in PCOS patients. (23). This has been confirmed by studies in which granulosa cells from PCOS women, cultured in vitro, were hyperresponsive to FSH, producing 6- to 10-fold more estrogens in response to FSH than normal cells (24-25). The origin of the decreased FSH sensitivity and follicular arrest may be due to a combination of factors. A neuroendocrine hallmark of PCOS is the increased GnRH pulse frequency leading to excess luteinizing hormone (LH) secretion (26). Combined with the premature acquisition of LH receptor expression by growing follicles at too early stages, this in turn causes increased ovarian androgen production, leading to a premature maturation of follicles (26). Eventually, there is a block in folliculogenesis leading to an accumulation of small antral follicles and thereby an increased Antral Follicle Count (AFC).

1.2.2 *The metabolic phenotype in PCOS*

Besides having a reproductive phenotype, PCOS patients also frequently display metabolic derangements, which closely resemble the metabolic syndrome. The metabolic syndrome is a cluster of risk factors for the development of cardiovascular diseases and according to the National Cholesterol Education Adult Treatment Program (27) comprises: diabetes and prediabetes, abdominal adiposity, high cholesterol and high blood pressure. Indeed, 38-88% (depending on the study) of the PCOS women are obese (28-29) and 50-70% are insulin resistant (30-32). Women with PCOS predominantly show abdominal obesity (33). This central or visceral adiposity is highly associated with insulin resistance (34). Also, a positive correlation is present between adiposity and hyperandrogenism. The androgen receptor is present in pre- and mature adipocytes of subcutaneous and abdominal fat of both men and women (35). Furthermore, the expression of androgen receptors is higher in intra-abdominal preadipocytes compared to other fat depots (36). Thus the high androgen levels present in PCOS women can have a direct effect on abdominal fat tissue, leading to abdominal adiposity. Furthermore, in vitro experiments using adipocytes from healthy premenopausal women revealed impaired insulin-mediated glucose uptake when adipocytes were exposed to testosterone (37). The link between obesity and hyperandrogenism can also partly be explained by hyperinsulinism, which is often present in PCOS. Although other metabolic tissues become insulin resistant in PCOS, the theca cells of the ovary partly remain insulin sensitive (38). As a result, high insulin levels stimulate the theca cells of the ovary to produce an-

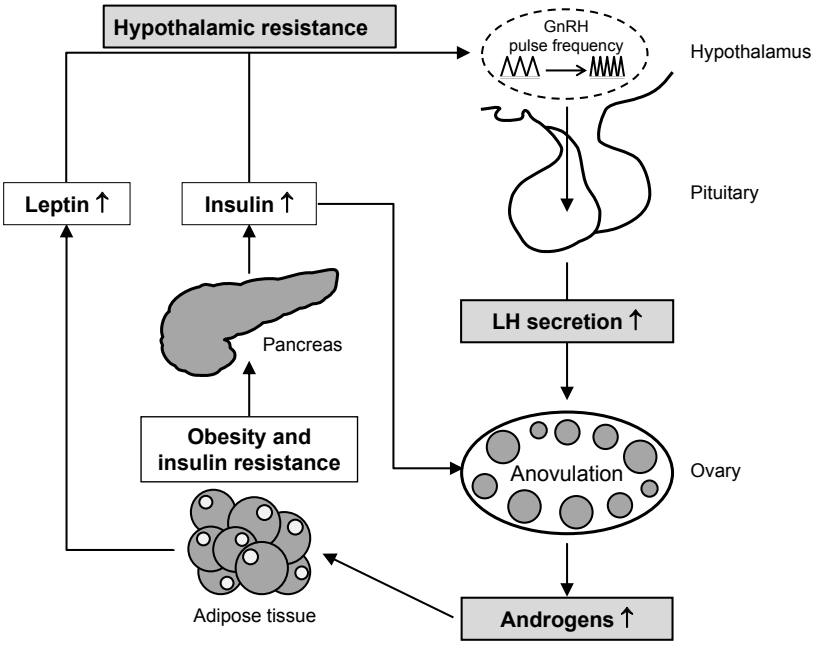

FIGURE 2. Schematic model of the pathophysiology in PCOS. GnRH pulse frequency is often increased in women with PCOS, leading to an increased LH secretion. LH stimulation of the ovary results in increased androgen production, which in turn has an effect on adipose tissue, causing obesity, insulin resistance and high leptin levels. Insulin resistance in turn leads to compensatory high insulin levels. These high insulin levels cause further elevation of androgen levels, since insulin in synergy with LH stimulates the theca cells in the ovary. High insulin and leptin levels can lead to hypothalamic resistance. Hyperandrogenism and hyperinsulinemia thereby create a vicious circle in PCOS.
Adapted from Escobar-Morreale, Trends in endocrinology and metabolism 18(7): 266-72, 2007 & Franks, Fertility and Sterility 97(1): 2-6, 2012.

drogens in synergy with LH (39). Furthermore, insulin inhibits the expression of Sex Hormone Binding Globulin (SHBG), a protein produced by the liver that binds sex steroid hormones. When SHBG levels decrease, more unbound or free testosterone is present in the circulation, which further contributes to hyperandrogenism. Also, high insulin levels can lead to an arrest of follicular development in ovaries of PCOS women (40). As a consequence, a detrimental vicious circle between high insulin levels, hyperandrogenism, abdominal adiposity and the ovaries is established, leading to a worsening of the PCOS phenotype (Figure 2), possibly explaining the beneficial effect of an insulin-sensitizing drug such as metformin. Weight loss in combination with metformin not only induces a decrease in serum insulin levels, but also of testosterone levels and an increase in serum SHBG levels and as a result significantly normalizes the menstrual cycle (30, 32).

1.3 ANIMAL MODELS FOR PCOS

So far the etiology of PCOS is still unknown because PCOS is a complex genetic disease (41-42) and has a heterogeneous presentation as discussed above. Therefore, animal models of PCOS may help to better understand the development of PCOS-related pathologies. Furthermore, they may contribute to our knowledge about the long term health consequences of PCOS. An ideal animal model should replicate most common clinical features of PCOS, i.e. the reproductive and metabolic abnormalities. Naturally occurring animal models for PCOS are unknown. However, in the past decade several animal models of PCOS have been developed. Most of these models are based on hyperandrogenism induced pre- or postnatally or during adulthood, because it is generally agreed that elevated androgens are the main culprit of PCOS. Although hyperinsulinemia is known to be of importance in the etiology of PCOS, previous studies in rats showed that experimentally high insulin levels are not sufficient to induce PCOS (43). Possibly, the application of insulin to induce PCOS in animal models has therefore not been further explored.

Prenatally induced hyperandrogenism in non-human primates and sheep has resulted in the most suitable animal model, displaying many of the characteristics of PCOS, such as cystic ovaries, enhanced androgen production by theca cells, increased visceral fat mass, and insulin resistance (44-48). In both sheep and non-human primate models of PCOS, androgens have most often been administered in utero, varying from early prenatal androgenization to late prenatal androgenization. In sheep androgenization during early gestation has been induced by either testosterone or dihydrotestosterone (DHT). After 60 days of in utero treatment, ovaries of the ewes contained a significantly lower proportion of primordial follicles and more growing follicles (49-50), due to an increased follicular recruitment. Androgenization during mid gestation did not alter ovarian morphology. However, there was evidence for an enhanced androgen production by theca cells, proving that a thecal defect can be induced during fetal life (46). Recabarren and colleagues (51) showed that sheep prenatally treated with testosterone had a reduced body weight and alterations in their insulin sensitivity, but these effects disappeared in later life. In contrast, Padmanabhan *et al.* showed that insulin resistance was still evident in adult life at 22 months (48), nevertheless dosages where higher in the latter study (100 mg vs 60 mg). Other studies using the 100 mg dosage of prenatal testosterone showed that sheep were hypertensive, had hyperglycaemia and were insulin resistant (47).

In non-human primates androgenization during early gestation resulted in both the reproductive as well as the metabolic phenotype of PCOS (44) with traits like PCO, insulin resistance and abdominal adiposity. Also in non-human primates, the difference between androgenization during early and late gestation was studied. Both

groups developed a metabolic phenotype, with an impaired insulin response. However, androgenization during early gestation led to an impaired insulin response due to impaired beta-cell function, whereas androgenization during late gestation caused an impaired insulin response due to an increase in the amount of visceral fat (45). This may suggest that the timing of androgen treatment is very important in order to create a permanent metabolic phenotype later in life in both sheep and non-human primate models of PCOS.

Although prenatally androgenization of non-human primates and sheep closely resembles PCOS, these models have the disadvantage that they are quite expensive and have a relatively long reproductive lifespan and gestational cycle. Therefore, many researchers, including ourselves, use rodents as a model of PCOS, since rodents have the advantage of being affordable, having a shorter reproductive lifespan, easier handling, and having stable genetic backgrounds. Particularly, the use of mice provides the possibility of genetic manipulation and availability of various transgenic lines already generated.

1.3.1 *Induced hyperandrogenism in mouse models of PCOS*

The excess of androgens is considered the main cause of PCOS. Therefore, not surprisingly, most PCOS animal models have been induced with androgens. Most commonly used androgens in mouse models of PCOS are testosterone, DHEA and DHT. However, also estrogen treatment has been applied. The timing of androgen exposure varies widely, starting as early as prenatal exposure. But also neonatal, prepubertal and adult androgenization of mice has been applied. Since clinical symptoms of PCOS often start during puberty (52), treatment of mice before adulthood will likely more closely resemble PCOS in human.

Dihydrotestosterone

Treatment with DHT is the most frequently used approach to induce a PCOS-like phenotype in rodents. This can be explained by the fact that DHT is a non-aromatizable androgen and therefore, in contrast to testosterone, is not converted into estradiol. Therefore, results obtained with DHT treatment can be fully attributed to DHT. However, data show that DHT can be metabolized to 5α-androstane-3β,17β-diol, which can bind to the estrogen receptor β (53). Nevertheless, DHT has a higher affinity for the androgen receptor than testosterone, making it a more potent androgen.

Prenatal treatment of female mice with 250 μg DHT per day on days 16-18 of pregnancy resulted in reproductive abnormalities in their female offspring (54-57). At an adult age, these prenatally DHT-exposed mice had a disrupted estrous cycle, and their ovaries contained fewer corpora lutea and an increased number of small antral

follicles. Follicles had a thinner granulosa cell layer and a slightly thickened theca cell layer (54, 57). However, the formation of cyst-like follicles was not reported. Serum LH and testosterone levels were elevated compared to vehicle-treated mice (57). Interestingly, in this neonatally-induced model of PCOS, the authors investigated the effect of androgens on GnRH pulsatility. In PCOS, GnRH pulse frequency is increased, which has prompted researchers to suggest that androgens may reprogram the steroidal feedback on GnRH neurons, resulting in GnRH neuron hyperactivity (58). Using transgenic mice with GFP targeted to GnRH neurons, allowing easy identification of these neurons, it was shown that prenatal DHT treatment resulted in increased GABAergic drive to GnRH neurons and increased GnRH neuron activity (55, 57). Importantly, treatment of these DHT-exposed mice with metformin, an insulin sensitizing agent frequently used to treat women with PCOS, during adulthood restored GnRH firing activity (55). These results suggest that metformin may have beneficial effects in PCOS at multiple sites. Prenatal DHT-treatment also induces several metabolic disturbances. Although the body weight and percentage fat mass did not differ between 5-month-old prenatally androgenized and control mice, visceral adipocytes of DHT-treated mice were increased in size. Serum adipokine levels were not different between treated and control mice. Nevertheless, prenatally DHT-treated mice had increased fasting glucose levels and reduced glucose tolerance, which may result from an impaired pancreatic β cell function or peripheral insulin resistance (56).

In contrast to human, formation of individual murine primordial follicles and initiation of follicular growth occurs after birth (59). Studies have shown that single oocyte formation is regulated by sex steroids (60). Hence we reasoned that androgen exposure either prenatally or neonatally might disrupt this process. Therefore, in our studies, we have chosen to develop a mouse model of PCOS, in which DHT treatment is initiated during puberty when all stages of follicle growth have been initiated. The results of this study are described in chapter 2 of this thesis.

Testosterone

Although testosterone has often been used in rat models of PCOS (61-64), it has been less frequently administered to mice. In mice, only models in which testosterone was administered neonatally have been described. Treatment of female mice with 100 μg testosterone or testosterone propionate during the first three days of life resulted in anovulation and polyfollicular ovaries (65). However, the ovarian and metabolic phenotype of this model was not studied in detail, since the focus was on the behavioral effects of steroids. A study in which a single injection of a very high dose of testosterone propionate (1 mg) was given to 5-day-old female mice reported the presence of cyst-like follicles and the absence of corpora lutea at 9 weeks of age (66). Furthermore, in vitro culture of tertiary follicles showed that follicles from

these testosterone-treated mice failed to show a dose-dependent increase in follicular diameter in response to hCG, whereas progesterone production was significantly increased (67). These results suggest that neonatal testosterone treatment of mice induces changes in follicular function later in life.

Dihydroepiandrosterone

DHEA, an androgen of mainly adrenal origin, is often increased in women with PCOS (68). Therefore, not surprisingly, DHEA has been used to induce PCOS in different rodent models. Interestingly, prenatal DHEA androgenization of mice has not been reported, probably because prenatal DHEA treatment during early pregnancy (up to day 7 of pregnancy) causes embryonic resorption (69). Whether prenatal administration of lower dosages of DHEA or administration at a later stage of pregnancy would prevent embryonic resorption and could induce PCOS has not been studied. Therefore, in most studies, prepubertal treatment with DHEA was applied. In the described studies, DHEA treatment was initiated during puberty. Motta and colleagues have treated 25-day-old mice for 20 days with daily injections of 6 mg/100 g body weight DHEA (70-72). Treated mice are infertile and their ovaries contained more atretic follicles and follicular cysts, although not more than two per ovary. These cysts had a thin theca layer and a compact layer of granulosa cells. Serum estradiol and progesterone levels were increased by at least 3-fold (71, 73). Subsequent studies showed that these DHEA-treated mice had a normal body weight and normal fasting glucose levels; however, fasting insulin levels were increased (71). Treatment with a 10-fold lower concentration of DHEA did induce an increase in body weight, and also increased fasting insulin and glucose levels (72). This suggests that the dosage of DHEA used may differentially affect metabolic parameters. These studies also showed that DHEA treatment resulted in an increased number of T lymphocytes infiltrating the ovary (71-72). Although this observation requires further analysis, it may suggest that an altered immunoregulatory response may contribute to the ovarian pathology in PCOS. A possible role for the immune system in PCOS has been implicated by human studies in which altered T lymphocyte and leukocyte profiles were observed in theca cells and in follicular fluid of women with PCOS (74). Combined treatment of DHEA and the insulin sensitizing drug metformin prevented the development of endocrine and immune phenotypes (71), although it would be more interesting to study whether metformin can alleviate the phenotypes after being induced.

In conclusion, postnatal treatment of mice with DHEA induced some of the phenotypes of PCOS, such as anovulation and follicular cysts. However, whereas in women with PCOS cysts have a thickened theca cell layer, cysts from DHEA-treated ovaries have a thin layer. This suggests that DHEA treatment may not be an ideal approach to mimic PCOS.

Estrogens

Daily subcutaneous injections of 20 μg estradiol in neonatal mice at 5-7 days of age have been applied to develop a PCOS model (75). Analysis of the ovaries of 100-day old mice revealed that this short term estradiol treatment resulted in the presence of follicular cysts and a complete lack of corpora lutea (75). Interestingly, thymectomy prior to estradiol injection prevented the formation of cystic follicles and restored ovulation in nearly 50% of the animals, while in the other 50% of the animals the ovaries became dysgenic. This might indeed indicate that an altered immunoregulatory balance may contribute to the ovarian pathology, as was observed upon DHEA treatment of mice. Estrogens may have a stimulatory role in autoimmune disease, which is mediated through altered function of the thymus (76). The presence of autoantibodies in serum of women with PCOS suggests that autoimmune processes may contribute to PCOS (77). Although the results of Chapman *et al.* (75) suggest that an altered thymus function may play a role in ovarian cyst formation, further studies are necessary to confirm whether this is also true in women with PCOS. Particularly, since elevated estrogen levels are not a hallmark of PCOS. Therefore, an estrogen-induced mouse model of PCOS may also not be an ideal model to study PCOS.

1.3.2 *Transgenic mouse models of PCOS*

To date, several genetically modified mouse models have been described that exhibit features of the PCOS phenotype. Most of these models have been linked with PCOS because their ovaries had a polycystic appearance. In some of the described models these cysts had a hemorrhagic appearance, different from the follicles found in PCOS women. Particularly in various transgenic mice with chronically elevated gonadotropin levels, hemorrhagic cyst formation appears to be common. Besides cyst formation, ovaries of mice overexpressing human chorionic gonadotropin (hCG) or the LHβ subunit display a thickened theca cell layer and luteinization of stroma cells (78-80). Indeed, enhanced ovarian steroidogenesis is observed in these mice, with increased hCG/LH action, resulting in elevated testosterone levels, but also in elevated estradiol levels. Elevated circulating LH levels are also observed in mice lacking estrogen receptor α (*Esr1*) or aromatase (*Cyp19*), due to a lack of negative feedback by oestradiol on the hypothalamic-pituitary-gonadal axis (81-82). The ovarian phenotype of both *Esr1* and *Cyp19* knockout mice displays strong similarities with the hCG/LHβ overexpressing mice, including the presence of hemorrhagic cysts. Combined, these transgenic mice clearly indicate a crucial role for LH action in follicular growth beyond the antral stage. This is in agreement with the phenotype observed in women with PCOS, where the increased serum LH/FSH ratio resulting from the increased GnRH pulsatility has been suggested to result in follicular arrest. However, in the

transgenic mice with increased hCG/LH action additional pathological phenotypes were observed, which included pituitary adenomas, mammary gland tumors in mice at older ages, or even ovarian teratomas (80, 83). Depending on the promoter used to chronically overexpress hCG or LHβ, serum levels of hCG and LH were 5-fold to even 1000-fold increased (78, 80, 83). In contrast, women with PCOS only show a 3-fold increase in LH levels, which is observed in about 60% of the patients (7). Thus, the extent of LH elevation may explain the difference in phenotype observed in the transgenic mice and women with PCOS. Interestingly, female hCG overexpressing mice, that carry the transgene in low copy numbers, do not develop any ovarian histological abnormalities, although they do become infertile by 6-7 months (78). Thus, elevated LH action alone may not be the primary cause of PCOS. LHβ overexpressing mice become obese and are hyperphagic. The significantly increased white adipose tissue mass is reflected by increased leptin levels. Furthermore, insulin levels are increased but insulin tolerance appears to be normal (84). The authors concluded that the elevated androgens are responsible for the obese phenotype, however a contribution of adrenal steroids could not be ruled out since these mice also have high corticosterone levels (84). Also in women with PCOS, an adrenal contribution to the phenotype has been suggested (85). Thus, LHβ overexpressing mice may be an interesting model to gain insight into the metabolic alterations in PCOS.

Another model that may provide insight into the etiology of PCOS are transgenic mice overexpressing human plasminogen activator inhibitor-1 (PAI-1) under the control of murine preproendothelin 1 promoter, which targets gene expression to vasculature and epithelial surfaces (86). These mice fail to ovulate, and more than half of the animals develop ovarian cysts. In addition, overexpression of PAI-1 resulted in a hypertrophied theca cell layer and in a nearly two-fold increase in testosterone levels (86). PAI-1 is a member of the serine protease inhibitors (serpin) family and has been implicated in various diseases, including diabetes (87-88). Furthermore, PAI-1 levels have been proposed as a marker for metabolic and cardiovascular risk (87, 89). In women with PCOS increased levels of PAI-1 have been reported, although this may not be independent of the obesity status (90-92). Since PAI-1 plays a role in vascular integrity, PAI-1 may be one of the factors involved in the vascular endothelial dysfunction in PCOS, in addition to its role in the ovary. Interestingly, fibrillin-3, another factor involved in vascular matrix remodeling, and extracellular matrix (EMC) remodeling in general, has been implicated in the etiology of PCOS through genetic studies (93). This may suggest that changes in ECM elasticity may be involved in various aspects of PCOS.

Ovarian nerve growth factor (NGF) has also been implicated to play a role in the ovarian pathology of PCOS. It has been proposed that many of the features of PCOS are the result of sympathetic overactivity (94). The neurotropin NGF is a marker of

sympathetic nerve activity. Indeed, increased levels of NGF were measured in follicular fluid and granulosa cell cultures of PCOS women (95). In mice, overexpression of NGF under the control of the *Cyp17* promoter, which leads to overexpression in androgen producing cells, causes follicular arrest at the antral stage, leading to an accumulation of small growing follicles. NGF overexpressing mice have normal LH levels, and ovaries of these mice do not contain follicular cysts. However, ovaries of NGF-overexpressing mice are hyperresponsive to gonadotropins. A challenge with a single dose of pregnant mare serum gonadotropin significantly increased the steroidal output, and treatment with a low dose of hCG for seven days led to significantly more cysts in immature NGF-overexpressing mice than in wild type animals (95). These findings suggest that increased innervation of the ovary, as observed in the NGF transgenic mice, may contribute to the ovarian and possibly metabolic phenotype in PCOS. Whether NGF is a causative factor in PCOS remains to be determined. Since increased ovarian expression of NGF is also observed in the estradiol valerate-induced rat PCOS model (96), changes in NGF expression may be a secondary effect. However, a role for NGF in follicular arrest is evident because immunoneutralization of NGF partially restored follicular growth in this rat model (96).

In PCOS, both hyperandrogenism and hyperinsulinemia contribute to the ovarian and metabolic phenotypes. A role for the metabolic pathway in the development of the ovarian phenotype in PCOS was elegantly shown in mice lacking leptin receptors and insulin receptors selectively in pro-opiomelanocortin (POMC) neurons of the hypothalamus (IR/LepR^{POMC}) (97). These mice were generated to study the hypothalamic contribution of leptin and insulin signaling in glucose homeostasis. As may have been expected, these mice develop a clear metabolic phenotype, with increased basal insulin levels, glucose intolerance, and insulin resistance. At 6 months of age, these IR/LepR^{POMC} mice had an increased body weight, reflected by increased white adipose tissue mass with hypertrophied adipocytes (98). Although the penetrance was incomplete, in mice older than 4 months 45% of the animals were anovulatory and histological analysis of their ovaries revealed the presence of occasional cysts (97-98). LH levels were increased by two-fold, and also testosterone levels were significantly increased (97). Previous studies have already shown that both leptin and insulin signaling are important for reproduction by regulating GnRH release. Indeed, POMC neurons directly project to GnRH neurons (reviewed in (99)). Furthermore, it was shown that in obese patients resistance of leptin and insulin can develop (100). The results from the IR/LepR^{POMC} mice suggest that hypothalamic resistance to leptin and insulin may contribute to the multiple phenotypes of PCOS.

Animal models may be useful to study the association between the reproductive and metabolic phenotype observed in PCOS, and to gain more insight into the pathophysiology of this syndrome. However, PCOS is a heterogeneous disorder

making it difficult to generate a model that reflects all phenotypes. Indeed, the various mouse models reflect the PCOS phenotypes only to a certain degree. The current models also reveal that PCOS can be induced at various levels, as schematically shown by the light grey boxes in Figure 2. This may suggest that several etiologies are involved in PCOS. Development of both reproductive and metabolic disturbances is predominantly seen upon androgen treatment. However, it is unknown whether besides androgens other factors secreted by the increased follicle pool contribute to the reproductive and metabolic phenotypes of PCOS.

1.4 TRANSFORMING GROWTH FACTOR β SUPER FAMILY

The Transforming Growth factor β (TGFβ) superfamily plays an important role in the ovary, regulating various stages of folliculogenesis (101) and, therefore, will be discussed in more detail. TGFβ family members signal through a receptor complex consisting of type I and type II serine/threonine kinase receptors. Upon ligand binding, the type II receptor activates the type I receptor through phosphorylation, which in turn phosphorylates and activates the downstream Smad proteins. Based upon the downstream Smad family members involved, two main signaling pathways can be identified: the TGFβ/activin-like signaling pathway, signaling through Smad2 and -3; and the BMP-like signaling pathway, signaling through Smad1, -5 and -8 (102).

1.4.2 *Bone Morphogenetic Proteins*

The Bone Morphogenetic Proteins (BMPs) and Growth Differentiation Factors (GDFs) form the largest group within the TGFβ family. In the ovary, GDF9 and BMP15, also known as GDF9B, are specifically expressed by oocytes of all follicular stages (103-107) and inhibit FSH receptor expression and stimulate granulosa cell mitosis (108). Furthermore, GDF9 and BMP15 are fundamental for the activation of primordial follicles and subsequently participate in all stages of follicular development (109). In sheep, naturally occurring inactivating BMP15 mutations have been identified which demonstrated a role for BMP15 in the regulation of ovulation rate. Sheep heterozygous for the BMP15 mutation have an increased ovulation rate, while sheep with a homozygous mutation are infertile, due to the absence of follicular development, in spite of the apparently normal activation of the oocyte and expression of a number of oocytes specific genes (110). In addition, likewise to the BMP15 mutation, sheep which carry a heterozygous mutation in GDF9 have increased ovulation rates and sheep with a homozygous mutation are infertile due to streak ovaries (111).

BMP6 has an oocyte/granulosa cells expression pattern in various species and inhibits FSH-induced progesterone synthesis by granulosa cells (112). BMP2 and BMP5 are expressed by granulosa cells and BMP4 and BMP7 are expressed by theca cells in rats. The first report of the existence of a functional BMP system in the ovary showed that BMP4 and BMP7 regulate FSH-induced estradiol and progesterone production in granulosa cells (113). Rats injected with BMP7 had increased numbers of primary, preantral, and antral follicles but significantly fewer ovulated oocytes compared to control rats (114). Furthermore, it was found that BMP7 is involved in the selection of bovine follicles (115). Thus, BMP7 promotes the recruitment of primordial follicles into the growing follicle pool, while inhibiting ovulation and progesterone production. It has also been reported that BMP2 and BMP4 regulate FSH-induced steroidogenesis by stimulating estradiol production and reducing progesterone synthesis (116).

Little is known about the expression of BMPs in ovaries of PCOS women. Interestingly, BMP6 expression levels in granulosa cells of women with PCOS are higher compared with granulosa cells from normal healthy women, possibly adding to the FSH insensitivity of PCOS follicles (117). Furthermore, decreased GDF9 expression has been observed in developing PCOS oocytes compared to normal oocytes (118). These findings indicate that TGFβ superfamily members may contribute to an altered intra-ovarian environment in PCOS, however, whether they play a role in the pathophysiology of PCOS, has not been investigated in detail.

1.4.3 *Anti-Müllerian Hormone*

Anti-Müllerian hormone (AMH), or Müllerian inhibiting substance (MIS), has long been known for its involvement in the sexual differentiation of the male embryo. AMH is secreted by the Sertoli cells of the fetal testis and induces regression of the Müllerian duct, the anlagen of the female reproductive tract (119-120). Although initially not expressed in the ovary, ovarian AMH expression starts after birth in mice and from the 36^{th} week of gestation onwards in human (121-122). In the human ovary, AMH is expressed in granulosa cells as soon as follicles are recruited from the primordial follicle pool. Expression is highest in follicles smaller than 4mm (preantral and small antral follicles) and is nearly lost in follicles larger than 8mm (123). Also in rodents, maximum expression is reached during the preantral and small antral stage. AMH expression decreases once FSH-dependent follicular growth has been initiated (121). Thus, AMH is expressed by those growing follicles in between initial recruitment and cyclic selection (Figure 3). The similarity of expression pattern in human and mice suggests that AMH may have a comparable role in both species.

AMH, signaling through its specific type II receptor (AMHRII), was shown to activate a BMP-like pathway (reviewed in (124)). Furthermore, in studies focusing

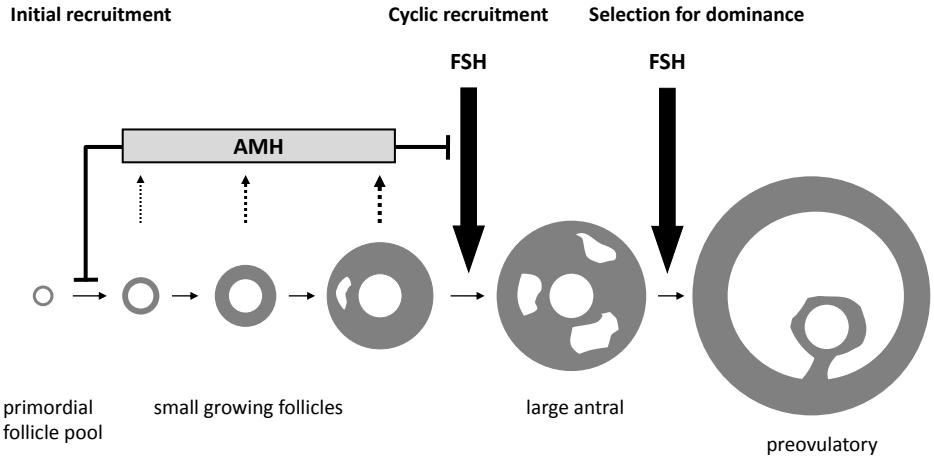

FIGURE 3. Follicular development and the role of AMH. During folliculogenesis, follicles progress through various developmental stages. AMH is expressed by the small growing follicles and has an inhibitory effect on primordial follicle recruitment and FSH sensitivity.
Modified from Visser et al, Reproduction 131(1): 1-9, 2006.

initially on Müllerian duct regression, the BMP type I receptors ALK2 and ALK3 were identified as AMH type I receptors. Antisense ALK2 treatment of urogenital ridge organ cultures resulted in inhibition of AMH-induced Müllerian duct regression (125). Targeted disruption of ALK3 signaling in the Müllerian duct also abolished regression (126). Interestingly, this phenotype could be rescued in the presence of increased AMH levels (127), suggesting the involvement of an additional type I receptor, such as ALK2. Finally, based on the AMH-induced interaction between AMHRII and ALK6, also ALK6 has been implicated as an AMH type I receptor (128). In rats, the AMHRII has a similar expression pattern to AMH (129). The three AMH/BMP type I receptors are also expressed in the ovary (130).

Insight into the role of AMH in folliculogenesis came from studies performed in the AMH knockout (AMHKO) mice. In these studies, the complete follicle population was determined in AMHKO and wild-type mice. At 4 months of age, ovaries of AMHKO mice contained more growing follicles and less primordial follicles compared to wild-type mice. The increased recruitment in the absence of AMH resulted in a much faster depletion of the primordial follicle pool, evident at 13 months of age when ovaries of AMHKO mice were nearly devoid of primordial follicles whereas wild-type ovaries still contained primordial follicles (131). These results strongly suggested that AMH has an inhibitory role in primordial follicle pool recruitment. This conclusion was confirmed in a neonatal ovary culture system in which ovaries of 2-day-old mice were cultured in the presence or absence of AMH. In the presence of AMH, cultured ovaries contained 40-50% less growing follicles compared with control ovaries (132).

Similarly, grafting of mouse neonatal ovaries beneath the chorioallantoic membrane of chick embryos, which results in exposure to high levels of AMH secreted by the chick gonads, suppressed primordial follicle recruitment. Interestingly, follicle recruitment was not suppressed when neonatal ovaries of AMHRII null mice were grafted (133). Similar results were obtained using bovine ovarian cortical strips. In vitro culture of cortical strips in the presence of AMH inhibited follicle recruitment, whereas recruitment occurred in bovine ovarian cortex grafted in gonadoectomized chicks (133). Likewise, treatment of human ovarian cortical strips with AMH for 7 days in vitro suppressed the initiation of primordial follicle growth (134). In contrast, Schmidt *et al.* (135) reported that follicle growth was more advanced in the presence of AMH in cryopreserved human ovarian cortical tissue cultured for 4 weeks. The difference in material (fresh vs. frozen-thawed) and the duration of culture (7 days vs. 4 weeks) perhaps explains these conflicting results.

The specific window of AMH expression, i.e. in between the two major regulatory steps of folliculogenesis, suggests that in addition to primordial follicle recruitment, AMH may also regulate FSH-dependent cyclic selection. The increase in FSH during each cycle results in the selection of a limited number of follicles from the cohort of small growing follicles. For this cyclic selection of follicles, FSH levels need to rise to a certain threshold concentration to prevent follicles becoming atretic (2). Studies in mice and human suggest that AMH is one of the intra-ovarian growth factors contributing to the establishment of this threshold. In the AMHKO mice, more growing follicles were observed despite lower FSH levels (131). Detailed analysis throughout the estrous cycle revealed that the FSH surge was blunted in AMHKO mice. Nevertheless, the FSH-dependent selection of small antral follicles was more pronounced in the absence of AMH. In addition, recruitment of large preantral follicles was observed in AMHKO mice at estrous, whereas in wild-type mice these follicles are not sensitive to FSH (136). Thus, in the absence of AMH follicles display an enhanced and premature sensitivity to FSH.

In mice serum AMH levels declined gradually with increasing age, whereas the expression of AMH in individual growing follicles did not change. This decline in serum AMH correlated strongly with the decreasing number of growing follicles, and more importantly, with the decline in number of primordial follicles (137). Similarly, in adult women serum AMH levels declined with increasing age to undetectable levels after menopause (138-139). AMH levels correlated strongly with antral follicle count (AFC), but also with other markers for ovarian aging such as FSH and inhibin B on cycle day 3 (138). Interestingly, AMH levels remained relatively stable during the menstrual cycle and also do not vary significantly between cycles (140-141), suggesting that AMH expression is not regulated by gonadotropins. This may explain why AMH is considered one of the earliest markers for ovarian reserve. In young

normo-ovulatory women AMH levels decreased over a 3-years interval, whereas serum levels of FSH and inhibin B and AFC did not change. In agreement, analysis of normal women at an on average 4-years interval revealed that of these markers, serum AMH level is the best predictor for the occurrence of menopausal transition (142). Furthermore, in a study by van Disseldorp *et al.* (143), in which AMH levels of a cohort of normal women were related to the observed age at menopause distribution of a prospective cohort, it was observed that AMH is a much better marker to predict a woman's reproductive age than chronological age.

1.5 ANTI-MÜLLERIAN HORMONE AND PCOS

Besides the effect of androgens on the etiology of PCOS, a role of ovarian growth factors, such as AMH and BMPs, on the pathophysiology of PCOS cannot be ruled out.

As mentioned above, serum AMH levels correlate with AFC. Several studies showed that serum AMH levels are 2-3 fold increased in PCOS women, and correlate with the increased follicle number (144-147). In addition, serum AMH levels are positively correlated with androgen levels. Increased AMH levels were also observed in follicular fluid of PCOS women (144, 148). This suggests that not only the increased number of follicles contribute to the elevated serum AMH levels, but also that the production per granulosa cell is increased. Indeed, Pellatt *et al.* (148) observed that AMH expression in granulosa cells of PCOS patients was nearly 75-fold higher than in those of control women. Interestingly, AMH levels are highest in those PCOS patients with the more severe phenotype. PCOS women with PCO had higher AMH levels than those without (146). Likewise, subdividing PCOS women in ovulatory and anovulatory women showed that anovulatory PCOS women had increased AMH levels (149). Women with both PCO and hyperandrogenism had higher AMH levels than women with PCO only, although the number of small antral follicles was not different between the two groups (150). A relationship with insulin resistance is less clear. A lack of correlation between serum AMH levels and insulin levels or BMI has been reported (146-147), whereas other studies did observe a positive correlation between the Homeostasis Model Assessment (HOMA) index and higher AMH levels in insulin-resistant PCOS women than in PCOS women with normal insulin sensitivity (151-152). Nevertheless, treatment with insulin lowering drugs such as metformin, only weakly lowers AMH levels and only after a prolonged treatment period (153-154), suggesting that insulin does not directly regulate AMH production.

1.6 AIM AND SCOPE OF THIS THESIS

In PCOS, androgens are seen as the main culprit of the syndrome, exerting an effect on both the ovary as well as on the metabolic tissues. Besides androgens, other growth factors are also secreted by the ovary. We postulated that ovarian growth factors contribute to the PCOS phenotype, especially since there are ovarian growth factors which are increased in PCOS, like AMH. In this thesis, we describe studies on the role of ovarian growth factors in the regulation of metabolism in PCOS. In chapter 2 of this thesis we describe the development of a mouse model of PCOS, which exhibited both the ovarian as well as the metabolic characteristics seen in PCOS women in order to learn more about the pathophysiology of PCOS. In chapter 3 we examined the contribution of ovarian growth factors on the metabolism in PCOS by removing the ovaries of PCOS mice. In chapter 4 we studied the contribution of an increase in ovarian growth factors on the metabolic phenotype, using anti-Müllerian hormone knock out (AMHKO) and anti-Müllerian hormone type II receptor knock out (MRKI) mice as models of an increased number of growing follicles. In chapter 5 we investigated whether serum BMP levels could be used as a marker of ovarian function in women with PCOS. In chapter 6 we examined the relationship of serum AMH with the metabolic syndrome in a large cohort of women with PCOS. Finally, in the general discussion, the findings of this thesis are discussed in a general perspective and some directions for future research are given.

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Chapter 2

Reproductive and metabolic phenotype of a mouse model of PCOS

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ABSTRACT:

Polycystic ovary syndrome (PCOS), the most common endocrine disorder in women in their reproductive age, is characterized by both reproductive and metabolic features. Recent studies in human, nonhuman primates and sheep suggest that hyperandrogenism plays an important role in the development of PCOS. We investigated whether chronic DHT exposure in mice reproduces both features of PCOS. Such a model would allow us to study the mechanism of association between the reproductive and metabolic features in transgenic mice.

In this study, prepubertal female mice received a 90 days continuous release pellet containing the non-aromatizable androgen dihydrotestosterone (DHT) or vehicle.

At the end of the treatment period, DHT-treated mice were in continuous anestrous, their ovaries contained an increased number of atretic follicles, with the majority of atretic antral follicles having a cyst-like structure. Chronic DHT-exposed mice had significantly higher body weights (21%) than vehicle-treated mice. In addition, fat depots of DHT-treated mice displayed an increased number of enlarged adipocytes ($P<0.003$). Leptin levels were elevated ($P<0.013$), adiponectin levels were diminished ($P<0.001$), and DHT-treated mice were glucose intolerant ($P<0.001$).

In conclusion, a mouse model of PCOS has been developed showing reproductive and metabolic characteristics associated with PCOS in women.

INTRODUCTION

PCOS is the most common endocrine disorder in women in their reproductive age and affects about 6-8% of women worldwide (1). Based on the Rotterdam criteria, PCOS is diagnosed by the presence of at least two of the following three criteria: clinical or biochemical hyperandrogenism, oligo- or amenorrhea, and the presence of polycystic ovaries (PCO) by ultrasound (2).

Although PCOS is a complex and multifactorial disorder, the failure in dominant follicle selection leading to an accumulation of small antral follicles suggests an altered FSH sensitivity in PCOS ovaries. Furthermore it is generally agreed that elevated androgens are the main culprit of the syndrome. Indeed, prenatal exposure of female rhesus monkeys to testosterone induces PCOS (3). Similarly, in sheep in utero exposure to testosterone resulted in increased LH secretion and cycle disturbances, resembling PCOS (4). Clinical evidence of the effects of early androgen exposure was observed in daughters of PCOS women. These girls are at high-risk for reproductive and metabolic abnormalities. The percentage of girls with increased ovarian volume and elevated androgen and insulin levels was significantly higher in Tanner IV and V stages for breast development for daughters of PCOS women compared to daughters of normo-ovulatory women (5). In addition to the ovarian phenotype, PCOS is also associated with a metabolic phenotype. A large proportion of PCOS women are obese (38-88%, depending on the study (5-6)), with predominantly abdominal adiposity, which is considered a risk factor for metabolic disease (7-8). Indeed, 50-70% of women with PCOS are insulin resistant resembling type 2 Diabetes Mellitus (8-10). Interestingly, the presence of hyperinsulinemia in women with PCOS appears to be independent of obesity, although only obese PCOS women are at risk of becoming glucose intolerant. A role for metabolic features in the pathogenesis and/or severity of PCOS is emphasized by studies showing that weight loss in combination with an insulin-sensitizing drug such as metformin not only induces a decrease in serum insulin but also in testosterone and significantly normalizes the menstrual cycle (9). The beneficial effect of lowered insulin levels on ovarian function is explained by the synergistic effect of insulin with LH on theca cells to produce androgens (10). In addition, hyperinsulinemia in the ovary can directly lead to an arrest of follicular development in women (11), which improves after metformin treatment. Consequently, the interplay between high insulin levels, hyperandrogenism, and abdominal adiposity may lead to an exaggerated PCOS phenotype (12-13).

Since naturally occurring animal models for PCOS are not known, several models have been generated. Prenatal or neonatal androgen exposure or treatment with an aromatase inhibitor was most often used to induce a polycystic phenotype (14-16).

In most of these studies the emphasis has been on the ovarian phenotype rather than the metabolic consequences of androgen treatment, although lately also the metabolic phenotype is receiving increased attention. Recently, a rat model has been described in which prepuburtal administration of dihydrotestosterone (DHT), the active metabolite of testosterone, induced both the ovarian and metabolic phenotype of PCOS (17). Besides acyclicity and the presence of cyst-like follicles, increased body weight and reduced insulin sensitivity were observed in DHT-treated rats (17). Since the use of mice has the advantage of the availability of the plethora of transgenic lines, we have used a prepubertal DHT-treatment approach to develop a mouse PCOS model.

Our data demonstrate that chronic exposure of mice to DHT for 90 days leads to acyclicity, large cyst-like follicles, and hormonal and metabolic disturbances resembling the features found in women with PCOS.

MATERIALS AND METHODS

Animals

C57BL/6J wild-type female mice were obtained from the Animal Facility of the Erasmus MC (Rotterdam, The Netherlands) and were kept under standard animal housing conditions in accordance with the National Institutes of Health guidelines for the Care and Use of Experimental Animals. The experiments were performed with permission of the local ethics committee.

At postnatal day 19, mice of comparable body weight were randomly divided over two treatment groups (DHT and control; n = 9-10 per group), and were implanted subcutaneously with a 90-day continuous DHT release pellet (Innovative Research of America, Sarasota, FL, USA). These pellets contained 2.5 mg DHT (daily dose 27.5 μg). Control mice received a placebo pellet. Mice were sacrificed at the end of the treatment period (90 days). To determine the stage of the estrous cycle, daily vaginal smears were taken ten days before the animals were sacrificed, and examined as described previously (18). Body weight was determined at the start and end of treatment. In addition, at the end of the 90-day treatment period blood samples and tissues were isolated. Blood samples were collected by orbital puncture after mice were anesthetized with isoflurane. Ovaries and uterus were isolated, weighed, and fixed overnight in Bouin's fluid. Inguinal, retroperitoneal and gonadal fat depots were isolated and fixed overnight in 4% paraformaldehyde. The inguinal fat depot is located anterior to the upper segment of the hind limbs, and represents subcutaneous adipose tissue in mice. The gonadal fat depot is located at the level of the ovaries, and

is considered to represent visceral fat. The retroperitoneal fat depot is located to the dorsal wall of the back (19). In addition, isolated tissues were snap frozen in liquid nitrogen and stored at −80°C until further processing.

Ovarian histology and follicle counting

For histological examination of ovarian morphology, one fixed ovary was embedded in paraffin. After routine histological procedures, 8 μm sections were mounted on glass slides and stained with hematoxylin and eosin. Follicle count was performed as described previously (18). In brief, based on the mean diameter of the follicle, growing follicles were divided into four classes: small preantral (20-170 μm), large preantral (171-220 μm), small antral (221-310 μm), and large antral (>311 μm). Nonatretic and atretic growing follicles were counted in every fifth section. Primordial follicles were counted in every second section. In addition, the presence of recent corpora lutea was determined.

Immunohistochemistry

Immunohistochemistry was performed as described previously (20). Briefly, sections were mounted, deparaffinized, blocked for endogenous peroxidase activity, washed with water and transferred to PBS. After antigen retrieval was performed on the sections, they were cooled down, rinsed in PBS and incubated with a biotinylated anti-Müllerian Hormone (AMH) mouse monoclonal antibody (antibody 5/6A, MCS2246; Serotec; (21)). Sections were diluted 1:100 at 4°C overnight and washed with PBS. Then the sections were incubated for 30 min at room temperature with streptavidin-biotinperoxidase complex (ABC; diluted 1:200 in PBS; Dako, Glostrup, Denmark), washed with PBS three times and the peroxidase activity was developed with 0.07% 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Finally, all sections were counterstained with hematoxylin.

Real time PCR

Total RNA was isolated per whole ovary using TriPure Isolation Reagent (Roche Applied Sciences, Almere, The Netherlands) according to the manufacturer's instructions. After DNase treatment (Promega Benelux BV, Leiden, The Netherlands), samples were reverse transcribed using a cDNA Synthesis Kit (Roche Applied Sciences, Almere, The Netherlands). Real-time PCR (RT-PCR) was performed with SYBR Green (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) using an ABI PRISM® 7900HT Sequence Detection System. The expression of the target

gene was normalized according to the average expression level of three housekeeping genes (β2-microglobulin, HPRT, and cyclophilin A) using the $2^{-\Delta\Delta Ct}$-method (22).

IPGTT

At the end of the treatment period mice were fasted overnight (16h) prior to an ip glucose tolerance test (IPGTT). Glucose levels were measured by tail vein blood sampling using a Freestyle mini glucometer and test strips (Abbot, Alameda, CA, USA). Following a baseline (fasted) measurement, mice were injected intraperitoneally with glucose (2 g/kg as a 20% glucose solution) and tail sampling was performed at 15, 30, 60, and 120 minutes. In addition, at baseline a 50 µl sample was drawn into EDTA microvettes (Sarstedt, Nümbrecht, Germany) for insulin measurements. After centrifugation at 3000 rpm, 4°C, plasma was removed and stored at −20°C.

Hormone measurements

AMH levels were measured with an in-house AMH ELISA assay (commercially available through Beckman Coulter, Woerden, The Netherlands) (20). Plasma insulin levels were determined using an ultrasensitive mouse insulin ELISA (Alpco Diagnostics, Salem, NH, USA). Total adiponectin serum levels were measured with an Adiponectin (Mouse) Total, HMW ELISA (Alpco Diagnostics). Leptin levels were measured with a Leptin (Mouse/Rat) ELISA (Alpco Diagnostics). All samples were measured in one assay. DHT levels were measured with a DHT ELISA (Diagnostics Biochem Canada Inc. Ontario, Canada). Serum LH levels were measured with a rodent LH+ ELISA (Endocrine Technologies, Newark, CA, USA).

Measurement of adipocyte size

Four mice per treatment group were randomly selected and fixed white adipose tissue was processed and analyzed. In brief, adipose depots were embedded in paraffin, and after routine histological preparation sectioned at 8 µm. Mounted sections were stained with hematoxylin and eosin. For each fat depot, micrographs were taken at 100x magnification. In three randomly selected micrographs the diameter of at least 130 adipocytes was measured using Image J software (version 2.0, http://imagejdev.org), and subsequently the number of adipocytes per similar size class with intervals of 2 µm was calculated.

Statistics

Data were statistically analyzed by Student's t-test, repeated measures ANOVA (IP-GTT, body weight), or two-way ANOVA (adipocyte size distribution) using SPSS 15.0 (SPSS Inc., Chicago, IL). Data were expressed as mean ± SEM and differences were considered significant at P<0.05.

RESULTS

DHT levels

To confirm that implantation of the DHT-releasing pellets resulted in elevated DHT levels, serum DHT was determined. Indeed, DHT levels in DHT-treated animals were significantly increased 6-fold compared to placebo-treated animals (2.82 ± 0.30 nmol/l *vs.* 0.44 ± 0.13 nmol/l, respectively, *P*<0.001).

Cyclicity and ovarian morphology

Cycle irregularity and the presence of polycystic ovaries are hallmarks of the reproductive abnormalities in PCOS. All placebo-treated mice had a regular cycle of 3-5 days and their ovaries contained fresh corpora lutea, indicative of recent ovulations. Vaginal smears of DHT-treated mice revealed that all mice were in continuous anestrous, suggesting that DHT-treated mice were acyclic. In agreement with the disrupted estrous cycle, none of the ovaries of DHT-treated mice showed signs of

TABLE 1. Hormone levels and ovarian weight in placebo- and DHT-treated mice

	Placebo	DHT
Ovarian weight (mg)	7.59 ± 0.27	8.73 ± 1.05
LH (ng/ml)	0.19 ± 0.05	0.09 ± 0.02
AMH (ng/ml)	6.01 ± 1.44	8.16 ± 1.26
Leptin (ng/ml)	2.14 ± 0.35	5.17 ± 1.09[a]
Adiponectin (µg/ml)	22.51 ± 2.28	12.75 ± 2.28[b]
Insulin (ng/ml)	0.49 ± 0.06	0.43 ± 0.07

Ovaries and serum samples were collected at the end of the 90-day treatment period. Serum samples were used for hormone measurements. For fasting insulin levels, plasma samples were collected at the start of the ip glucose tolerance test, performed at the end of the 90-day treatment period. Values represent mean ± SEM (n = 9-10 mice per group, except for LH: n = 6 mice per group). [a] *P*<0.05, [b] *P*<0.01 (placebo *vs*. DHT).Values represent mean ovarian weight of both ovaries ± SEM (n = 9-10 mice per group).

ovulations, *i.e.* corpora lutea. Serum LH levels did not differ significantly between the two groups, although LH levels tended to be decreased in the DHT-treated mice (Table 1). Particularly, in the placebo-treated mice LH levels showed a wide variation, which could be explained by sampling exactly on day 90 of the experiment, irrespective of their estrous cycle, whereas all DHT-treated mice were anestrous. Ovaries of

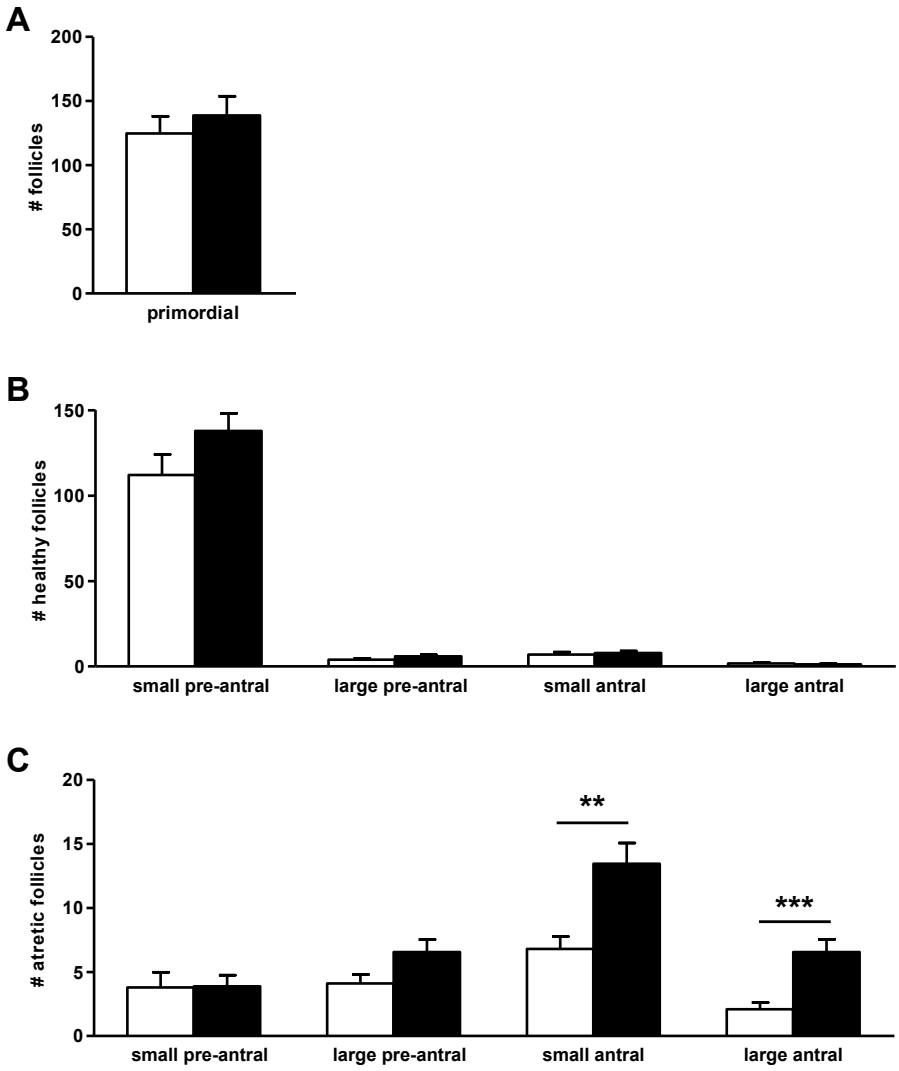

FIGURE 1. Effect of DHT-treatment on follicle numbers. Mice were treated for 90 days with vehicle (open bars) or DHT (closed bars) and ovaries were isolated at the end of the treatment period (90 days). The number of follicles in different size classes was determined per ovary. A) Number of primordial follicles; B) Number of healthy growing follicles; C) Number of atretic follicles. Data represent mean ± SEM (n = 9-10 mice), ** $P<0.01$, and *** $P<0.001$.

DHT-treated mice tended to have an increased weight, although this failed to reach significance (data not shown). Ovaries of DHT-exposed mice contained a similar number of primordial and healthy growing follicles to placebo-treated mice (Figure 1A and B). However, the number of atretic follicles, in particular the number of atretic small antral and large antral follicles was increased by 2-4 fold (Figure 1C). The majority of these atretic follicles had a cyst-like appearance, which was never observed in the placebo-treated mice (Figure 2A and D). Compared to atretic follicles in placebo-treated mice (Figure 2B), these cystic follicles were larger and had a larger fluid-filled antrum with a thin granulosa cell layer containing several detached pyknotic granulosa cells (Figure 2E). Atretic follicles in placebo-treated mice had a compact theca cell layer (Figure 2C), whereas in DHT-treated mice the theca cell layer of the cystic ovaries was dispersed and contained cells that resemble interstitial tissue, giving the theca cell layer a hyperplastic appearance. For several of these cyst-like follicles the difference between theca cell layer and stroma was not distinct (Figure 2F). Immunohistochemistry showed that DHT did not have an effect on the expression pattern of the important ovarian regulator AMH (Supplemental Fig.1). In agreement, serum AMH levels did not differ between placebo- or DHT-treated mice (Table 1). Aromatase mRNA expression was not significantly different between the two groups (Supplemental Fig. 2)

Body weight of DHT-treated mice

In addition to the ovarian morphology, we also analyzed several metabolic characteristics. At the start of treatment, there were no differences in body weight present between the placebo- and DHT-treated mice (Figure 3). However, DHT-treated mice gained significantly more weight than placebo-treated mice ($P<0.0001$). As a result, DHT-treated mice had a 21% higher body weight at the end of the treatment period, compared to the placebo treated mice ($P<0.0001$) (Figure 3).

Adipocyte size in DHT-treated mice

Although body composition or weight of the adipose depots were not analyzed, gross morphological analysis of the mice suggested that adipose depots of DHT-treated mice were enlarged. Histological analysis of retroperitoneal, inguinal, and gonadal white adipose tissue (WAT) revealed the presence of enlarged adipocytes in DHT-treated mice (Figure 4A). Detailed analysis of the adipocyte size revealed that all adipose depots of DHT-treated mice contained a significantly increased number of enlarged adipocytes, reflected by the right-sided shift in the size distribution ($P<0.001$) (Figure 4B). In agreement, in DHT-treated mice the mean adipocyte size

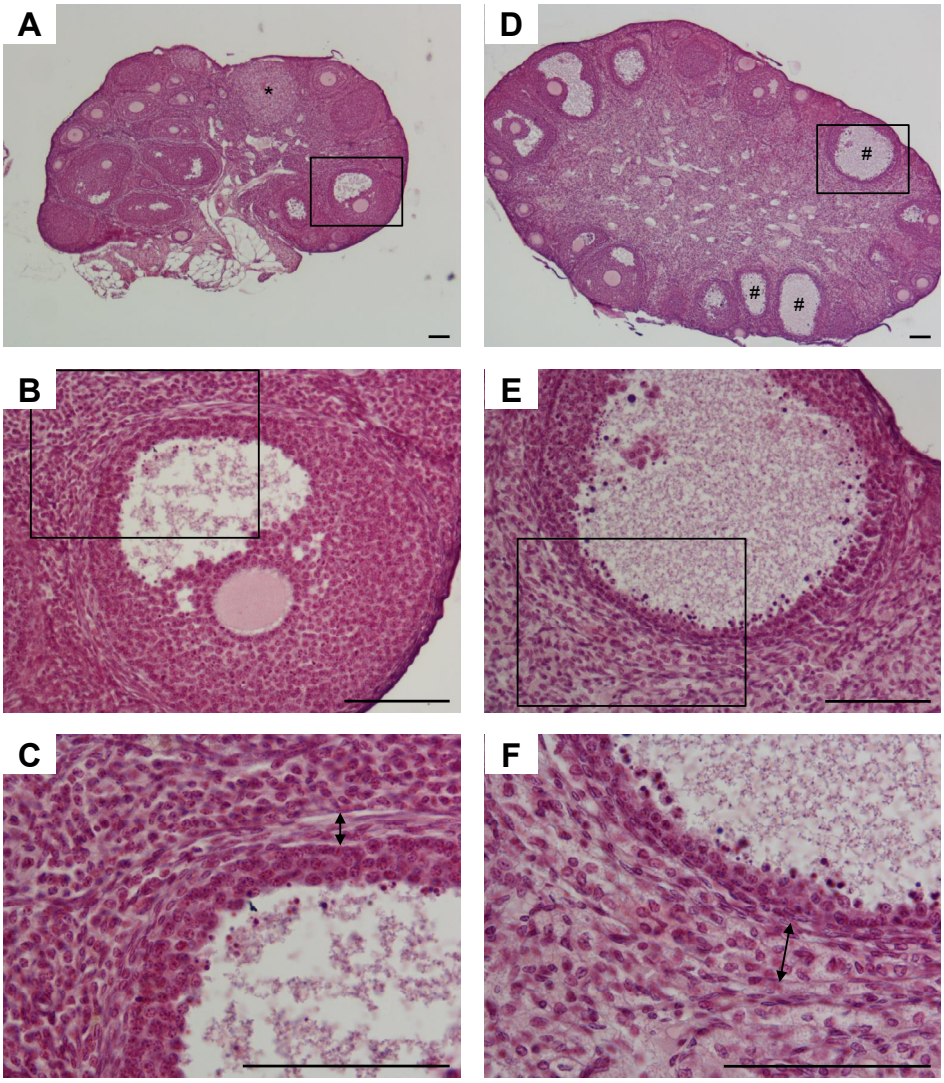

FIGURE 2. Ovarian morphology of placebo- and DHT-treated mice. Ovaries were isolated at the end of the 90-day treatment period. A) HE-stained ovarian section of a normal cycling placebo-treated mouse with healthy growing follicles and a corpora luteum (*) Magnification 40x. B) Higher magnification (200x) of the boxed area in A, showing an atretic antral follicle. C) Higher magnification (400x) of the boxed area in B, showing a compact theca cell layer (double-sided arrow). D) HE-stained ovarian section of an acyclic DHT-treated mouse with several cyst-like follicles (#). E) Higher magnification (200x) of the boxed area in D, showing an atretic cyst-like follicle with a large fluid filled antrum and detached pycnotic granulasa cells. F) Higher magnification (400x) of the boxed area in E, showing a dispersed theca cell layer containing cells that resemble interstitial tissue (double-sided arrow). Scale bar = 100 μm.

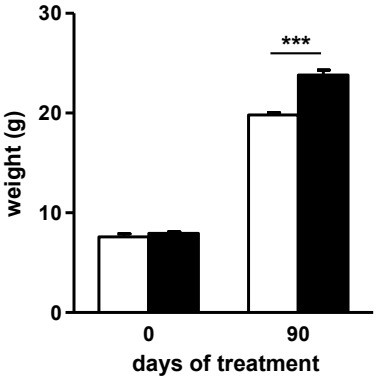

FIGURE 3. Effect of DHT-treatment on body weight. Body weight was determined at start (0 days of treatment) and at the end of the experiment (90 days of treatment) in mice exposed to vehicle (open bars) or DHT (closed bars). Data represent mean ± SEM (n = 9-10), *** $P<0.001$.

TABLE 2. Average diameter of adipocytes per fat depot.

	Gonadal	Retroperitoneal	Inguinal
Placebo (µm)	12.83 ± 1.10	13.53 ± 1.28	8.38 ± 0.37
DHT (µm)	23.89 ± 0.67*	29.45 ± 1.71*	23.44 ± 1.83*

Fat depots were isolated at the end of the 90-day treatment period. The diameter of a total of 130 adipocytes was measured in three randomly selected sections per fat depot per mouse. Values represent mean ± SEM (n = 4 mice per group). * $P<0.01$ (placebo *vs.* DHT).

was significantly increased in all WAT depots analyzed compared to placebo-treated mice ($P<0.01$) (Table 2).

Adipokine levels and glucose tolerance in DHT-treated mice

Obesity has been shown to be associated with changes in serum adipokine levels (23). DHT treatment resulted in 2.5-fold higher leptin ($P=0.013$) and 1.5-fold lower adiponectin ($P=0.001$) levels in DHT-treated compared to placebo-treated mice (Table 1). When corrected for body weight, the difference in leptin levels just failed to reach significance ($P=0.05$), whereas adiponectin levels remained highly significantly different between placebo- and DHT-exposed mice ($P=0.004$).

To investigate whether the DHT-treated mice showed an altered glucose tolerance, an ip glucose tolerance test was performed at the end of the treatment period. While fasting glucose levels were unchanged, DHT treatment resulted in significantly elevated glucose levels following ip glucose administration at all time points determined ($P<0.001$) (Figure 5A) and a significantly increased 0-120 min AUC value ($P<0.001$) (Figure 5B). Fasted insulin plasma levels did not differ between DHT- and placebo-treated mice (Table 1).

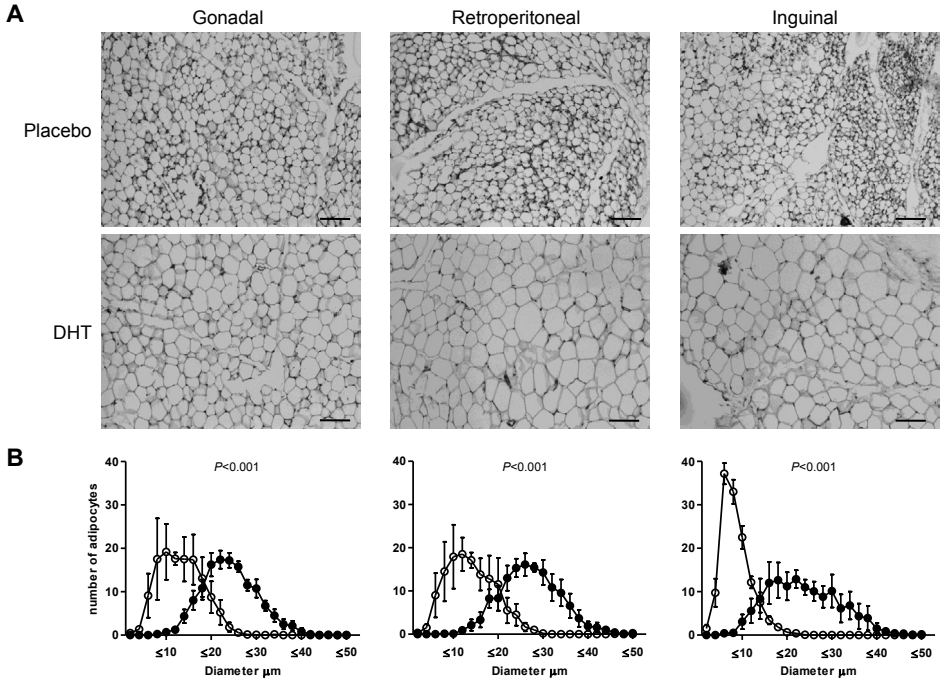

FIGURE 4. Increased adiposity upon chronic DHT treatment. Adipose depots were isolated at the end of the 90-day treatment period. A) HE-stained section of gonadal, retroperitoneal, and inguinal adipose depots in placebo- and DHT-treated mice. Scale bar = 100 µm. B) Adipocyte size distribution in placebo- (open circles) and DHT-treated (closed circles) mice. In each group, the diameter of a total of 130 adipocytes was measured in three randomly selected sections per fat depot per mouse. Data represent mean ± SEM (n = 4 mice per group), $P<0.001$.

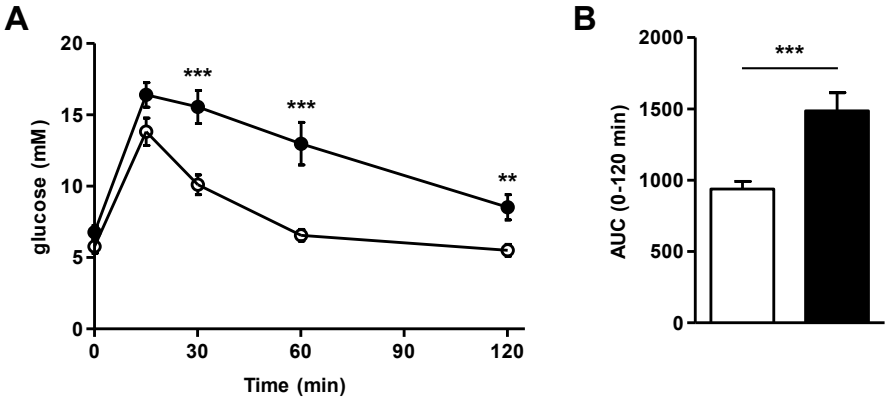

FIGURE 5. DHT treatment induced glucose intolerance. The ip glucose tolerance test was performed at the end of the 90-day treatment period. A) Ip glucose tolerance test in placebo- (open circles) and DHT-treated (closed circles) mice. B) Corresponding 0-120 minutes AUC for placebo- (open bars) and DHT-treated (closed bars) mice. Data represent mean ± SEM (n = 9-10), *** $P<0.001$.

DISCUSSION

This study shows that chronic exposure to DHT in prepubertal female mice induces a reproductive phenotype resembling features observed in women with PCOS, such as acyclicity and large atretic follicles with a cyst-like structure. Furthermore, our results show that the mice had an increased body weight and their WAT depots contained significantly more enlarged adipocytes. Leptin levels were elevated, adiponectin levels were decreased, and DHT-treated mice were glucose intolerant. Thus, our DHT-treated mice also display the metabolic disturbances often associated with PCOS.

Women with PCOS suffer from oligo- or anovulation and their ovaries contain large cyst-like follicles. In our study, all mice treated with DHT were acyclic and their ovaries were devoid of corpora lutea, showing that these mice lacked ovulations. Furthermore, the ovaries of DHT-treated mice contained many large follicles with a cyst-like appearance. The hyperplastic appearance of the theca cell layer of the cystic follicles, which contained cells that resembled interstitial cells, is in agreement with previous studies showing that androgen treatment in rats resulted in a pronounced thickening of the theca cell layer (17, 24). However, we cannot rule out that the phenotype of the granulosa and theca cell layer of the cystic-follicles also represents an advanced apoptotic stage, resulting in a degenerating follicular integrity. Interestingly, Manneras *et al.* (17) reported that DHT-treated rats, which our mouse model is based on, had irregular cycles and the ovarian weights of the DHT-treated rats were decreased compared to control rats, whereas in our DHT-treated mice ovarian weights tended to be increased (results not shown). A possible explanation of this discrepancy may be the dosage of DHT used. The DHT-treated mice showed 6-fold increased DHT serum levels compared to controls. In normo-ovulatory women, mean DHT levels range from 8.1 to 8.5 ng/dL during their cycle (25). In women with PCOS this can increase 3-fold to 24 ng/dL (26), which is less high as in the DHT-treated mice. Although, unfortunately the DHT levels in the rat model were not reported (17), it may be suggested that the more severe hyperandrogenic state in the DHT-treated mice may be responsible for the more severe reproductive phenotype. Indeed, also in women with PCOS higher androgens levels are associated with a worse phenotype (27).

Although the ovaries of DHT-treated mice contained cyst-like follicles, the follicle distribution did not completely resemble the one found in ovaries of PCOS women. Women with PCOS have an increased antral follicle count with accompanying increased AMH levels, with AMH reflecting the increased number of small antral follicles (28-29), while ovaries of DHT-treated mice had a comparable number of growing follicles and AMH levels to control mice. Furthermore, the AMH expression pattern as determined by immunohistochemistry was not different from control animals,

whereas a reduced AMH expression in transitional primordial follicles accompanied with an increased expression in growing follicles has been observed in human polycystic ovaries compared to normal ovaries (30). This suggests that AMH expression, at least in mice, is not regulated by 5α-reduced androgens. The unchanged follicle distribution was unexpected, since it has been suggested that androgens increase early follicle selection and growth in primates (31-32). Also in sheep, *in utero* exposure to testosterone or DHT also resulted in an increased recruitment of primordial follicles in the fetal ovary (33-34). However, postnatally, this effect was dependent on whether testosterone or DHT was administered prenatally. Only prenatal testosterone-treated sheep displayed an increased number of growing follicles, whereas animals treated with the non-aromatizable DHT were similar to controls. Smith *et al.* (33) concluded that estrogenic programming affects postpubertal follicular recruitment and growth, and androgenic programming only plays a role prenatally. However, in rats postnatal exposure to high testosterone levels lead to extensive follicle loss, whereas low doses of testosterone had no effect on follicular morphology (24). These results suggest that depending on the concentration of androgens used, opposing effects on follicle growth can be observed. In our study, mice were exposed to relatively high DHT levels, which might explain the lack of increased follicle growth. In addition, one cannot exclude that androgens have a different effect on folliculogenesis in poly-ovulatory species, such as rodents, than in species with single birth and twin/triplet births, such as human and sheep. We did observe that ovaries of DHT-treated mice contained a significantly higher number of atretic small and large antral follicles. Follicular arrest and atresia of these follicular stages suggests the presence of an insufficient FSH surge or a reduced sensitivity of these follicles to FSH. The latter has been proposed as the main cause of the follicular arrest in PCOS, and is explained by the antagonistic effect of androgens, particularly 5α-reduced androgens, on FSH-induced follicular growth (35-36). DHT administration reduced FSH-induced estradiol production in primates and it inhibited FSH-induced granulosa cell proliferation and induced atresia in rats (37-38). Although FSH levels were not determined in our mice, a previous study in rats suggests that DHT treatment does not alter the FSH surge and that DHT directly acts on the follicles by decreasing granulosa cell number and aromatase activity, resulting in reduced estrogen production and increased atresia (39). However, in our DHT-treated mice, aromatase mRNA expression did not differ from placebo-treated mice.

PCOS is often associated with metabolic dysfunction such as abdominal obesity and impaired insulin sensitivity and, similar to the reproductive disturbances, androgens have also been implied in the development of metabolic abnormalities in PCOS. Indeed, our results showed that prepubertal mice treated with DHT had an increased body weight and that their WAT depots contained significantly more enlarged adipocytes.

Increased adiposity with adipocyte hypertrophy is known to cause altered levels of adipokines (40). Adipokines, adipose secreted factors that play an important role in metabolic disorders, may be involved in both metabolic and reproductive characteristics of PCOS. Leptin levels have been shown to increase with increasing adiposity, whereas adiponectin levels decrease (41-42). The increase of leptin in PCOS women is believed to be the result of their obesity. When leptin levels in PCOS women were corrected for BMI, levels did not differ from weight matched controls (43). Also in our DHT-treated mice, a body weight-dependent increase in leptin was found. In addition to the increased adiposity, the decreased adiponectin levels may also reflect a direct effect of DHT on adipocytes, since in cultured rat adipocytes, androgens have been shown to suppress the secretion of adiponectin (44). Lower adiponectin levels, associated with impaired glucose tolerance, were observed in PCOS women compared to BMI matched controls (45-46). In agreement, in our DHT-induced PCOS model, mice displayed decreased adiponectin levels independent of body weight. Furthermore, DHT-treated mice were indeed glucose intolerant. Impaired glucose tolerance can be interpreted as a metabolic state intermediate between normal glucose homeostasis and diabetes, and is referred to as prediabetes (47). Thus, DHT-treated mice are in a prediabetic state, similar to women with PCOS.

Surprisingly, basal fasting insulin levels were not affected by DHT treatment, whereas previous studies detected an increase in circulating insulin levels in sheep (48), rats (15) and rhesus monkeys (49) upon prenatal androgen exposure. The absence of changed insulin levels in the DHT-treated mice may be due to the timing of androgen administration; prenatal androgen treatment may result in an altered programming of glucose homeostasis, whereas postnatal treatment may have no effect. Since our DHT-treated mice were unable to suppress glucose levels and probably are insulin-resistant to a certain degree, it will be of interest for future studies using our DHT-treated mouse model to incorporate insulin tolerance tests.

The effects of DHT on adipocytes and glucose tolerance seen in our mouse model resemble the androgenic effect seen in other animal models with increased adiposity upon *in utero* or postnatal exposure to testosterone or DHT. The pro-adipogenic and metabolic effects of DHT in our mouse model may also be partly explained by the absence of estrogen production by the non-cycling ovaries, since it has been shown that a lack of estrogens or estrogen receptor-α (ERα) signaling results in adipocyte hyperplasia, hypertrophy, insulin resistance and glucose intolerance (50). Also, estrogen receptor-β (ERβ) KO mice, fed a high fat diet, showed increased adiposity, although these mice where protected against diet-induced glucose intolerance. Thus, based on these results the metabolic phenotype in our DHT-treated mice could be explained by a direct androgenic effect, reduced estrogen signaling, or a combination of both.

In conclusion, chronic exposure to DHT excess in prepubertal mice induces a reproductive and metabolic phenotype with features in common with PCOS. In the future, this treatment regimen can be applied to transgenic mice to study the contribution of ovarian growth factors to the reproductive and metabolic phenotypes of PCOS.

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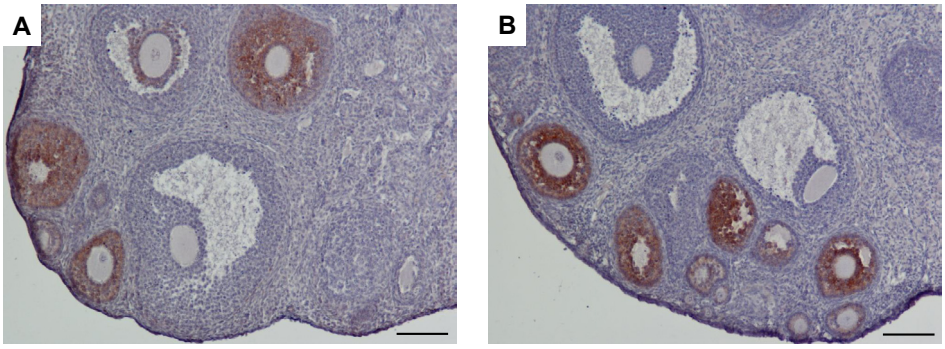
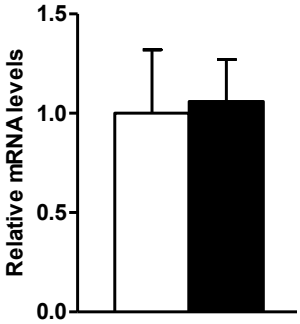
SUPPLEMENTAL DATA



SUPPLEMENTAL FIG. 1. Immunohistochemical analysis of AMH expression in ovaries of placebo- and DHT-treated mice. AMH is expressed in granulosa cells of healthy growing follicles. Expression disappears in small antral atretic follicles and is absent in the large antral atretic follicles. The pattern and intensity of AMH expression did not differ between A) placebo-treated mice and B) DHT-treated mice, analyzed at the end of the 90-day treatment period. Scale bar = 100 μm.



SUPPLEMENTAL FIG. 2. Aromatase mRNA expression in ovaries of placebo- and DHT-treated mice. Aromatase mRNA expression did not differ between placebo- (open bar) and DHT-treated mice (closed bar) after the 90-day treatment period.

Chapter 3

Ovarian growth factors contribute to the metabolic dysregulation in a mouse model of Polycystic Ovary Syndrome

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To be submitted

ABSTRACT

Androgens are known to play an important role in the development of polycystic ovary syndrome (PCOS). Indeed, dihydrotestosterone (DHT) treatment of prepubertal mice induces reproductive and metabolic characteristics resembling those of women with PCOS. However, little is known about the contribution of ovarian growth factors to the metabolic phenotype of PCOS. In order to study their contribution, we ovariectomized DHT-treated mice and compared their metabolic phenotype to intact DHT-treated mice.

Prepubertal female mice received a 90 days continuous release pellet containing DHT or vehicle. At day 45 of the treatment period, mice were ovariectomized (OVX) or received a sham surgery. Mice were analyzed after 90 days of DHT treatment.

DHT treatment and ovariectomy both resulted in an increase in body weight. The combined treatment resulted in a further gain in body weight. DHT treatment reduced glucose tolerance, while ovariectomy alone had no effect. Interestingly, ovariectomy of DHT-treated mice normalized the glucose tolerance. DHT treatment and ovariectomy both increased the weight of white adipose tissues, adipocyte size, and lipid accumulation in brown adipose tissue (BAT). Ovariectomy did not alter the DHT-induced white adipose tissue (WAT) characteristics. However, in BAT of DHT-OVX mice lipid accumulation was less pronounced than in DHT-sham mice, which is also reflected by the decrease in Hormone Sensitive Lipase (HSL) and the increase of Lipoprotein Lipase (LPL) mRNA expression levels in BAT after ovariectomy in DHT-treated mice.

In conclusion, androgens are a dominant factor in the development of the metabolic phenotype in our DHT-induced PCOS mouse model. However, removal of the ovaries results in a normal glucose tolerance and reduced lipid accumulation in BAT. These results suggest that, in addition to androgens, ovarian growth factors may contribute to the metabolic phenotype of PCOS.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is one of the most common endocrine disorders in women in their reproductive years affecting 6.5-8% of women (1). According to the Rotterdam Criteria, two out of the following three criteria diagnose PCOS: ovarian dysfunction characterized by oligo- or anovulation, hyperandrogenism, and the presence of polycystic ovaries on ultrasound (2). In addition to the ovarian phenotype, PCOS is associated with a metabolic phenotype: 38-88% of PCOS women are obese and 50-70% of women with PCOS are insulin resistant (3-7).

In women with PCOS, there is a failure in the selection of a dominant follicle. Combined with an accelerated early follicular growth, this leads to an accumulation of small antral follicles. Although the etiology of PCOS is unknown, the failure in dominant follicle selection suggests an altered FSH sensitivity in PCOS ovaries. This change in FSH sensitivity can partly be explained by an increased LH/FSH-ratio (8). As a consequence, growing follicles are prematurely stimulated by LH to produce androgens. Hyperandrogenism is considered to play a significant role in the pathophysiology of PCOS (9), since there is a strong correlation between hyperandrogenism and phenotype severity. Hyperandrogenic PCOS women have a higher body weight, BMI, and waist-hip ratio. They have higher blood glucose levels and are more frequently insulin resistant compared to normoandrogenic women with PCOS (10). Furthermore, many animal models of PCOS demonstrated that administration of androgens induces a reproductive PCOS phenotype but also metabolic disturbances (11-13). Indeed, recently we showed that prepubertal dihydrotestosterone (DHT) exposure in female mice led to both reproductive and metabolic phenotypes, resembling those observed in women with PCOS. Mice treated with DHT for 90 days were in continuous anestrous and their ovaries contained an increased number of atretic follicles with the majority having a cyst-like structure. In addition, DHT-treated mice had increased body weight, increased adiposity, elevated serum leptin levels, and decreased serum adiponectin levels. Furthermore, DHT-treated mice were glucose intolerant (14). Thus, androgens play an important role in the development of the phenotypic appearance of PCOS. However, it is unknown whether, besides androgens, also other growth factors or hormones secreted by the increased follicle pool contribute to the metabolic phenotype of PCOS. Previous studies have shown that serum levels of the transforming growth factor β (TGFβ) family member anti-Müllerian hormone (AMH) are 2-3 fold elevated in women with PCOS (15-18). These increased serum levels are explained by the increased follicle numbers, but also by an increased expression per granulosa cell in women with PCOS (19). Although AMH is not expected to have a direct effect on metabolism because its receptors are not expressed beyond the reproductive system, other ovarian growth factors potentially

could. Receptors of the bone morphogenetic proteins are expressed in metabolic tissues, such as adipose tissue and pancreas (20-22). BMPs have been shown to play a critical role in white and brown adipogenesis (23-25). Furthermore, studies from knockout mice revealed that BMP4 signaling is required for insulin secretion (21). Interestingly, expression levels of BMP6 are increased in granulosa cells of PCOS women compared with normal healthy women (26), whereas the expression of the oocyte-specific GDF9 is decreased in PCOS women (27). However, whether this altered ovarian expression also leads to altered serum levels of BMPs is yet unknown. Unfortunately, the current sensitivity of the BMP assays, does not allow the detection of BMP levels in almost all PCOS patients, as we showed recently (28). In contrast, it has been shown that serum levels of Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF) are elevated in PCOS patients (29, 30). VEGF not only is secreted by granulosa cells of the ovary, but also is highly expressed in adipose tissue and levels are increased in overweight and obese subjects (31). Therefore, not surprisingly, a link between VEGF and metabolism has been established. Upregulation of VEGF-A in adipocytes improved their vascularization and induced the appearance of a metabolically favourable phenotype, i.e. the presence of brown adipocytes in white adipose tissue (WAT). In contrast, in a preexisting disturbed metabolic environment, such as in the obese leptin deficient mice, inhibition of VEGF-A can also lead to an improved metabolic phenotype (32). These findings suggest that the role of VEGF-A may depend on the metabolic environment. FGFs are expressed by ovarian granulosa cells (33), and similar to VEGF, play an important role in adipocyte differentiation. FGF2 is expressed in preadipocytes and during differentiation FGF2 protein levels dropped coincidental with the appearance of lipid droplets (34). These results suggest that factors secreted by the ovary also play a role in metabolic tissues, such as WAT. We hypothesize that, in addition to androgens, an altered production of ovarian growth factors in PCOS may modulate and contribute to the metabolic phenotype.

Removal of ovarian growth factors and hormones through ovariectomy results in body weight gain, increased fat accumulation, and also in a worsening of glucose tolerance. Although it is known that the acute loss of estrogens plays an important role in these metabolic changes, it is unknown to which extent the loss of other ovarian hormones and secreted growth factors contribute to these metabolic changes. In order to study the contribution of ovarian growth factors to the metabolic phenotype in PCOS, we analyzed the metabolic phenotype in our DHT-induced PCOS mouse model after ovariectomy.

MATERIALS AND METHODS

Animals

C57BL/6J wild-type female mice were obtained from the Animal Facility of the Erasmus MC (Rotterdam, The Netherlands) and were kept under standard animal housing conditions in accordance with the National Institutes of Health guidelines for the Care and Use of Experimental Animals. The experiments were performed with permission of the local ethics committee.

At postnatal day 19, mice of comparable body weight were randomly divided over two treatment groups (DHT and control), and were implanted subcutaneously with a 90-day continuous release pellet containing 2.5 mg of DHT or a placebo pellet (Innovative Research of America, Sarasota, FL, USA), as described previously (14). At day 45 of the treatment period, DHT- and placebo-treated mice were randomly divided over two intervention groups (ovariectomy (OVX) or sham surgery; n=6-10 per group). Mice were anesthetized with isoflurane and an incision was made in both flanks to remove the ovaries or as a sham surgery. Mice were sacrificed at the end of the treatment period (90 days). Body weight was measured every two weeks. At the end of the experiment blood samples were collected. Uteri and ovaries from the sham groups were isolated, weighed, and fixed overnight in Bouin's fluid or snap frozen in liquid nitrogen and stored at −80°C until further processing. In addition, white and brown adipose depots were isolated, weighed, and fixed overnight in 4% paraformaldehyde or stored at −80°C for further analysis.

Ovarian histology

Ovaries of ovariectomized placebo- and DHT-treated mice were fixed overnight in Bouin's fluid and processed as described previously (14). Eight μm sections were mounted on glass slides and stained with hematoxylin and eosin for histological examination.

Real time PCR

Total RNA was isolated from the different fat depots, using an RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA samples were treated with Dnase (Promega Benelux BV, Leiden, The Netherlands) prior to cDNA synthesis. cDNA was synthesized using a cDNA synthesis kit (Roche Applied Sciences, Almere, the Netherlands). Real-time PCR (RT-PCR) was performed as described previously (14). Briefly, RT-PCR was performed with SYBR Green (Applied

TABLE 1. Primer sequences

	Gene	Sequence (5′-3′)	Accession number
B2m	B2 microglobulin	F: atccaaagctgaagaacgg R: cagtctcagtgggggtgaat	NM_009735.3
Hprt	Hypocanthine-guanine phosphoribosyl transferase	F: gcagtacagccccaaaatgg R: aacaaagtctggcctgtatccaa	NM_013556.2
Rps29	Ribosomal protein S29	F: tgaaggcaagatgggtcac R: gcacatgttcagcccgtatt	NM_009093.2
HSL	Hormone Sensitive Lipase	F: cgctacacacaaaggctgctt R: ggatggcaggtgtgaactg	NM_001039507.2
ATGL	Adipose Triglyceride Lipase	F: gagccccggggtggaacaagat R: aaaaggtggtgggcaggagtaagg	NM_001039507.2
LPL	Lipoprotein Lipase	F: gctggtgggaaatgatgtg R: tggacgttgtctagggggta	NM_008509.2
CD36	Fatty Acid Translocase	F: cctccagaatccagacaacc R: cacaggctttccttctttgc	NM_008509.2

Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) using an ABI 7900 HT apparatus. The expression of the target genes was normalized to the average expression level of three housekeeping genes (β2-microglobulin, hypoxanthine-guanine phosphoribosyl transferase (HPRT), and ribosomal protein S29) using the $2^{-\Delta\Delta Ct}$ method (35). Primers of target and housekeeping genes are shown in Table 1.

Intraperitoneal glucose tolerance test

Glucose tolerance was measured using an intraperitoneal glucose tolerance test (IPGTT) as described previously (14). In short, after an overnight fast (16h), basal glucose levels were measured using a Freestyle mini glycometer and test strips (Abbott, Alameda, CA). Next, 2% glucose was injected intraperitoneally and glucose levels were measured 15, 30, 60 and 120 minutes after the injection.

Hormone measurements

Plasma insulin levels were determined using an ultrasensitive mouse insulin ELISA. C-peptide serum levels were measured using a Mouse C-peptide ELISA. Total adiponectin serum levels were measured with an Adiponectin (Mouse) Total, HMW ELISA. Leptin levels were measured with a Leptin (Mouse/Rat) ELISA (all assays, Alpco Diagnostics, Salem, NH, USA). All samples were measured in one assay. DHT levels were measured with a DHT ELISA (Diagnostics Biochem Canada Inc. Ontario, Canada). Triglycerides were measured with an ABX PENTRA Triglycerides CP reagent with associated calibrators (HORIBA ABX, Montpellier, France). Glycerol

and non-esterified fatty acids (NEFA) were measured by enzymatic calorimetric procedure using a glycerol or a NEFA-HR kit (both Instruchemie, Delfzijl, The Netherlands).

Measurement of adipocyte size and quantification of lipid droplets

To determine the adipocyte size distribution in white adipose tissues (WAT), four mice per group were randomly selected. Mounted sections of 8 μm, stained with hematoxylin and eosin (HE), were randomly selected per fat depot and three micrographs were taken with a 100x magnification. Next, the diameter of at least 130 adipocytes per micrograph was measured using ImageJ software (version 2.0; http://imagejdev.org), followed by the calculation of the number of adipocytes per similar size class with intervals of 2 μm.

To determine the lipid droplet content in brown adipose tissue (BAT), three mice per group were randomly selected. HE-stained sections of 8 μm were randomly chosen and per mouse five micrographs were taken with a 100x magnification. ImageJ software was used to transform the micrographs into 8-bit grayscale images to calculate the lipid droplet content.

Immunohistochemistry

Immunohistochemistry was performed as described previously (14). Briefly, sections were mounted, deparaffinized, blocked for endogenous peroxidase activity, washed with water and transferred to PBS. After antigen retrieval, sections were incubated overnight with an Uncoupling Protein 1 (UCP1) rabbit polyclonal antibody (1:1000) (Abcam, Cambridge MA, USA). Next, sections were incubated with Brightvision poly-HRP-Anti Ms/Rb/Rt IgG kit (Immunologic, Duiven, The Netherlands) and the peroxidase activity was developed with 0.07% 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Finally, all sections were counterstained with hematoxylin.

Statistics

Data were statistically analyzed by two-way ANOVA to measure the effects of treatment (placebo vs. DHT) and procedure (sham vs. OVX) (treatment x procedure). When a significant difference was present, Student's t-test was performed as a posthoc test. Body weight gain and IPGTT were analyzed by two-way repeated measures (RM) ANOVA (treatment x procedure), followed by a one-way ANOVA analysis as a posthoc test. The adipocyte size distribution was analyzed by three-way ANOVA

(treatment x procedure x size). All statistical analyses were performed using SPSS, version 20.0 (SPSS, Inc., Chicago, IL). Data were expressed as mean ± SEM, and differences were considered significant at $P<0.05$.

RESULTS

Ovarian histology

Analysis of the ovaries removed during the ovariectomy procedure revealed that 45 days of DHT treatment was sufficient to induce the ovarian PCOS-like phenotype. After 45 days of treatment, ovaries of DHT-exposed mice already lacked corpera lutea and contained large cyst-like follicles with a hyperplastic theca cell layer and a thin granulosa cell layer. This ovarian phenotype was similar to that observed after 90 days of DHT treatment (Supplemental Figure 1).

Increased body weight but normal glucose tolerance after combined ovariectomy and DHT treatment

In our previous study we observed that DHT treatment resulted in increased body weight and reduced glucose tolerance (14). To characterize the contribution of ovarian hormones and growth factors to this metabolic phenotype, we determined the effect of ovariectomy on body weight in placebo- and DHT-treated mice. By two-way RM ANOVA analysis, there was a significant effect of both treatment and procedure on body weight (both $P<0.001$) (Figure 1A). DHT treatment resulted in a significant increase in body weight compared to placebo-treated mice ($P<0.001$, by post-hoc analysis). This increase in body weight in DHT-treated mice was already present prior to ovariectomy, i.e. day 45 of DHT treatment ($P<0.001$, by post-hoc analysis). Ovariectomy at day 45 of treatment resulted in a significant weight gain in both placebo- and DHT-treated mice ($P=0.006$ and $P<0.001$, respectively, by posthoc analysis) (Figure 1A). As a consequence, at the end of the treatment period DHT-sham and placebo-OVX mice had a comparable increased body weight, while DHT-OVX mice had the highest body weight.

In addition, we determined the glucose tolerance of these mice. Two-way RM ANOVA analysis indicated a borderline significant interaction between treatment and procedure ($P=0.053$). In agreement with our previous study, DHT-sham mice had an impaired glucose tolerance compared to placebo-sham mice ($P=0.012$, by posthoc analysis) (Figure 1B). Interestingly, DHT-OVX mice had a significantly better glucose tolerance than DHT-sham mice ($P=0.016$, by posthoc analysis), and did

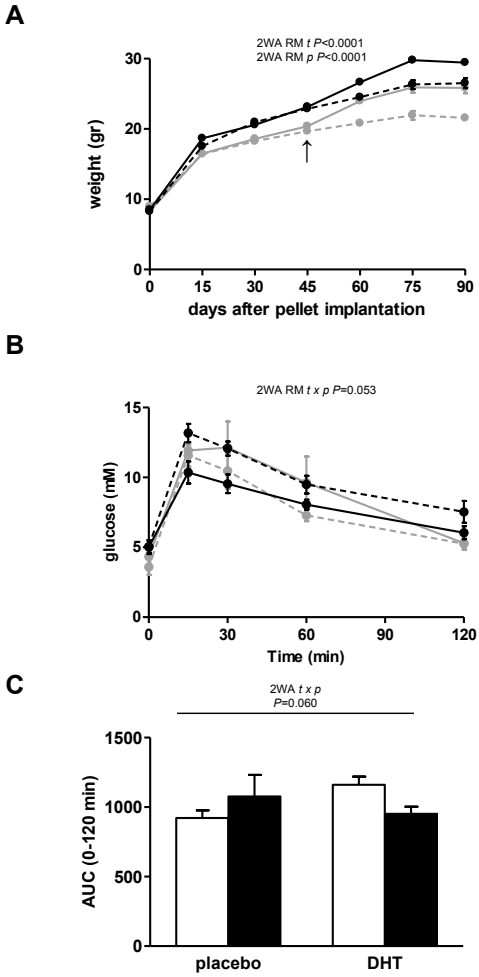


FIGURE 1. Ovariectomy increases body weight but normalizes glucose tolerance in DHT-treated mice.

A) Body weight of plc-sham (dotted grey line), plc-OVX (solid grey line), DHT-sham (dotted black line) and DHT-OVX (solid black line) mice was measured every 2 weeks, starting at 19 days of age (d0 of DHT treatment) until 90 days of DHT treatment. Ovariectomy was performed at day 45 of DHT treatment, indicated by the arrow. Two-way RM ANOVA revealed a significant effect of both treatment ($P<0.001$) and procedure ($P<0.001$) on body weight. B) The intraperitoneal glucose tolerance test was performed 5 days before sacrifice in plc-sham (dotted grey line), plc-OVX (solid grey line), DHT-sham (dotted black line) and DHT-OVX (solid black line) mice. Two-way RM ANOVA revealed a borderline significant interaction between treatment and procedure ($P=0.053$) on glucose tolerance. Post-hoc analysis indicated a significant effect of ovariectomy on glucose tolerance in the DHT-treated mice ($P=0.016$). C) Corresponding 0-120 minutes AUC values in sham (open bars) and ovariectomized (black bars) placebo- and DHT-treated mice. Two-way ANOVA indicated a borderline significant interaction between treatment and procedure ($P=0.060$). Data represent mean ± SEM (n=6-10).

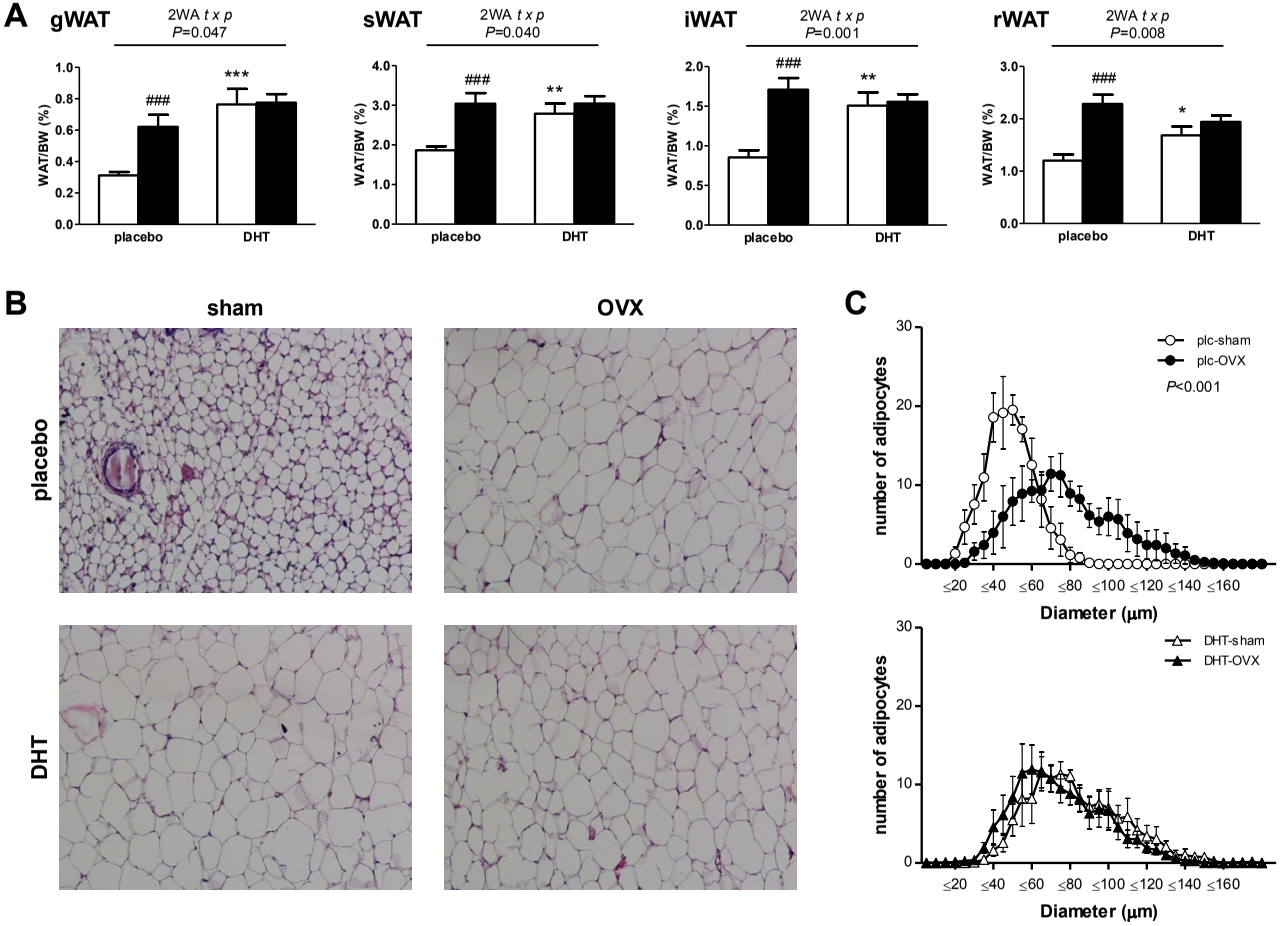
not differ from plc-sham mice. Ovariectomy had no effect on the glucose tolerance of placebo-treated mice (Figure 1B). Similarly, there was a borderline significant interaction between treatment and procedure by two-way ANOVA analysis for the 0-120 min AUC values (P=0.060) (Figure 1C).

Ovariectomy does not alter the adiposity of DHT-treated mice

To understand the normal glucose tolerance combined with the increased body weight in DHT-OVX mice, we next analyzed WAT depots. By two-way ANOVA, there was a significant interaction between treatment and procedure for the relative weight of all WAT depots (Figure 2A). Gonadal, subcutaneous, inguinal, and retroperitoneal WAT depots (gWAT, sWAT, iWAT, and rWAT) showed a significant increase in weight after DHT treatment in sham operated mice (P=0.001, 0.003, 0.004, and 0.033, respectively, by post-hoc analysis) (Figure 2A). Ovariectomy resulted in a significant increased weight of all WAT depots in placebo-treated mice (P<0.001 for all WAT depots, by post-hoc analysis) (Figure 2A). Interestingly, ovariectomy did not result in a further increase in WAT depot weight in DHT-treated mice, despite the increase in body weight in DHT-OVX mice.

Gross morphological analysis of the sWAT depot of DHT-sham mice showed that adipocytes were enlarged compared to placebo-treated mice (Figure 2B), which is in agreement with our previous study (14). Measurement of the adipocyte cell diameter confirmed that sWAT of DHT-sham mice contained more large adipocytes than placebo-sham mice (P<0.001, by posthoc analysis) (Supplemental Figure 2). This is also reflected by the increased average adipocyte diameter in DHT-sham

FIGURE 2. No effect of ovariectomy on adiposity of DHT-treated mice.
A) Percentage adipose depot weight relative to body weight in sham (open bars) and ovariectomized (black bars) placebo- and DHT-treated mice. Two-way ANOVA indicated a significant interaction between treatment and procedure for relative weight of gonadal WAT (gWAT) (P=0.047), subcutaneous WAT (sWAT) (P=0.040), inguinal WAT (iWAT) (P=0.001) and retroperitoneal WAT (rWAT) (P=0.008). Data represent mean ± SEM (n=6-10). Significant differences for the effect of DHT treatment in sham-operated mice by posthoc tests: *, P<0.05; **, P<0.01; ***, P<0.001. Significant effect of ovariectomy in placebo-treated mice: $^{###}$, P<0.001. B) Representative HE-stained section of sWAT of plc-sham, plc-OVX, DHT-sham and DHT-OVX mice. C) Adipocyte size distribution of sWAT. Upper graph: plc-sham (open circles) vs. plc-OVX (closed black circles) mice. Lower graph: DHT-sham (open triangles) vs. DHT-OVX (closed triangles) mice. Two-way ANOVA indicated a significant interaction between treatment and procedure (P<0.001). Posthoc analysis indicated a significant effect of ovariectomy on the adipocyte size in placebo-treated mice (upper graph) (P<0.001), but not in DHT-treated mice (lower graph). In each group the diameter of a total of 130 adipocytes was measured in three randomly selected sections per mouse. Data represent mean ± SEM (n=4 mice per group).


A
gWAT
2WA t x p
P=0.047
sWAT
2WA t x p
P=0.040
iWAT
2WA t x p
P=0.001
rWAT
2WA t x p
P=0.008
WAT/BW (%)
placebo
DHT
B
sham
OVX
placebo
DHT
C
plc-sham
plc-OVX
P<0.001
DHT-sham
DHT-OVX
number of adipocytes
Diameter (μm)
≤20 ≤40 ≤60 ≤80 ≤100 ≤120 ≤140 ≤160

mice (plc-sham: 46.0 ± 3.3 μm *vs.* DHT-sham: 80.8 ± 6.8 μm, *P*=0.004, by posthoc analysis). Morphological analysis of the sWAT depot revealed that ovariectomy alone increased adipocyte size but did not have an additional effect on adipocyte size in the DHT-treated mice (Figure 2B). The increase in adipocyte size in plc-OVX mice was confirmed by the right sided shift in the size distribution compared to plc-sham mice (*P*<0.001, by posthoc analysis) (Figure 2C), and by the increase in average adipocyte diameter (plc-sham: 46.0 ± 3.3 μm *vs.* plc-OVX: 74.8 ± 9.3 μm, *P*=0.027, by posthoc analysis). In contrast, ovariectomy did not affect adipocyte size distribution in DHT-treated mice (Figure 2C), which is also reflected by the comparable average adipocyte diameter in DHT-sham and DHT-OVX mice (DHT-sham: 80.8 ± 6.8 μm *vs.* DHT-OVX: 72.1 ± 5.9 μm, *P*=0.371, by posthoc analysis).

Ovariectomy does not alter leptin and C-peptide levels in DHT-treated mice

Next, we measured serum hormones, adipokines, and lipid levels to gain insight into the normalized glucose tolerance in the presence of unchanged fat depot weights upon ovariectomy in DHT-treated mice (Table 2). Two-way ANOVA analysis indicated a significant effect of both treatment (*P*=0.026) and procedure (*P*=0.03) on

TABLE 2. Hormone levels in sham and ovariectomized placebo- and DHT-treated mice

	Plc-sham	Plc-OVX	DHT-sham	DHT-OVX
Basal glucose levels (mM)	3.60 ± 0.57	4.33 ± 0.22	5.05 ± 0.44*	5.01 ± 0.50
Insulin (ng/ml)	0.37 ± 0.10	0.68 ± 0.08	1.37 ± 0.30*	1.41 ± 0.20¶¶
C-peptide	464.77 ± 70.16	807.48 ± 110.27	1087.97 ± 205.14*	1435.94 ± 300.17
Leptin (ng/ml)	1.46 ± 0.18	7.81 ± 2.04$^{\#\#}$	7.92 ± 1.92**	9.23 ± 1.81
Adiponectin (μg/ml)	29.32 ± 2.52	30.95 ± 2.83	14.71 ± 1.51***	12.14 ± 0.69¶¶¶
Triglycerides	0.74 ± 0.07	0.88 ± 0.05	1.02 ± 0.12	1.09 ± 0.12
Glycerol	0.42 ± 0.03	0.47 ± 0.02	0.45 ± 0.02	0.47 ± 0.03
NEFA	1.00 ± 0.14	1.04 ± 0.04	1.00 ± 0.08	0.98 ± 0.07

Nineteen-days-old female mice (T0) were treated with a DHT or placebo pellet for 90 days. Ovariectomy or sham surgery was performed on day 45 of the treatment period (T45). Serum samples were collected at the end of the 90-day treatment period (T90). For fasting insulin levels, plasma samples were collected at the start of the ip glucose tolerance test, which was performed at the end of the experiment. Two-way ANOVA indicated a significant effect of treatment on basal glucose, insulin, C-peptide, leptin, adiponectin, and triglyceride levels (2WA *t*: *P*=0.025, *P*<0.001, *P*=0.002, *P*=0.026, *P*<0.001, and *P*=0.015, respectively), and a significant effect of procedure on leptin levels (2WA *p*: *P*=0.03). Values represent mean ± SEM (n = 6-8 mice per group). Significant differences by post-hoc test between plc-sham and DHT-sham mice: *, *P*<0.05; **, *P*<0.01, ***, P<0.001; between plc-OVX and DHT-OVX mice: ¶, *P*<0.01; ¶, *P*<0.001; between placebo-sham and placebo-OVX: ##, P<0.01.

leptin levels. DHT treatment resulted in a 5.4-fold increase in leptin levels in the sham-operated mice (P=0.005, by post-hoc analysis). Ovariectomy significantly increased leptin levels in placebo-treated mice (P=0.008, by post-hoc analysis) to comparable levels as observed in DHT-sham mice, but did not further elevate leptin levels in DHT-treated mice (Table 2). Two-way ANOVA analysis indicated that DHT treatment significantly reduced adiponectin levels (P<0.001), while ovariectomy had no effect on adiponectin levels. Likewise, DHT treatment significantly increased triglyceride levels (P=0.015), while ovariectomy had no effect. There were no significant differences present in glycerol and NEFA profiles upon DHT treatment or ovariectomy. Two-way ANOVA analysis revealed a significant effect of treatment on insulin and C-peptide levels (P<0.001 and P=0.002, respectively), while ovariectomy tended to affect only C-peptide levels (P=0.07). C-peptide and insulin levels were significantly increased after DHT treatment in sham-operated mice (P=0.016 and P=0.012, respectively, by posthoc analysis) (Table 2).

Ovariectomy does not alter the decreased BAT activity upon DHT treatment

Brown adipose tissue (BAT) plays an important role in the regulation of energy expenditure and adiposity. Therefore, we determined whether BAT activity was affected in our mice. DHT treatment resulted in a significant increase in weight of the intrascapular BAT depot (P<0.001, by posthoc analysis), while ovariectomy had no effect on BAT weight in either placebo- or DHT-treated mice (data not shown). Morphological analysis of BAT of plc-sham mice showed a compact structure with the presence of multilocular lipid droplets (Figure 3A). DHT treatment resulted in a profound accumulation of lipids, resulting in the presence of unilocular lipid droplets in BAT (Figure 3A). Lipid accumulation also occurred after ovariectomy in BAT of placebo-treated mice, although the lipid droplets appeared smaller compared to DHT-treated mice (Figure 3A). In the DHT-treated mice, ovariectomy slightly decreased the DHT-induced lipid accumulation, since the lipid droplets in BAT of DHT-OVX mice appeared smaller compared to those of DHT-sham mice. Two-way ANOVA analysis of the total lipid content in BAT confirmed that there was a significant interaction between treatment and procedure (P<0.001). Indeed, DHT treatment induced a significant increase in BAT lipid content (P=0.001, by post-hoc analysis) (Figure 3B). Also ovariectomy increased the BAT lipid content of placebo-treated mice (P=0.001, by post-hoc analysis), but this increase was significantly less than in DHT-treated mice (P=0.02, by post-hoc analysis). In DHT-treated mice, ovariectomy slightly reduced the BAT lipid content, which was borderline significant (P=0.054, by post-hoc analysis) (Figure 3B).

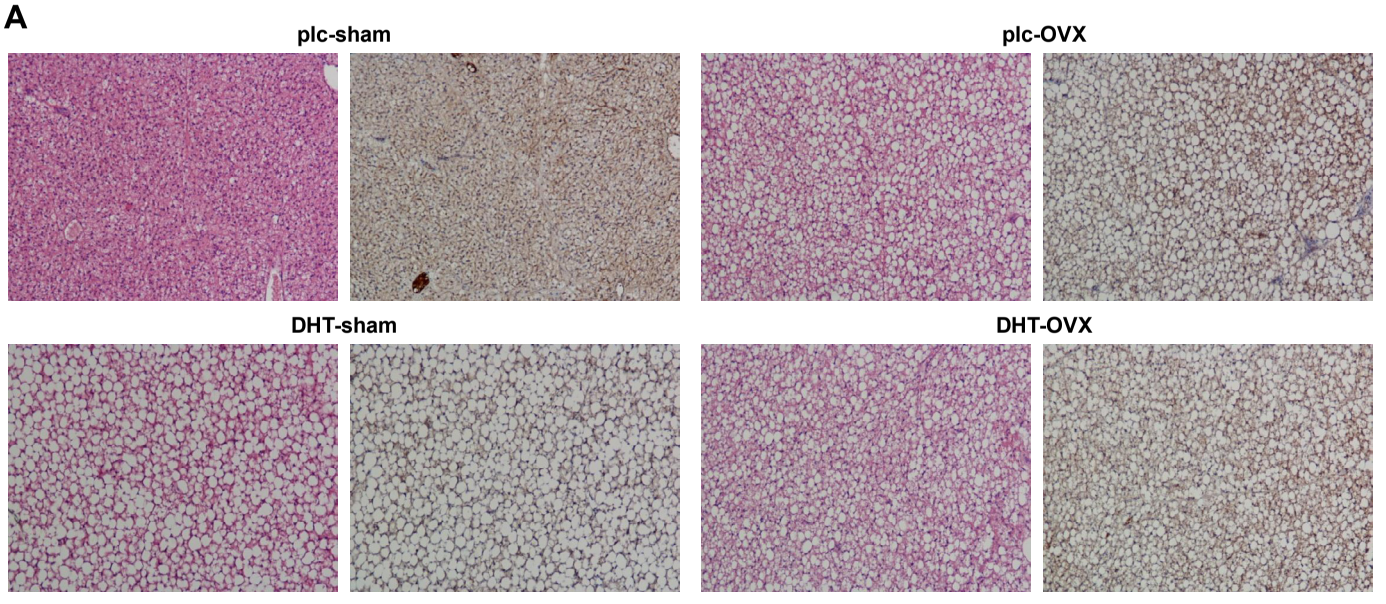

A
plc-sham
plc-OVX
DHT-sham
DHT-OVX
B
2WA t x p
P<0.001
% lipid droplets
60
40
20
0
###
***
Placebo
DHT

FIGURE 3. Ovariectomy partly protects against lipid accumulation and a decreased UCP activity in BAT of DHT-treated mice.
A) HE-stained section and adjacent section with immunohistochemical UCP1 expression in BAT of plc-sham, plc-OVX, DHT-sham and DHT-OVX mice. DHT-treatment increased lipid accumulation and decreased UCP1 expression in BAT. With the combined treatment lipid droplets appeared slightly smaller and UCP1 expression seemed stronger than upon DHT treatment alone. B) Percentage of lipid droplet content in BAT. Two-way ANOVA indicated a significant interaction between treatment and procedure on the lipid droplet content ($P<0.001$). DHT treatment increased the lipid content, while ovariectomy slightly reduced this increase ($P<0.001$ and $P=0.054$, respectively, by posthoc analysis). In each group, 3 mice were randomly selected and the lipid content was measured in five sections per mice. Data represent mean ± SEM (n=3 mice per group).

In addition, we performed immunohistochemistry for UCP1, a marker for BAT activity. UCP1 showed a decrease in staining intensity upon DHT treatment and ovariectomy, although the decrease seemed less strong upon ovariectomy. In agreement with the lower lipid accumulation, UCP1 staining intensity appeared to be slightly stronger in BAT of DHT-OVX mice compared to DHT-sham mice (Figure 3A).

DHT-treated ovariectomized mice do not exhibit an increased lipolysis

To determine whether the change in adipocyte size in WAT and lipid accumulation in BAT was accompanied by a change in lipolysis and lipogenesis, we determined mRNA levels of the lipolytic genes Hormone Sensitive Lipase (HSL) and adipose TG lipase (ATGL) and the lipogenic genes lipoprotein lipase (LPL) and CD36, also known as fatty acid translocase. In sWAT, DHT treatment, ovariectomy, or the combination, had no effect on mRNA expression of these lipolytic and lipogenic genes (data not shown). In BAT, however, analysis by two-way ANOVA revealed a significant interaction between treatment and procedure for both HSL and LPL mRNA expression ($P=0.008$ and $P=0.010$, respectively), while there was no significant effect on the mRNA expression of ATGL and CD36. HSL mRNA expression was increased in DHT-sham mice compared to plc-sham ($P=0.026$, by post-hoc analysis). Interestingly, upon ovariectomy HSL mRNA expression in the DHT-treated mice decreased to expression levels observed in placebo-sham mice ($P=0.028$, by post-hoc analysis) (Figure 4). LPL mRNA expression was decreased to a similar extend by DHT treatment ($P=0.045$, by posthoc analysis) and ovariectomy, although the latter only reached borderline significance ($P=0.057$, by posthoc analysis). In contrast, ovariectomy tended to increase LPL mRNA expression in DHT-treated mice ($P=0.056$, by post-hoc analysis) (Figure 4).

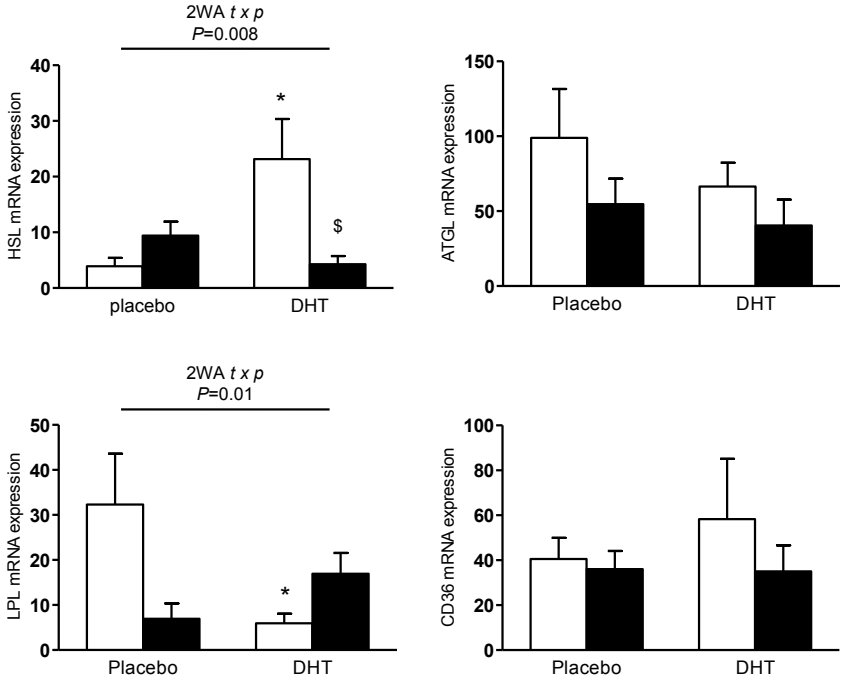


FIGURE 4. Analysis of lypolytic and lypogenic genes in BAT of ovariectomized DHT-treated. A) mRNA expression of the lipolytic genes HSL and ATGL and the lipogenic genes LPL and CD36 in BAT of sham and ovariectomized placebo- or DHT-treated mice. Two-way ANOVA indicated a significant interaction between treatment and procedure for the expression of HSL (P=0.008) and LPL (P=0.01). Significant effect of DHT treatment in sham-operated mice: *, P<0.05. Significant effect of ovariectomy in DHT-treated mice: $^{\$}$, P<0.05.

DISCUSSION

In this study, we investigated whether ovarian growth factors contribute to the metabolic phenotype observed in the DHT-induced PCOS mouse model. In agreement with our previous study (14), mice treated with DHT became glucose intolerant. However, we observed that ovariectomy prevented this DHT-induced glucose intolerance. This normalized glucose tolerance in DHT-OVX mice was surprising because ovariectomy did not affect the DHT-induced increase in adipose depot weights or adipocyte size. Basal glucose and insulin levels were not different between the two groups. However, C-peptide levels were increased, although not significantly, in the DHT-OVX mice indicating that the endogenous insulin production by the pancreas may be elevated, which might partially explain the improved glucose tolerance in DHT-OVX mice. In addition, we observed an increase in body weight without a further increase in adiposity in DHT-OVX mice, which might suggest an enhance-

ment of the anabolic effect of DHT after ovariectomy. This may lead to a stronger increase in lean body mass in DHT-OVX mice compared to DHT-sham mice, which might contribute to the improved glucose tolerance. However, we did not observe a difference in muscle or heart weight relative to body weight between the studied groups (data not shown). For future studies, DEXA scans, which were not available for the current study, are needed to determine whether indeed a change in lean body mass occurred with the combined treatment.

In contrast to our results, McInnes et al (36) showed that DHT treatment combined with ovariectomy in mice did lead to an increase in fat depot weight. However in their study, DHT treatment was initiated at the time of ovariectomy at an age of one year, when ovaries of mice are already nearly depleted of follicles (37), which makes the two models impossible to compare.

Ovariectomy did not appear to alter the function of DHT-exposed WAT. Leptin and adiponectin levels were not significantly different between the DHT-sham and DHT-OVX mice and also mRNA expression of lipolytic and lipogenic genes in WAT did not change between these two groups. In contrast, our results suggest that ovariectomy affects the function of BAT. BAT is specialized in energy expenditure and an increase in BAT activity can partially explain an improvement in metabolism. In this study, DHT treatment led to an increased BAT depot weight, due to an accumulation of unilocular lipid droplets and decreased UCP1 staining in BAT. These results are in agreement with results observed in female mice overexpressing LH (38), that have increased BAT depot weights combined with reduced thermogenic capacity, due to elevated androgens levels in response to the overexpressed LH. Similarly, ovariectomy increased lipid accumulation of BAT, although this effect seemed weaker compared to DHT treatment. Interestingly, also with the combined treatment, the increase in lipid accumulation and reduction in UCP1 expression was less pronounced than with DHT treatment alone. The combined treatment also normalized or tended to normalize the DHT-induced changes in HSL and LPL mRNA expression in BAT. These findings suggest that removal of the ovaries reduces the negative metabolic effect of DHT on BAT. Although ovariectomy only partly counterbalanced the effects of DHT on BAT, this improvement may be sufficient to lead to a better energy expenditure, and thereby also contribute to the normal glucose tolerance in DHT-sham mice compared to DHT treatment alone.

Ovariectomy, and thus the removal of estrogens and ovarian growth factors, has been extensively studied in various species like monkeys, rats, mice and sheep (39-43). Despite the difference in timing and duration of ovariectomy, most studies showed a worsening of metabolic parameters upon ovariectomy, such as body weight increase, glucose intolerance and higher levels of circulating lipids (44-46). In this study, ovariectomy led to metabolic disturbances, such as body weight increase,

increased adiposity and increased lipid accumulation in BAT. However, we did not observe a worsening in glucose intolerance. This discrepancy has also been reported in previous studies, in which glucose intolerance was only observed when ovariectomy was combined with an extra stressor such as a high fat diet (42, 47). It is well known that the loss of estrogens upon ovariectomy causes these changes in metabolism. After menopause, women gain body weight (48), but also have elevated plasma triglyceride concentrations (49). However, a loss of estrogens alone does not explain the difference in metabolic phenotype of DHT-sham and DHT-OVX mice because the DHT-treated mice lack preovulatory follicles and thus already have low estrogen levels. Indeed, uterine weight, which was measured as readout of estrogen action, was decreased to a similar extent in DHT-sham and DHT-OVX mice (results not shown). Therefore, we rationalize that, in addition to androgens, also ovarian growth factors contribute to the metabolic phenotype of DHT-treated mice. In contrast to the ovarian phenotype observed in women with PCOS, ovaries of the DHT-exposed mice do not contain more healthy growing follicles, but do have an increased number of atretic growing follicles (14). Therefore, it remains to be determined whether the growth factors influencing metabolism are secreted from the healthy or atretic growing follicle pool. Furthermore, it remains to be determined whether follicles of women with PCOS secrete the same factors and whether also in women with PCOS these factors contribute to the metabolic phenotype.

To conclude, our study demonstrates that androgens are a dominant factor in the development of the metabolic phenotype. However, our findings also show that removal of the ovary prevents or improves these metabolic alterations, suggesting that ovarian growth factors contribute to the metabolic phenotype observed in our DHT-induced mouse model. Whether these ovarian growth factors also contribute to the metabolic phenotype of women with PCOS will need to be determined in the future.

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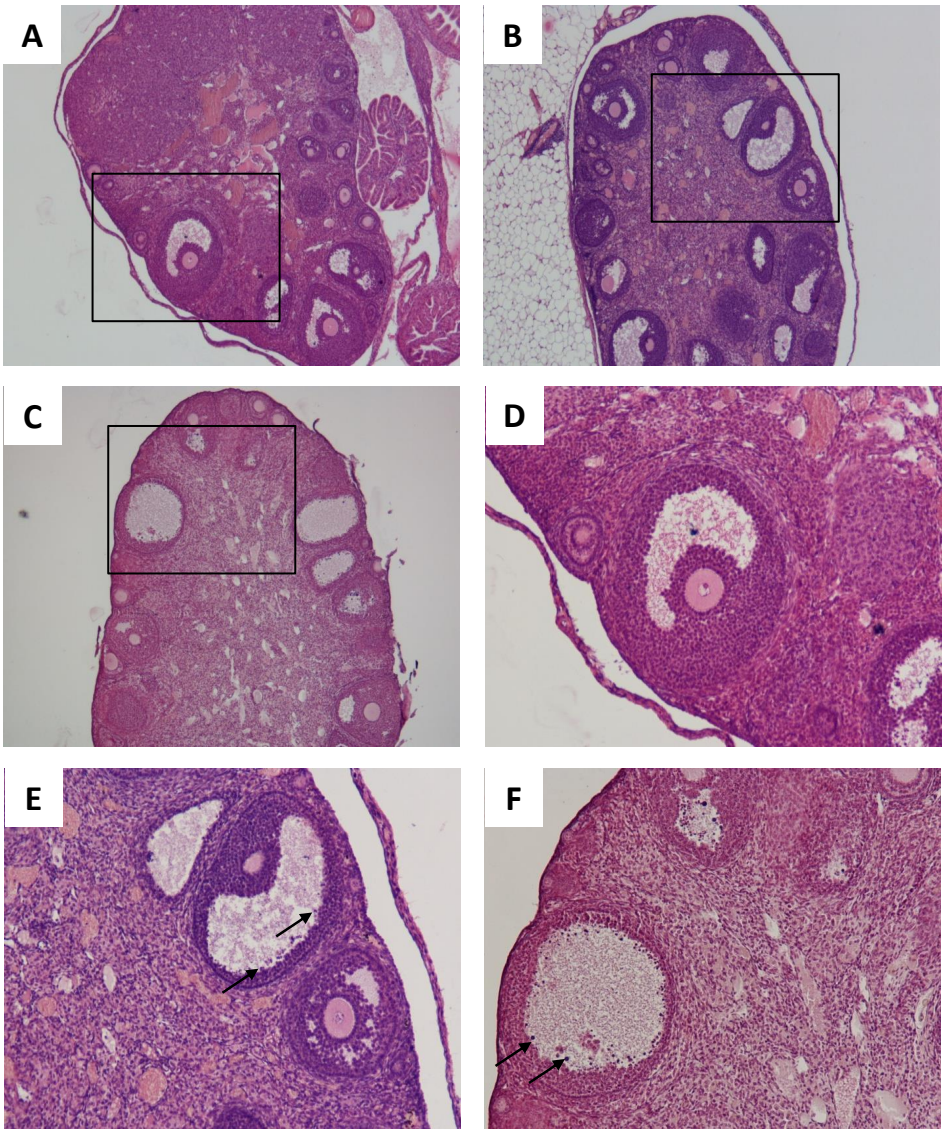
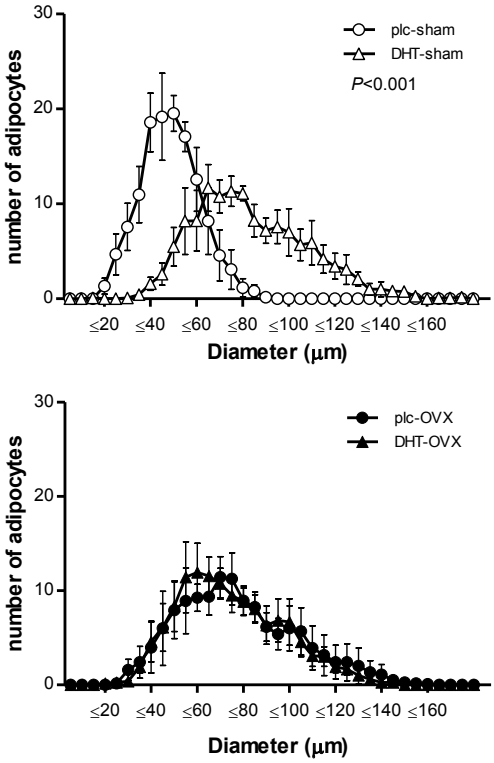
SUPPLEMENTAL DATA



SUPPLEMENTAL FIGURE 1. Ovarian morphology of placebo and DHT-treated mice.
HE-stained ovarian sections of A) a normal cycling placebo-treated mouse with healthy growing follicles and corpora lutea (CL); B) an acyclic DHT-treated mouse with several cyst-like follicles and absence of CL after 45 days of treatment; C) an acyclic DHT-treated mouse with several cyst-like follicles and absence of CL after 90 days of treatment. Magnification 40x. D, E, and F: Higher magnification (100x) of the boxed area in A, B, and C, respectively. Arrows indicate the presence of apoptotic granulosa cells in the cyst-like follicles.



SUPPLEMENTAL FIGURE 2. Increased number of large adipocytes upon DHT-treatment.
Adipocyte size distribution in sWAT. Upper graph: plc-sham (open circles) vs DHT-sham (open triangles) mice. Lower graph: plc-OVX (closed circles) vs DHT-OVX (closed triangles) mice. Two-way ANOVA indicated a significant interaction between treatment and procedure (P<0.001). Posthoc analysis indicated a significant effect of DHT treatment on the adipocyte size in sham-operated mice (upper graph) (P<0.001), but not in ovariectomized mice (lower graph). For clarity adipocyte size distribution graphs are presented per procedure. In each group the diameter of a total of 130 adipocytes was measured in three randomly selected sections per mouse. Data represent mean ± SEM (n=4 mice per group).

Chapter 4

Improved metabolic phenotype with increasing age in female mice lacking anti-Müllerian hormone signaling

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To be submitted

ABSTRACT

Polycystic ovary syndrome (PCOS) is a disorder associated with infertility and metabolic disturbances. Hyperandrogenism is an important hallmark of PCOS and contributes to the metabolic phenotype. An increased number of growing follicles is another important feature. However, the contribution of ovarian growth factors to the metabolic phenotype of PCOS has not been investigated. To determine the interaction between ovarian and metabolic function, we studied the metabolic phenotype of mice lacking anti-Müllerian hormone (AMH) signaling as a model for an altered profile in ovarian growth factors.

Female mice lacking AMH (AMHKO) or its type II receptor (MRKI) and wildtype (WT) littermates were analyzed at 4, 5, and 8 months of age. AMHKO and MRKI mice had a normal body weight, but displayed an improved glucose tolerance compared to WT mice at 5 months of age ($P<0.002$). AMHKO and MRKI mice had reduced adiposity, with white adipose tissue (WAT) depots containing smaller adipocytes ($P<0.001$). In agreement, leptin levels were 40-80% reduced in AMHKO and MRKI mice ($P<0.001$ and $P=0.007$, respectively). Interestingly, mice lacking AMH signaling had more active brown adipose tissue (BAT) and displayed increased browning of subcutaneous WAT, illustrated by an increased UCP1 staining, compared to WT mice. At 8 months of age, AMHKO mice continued to have smaller adipocytes ($P<0.001$), lower leptin levels ($P<0.05$), and tended to have an improved glucose tolerance ($P<0.07$) combined with lower insulin levels ($P<0.05$) compared to WT mice.

In conclusion, in the absence of AMH signaling mice are protected against the age-related decline in metabolism. This suggests that an altered profile in ovarian growth factors may affect metabolism and may modulate the effect of metabolic aging.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is the most common endocrinopathy in premenopausal women, with a prevalence of up to 10% in the general population (1). PCOS is characterized by two out of the following three criteria being present: hyperandrogenism, oligo/anovulation and polycystic ovaries (2). Ovaries of women with PCOS show an increased accumulation of small antral follicles and an aberrant selection of a dominant follicle, suggesting a decrease in FSH sensitivity (3). Although the etiology of PCOS is unknown, the excess in androgen levels is considered the main culprit in PCOS.

In addition to a reproductive phenotype, PCOS patients also often display a metabolic phenotype; 38-88% (depending on the study) of the PCOS women are obese and 50-70% are insulin resistant (4-8). Metabolic aberrations include glucose intolerance and type II diabetes mellitus. The excess in androgen levels is considered to induce multiple metabolic effects in women with PCOS (9). Indeed non-human primate and sheep animal models of PCOS demonstrated that prenatal exposure to testosterone resulted in metabolic disturbances (10, 11). In addition, we recently showed that prepubertal dihydrotestosterone (DHT) treatment of mice not only resulted in a reproductive but also in a metabolic phenotype resembling PCOS (12). However, several studies suggest that non-hyperandrogenic PCOS women may have an adverse metabolic phenotype compared to non-PCOS women. Non-hyperandrogenic PCOS women have an increased waist-hip-ratio compared to BMI-matched non-PCOS women (13). Furthermore, when matched for age and BMI, non-hyperandrogenic PCOS women remain to have elevated insulin levels and HOMA-IR (14), suggesting that androgens are not the sole driver of the metabolic phenotype in PCOS. Since ovaries of women with PCOS contain an increased number of small growing follicles, a contribution of an altered ovarian growth factor profile to the metabolic phenotype of PCOS cannot be ruled out. Studies have already shown that ovarian growth factors, especially members of the transforming growth factor β (TGFβ) family, may play a role in the ovarian pathogenesis of PCOS. For example, the mRNA expression level of growth differentiation factor 9 (GDF9) is reduced in PCOS (15), while expression of anti-Müllerian hormone (AMH) and bone morphogenetic protein 6 (BMP6) is increased in granulosa cells of PCOS women (16-18). Furthermore, reflecting the increased number of growing follicles and the increased expression per granulosa cell, serum levels of AMH are elevated in women with PCOS (19, 20). This may suggest that serum levels of other ovarian growth factors are also altered in women with PCOS.

To determine whether an altered profile of ovarian growth factors indeed contributes to the regulation of metabolism, we have used mice lacking AMH (AMHKO)

or its specific type II receptor (MRKI) as a model resembling the increased number growing follicles in women with PCOS. AMH is solely expressed in the ovary and in the absence of AMH, mice display enhanced recruitment of primordial follicles (21). As a result, ovaries of AMHKO mice contain 3- to 4-fold more growing follicles compared to wild type mice (22). Mice lacking AMH signaling have a normal ovulation rate and a normal estrous cycle, allowing us to investigate the effect of ovarian growth factors secreted by the increased number of growing follicles on metabolism independent from cycle irregularities.

Our results revealed that female mice lacking AMH signaling have reduced adiposity, with increased browning of white adipose tissues, and an improved glucose tolerance with increasing age.

MATERIALS AND METHODS

Animals

AMH knockout mice (AMHKO) and AMH type II receptor, or Müllerian inhibiting substance (MIS) receptor, knockout mice (MRKI) were generated as described previously (22,23). The MRKI mice were kindly provided by Dr. R. Behringer. Female AMHKO and MRKI mice and wildtype littermates on a C57BL/6J background were obtained from the Animal Facility of the Erasmus MC and were group-housed under standard animal housing conditions in accordance with the National Institutes of Health guidelines for the Care and Use of Experimental Animals. The experiments were performed with permission of the local ethics committee. Mice were analyzed at 4, 5 and 8 months of age.

To determine the stage of the estrous cycle, daily vaginal smears were taken two weeks before the animals were sacrificed and examined as described previously (24). Body weight was measured every two weeks. At the end of the experiment ovaries and uterus were isolated, weighed, and fixed overnight in Bouin's fluid or snap frozen in liquid nitrogen and stored at −80°C until further processing. In addition, white and brown adipose depots were isolated, weighed and either fixed overnight in 4% paraformaldehyde or stored at −80°C for further analysis.

Follicle count

After routine histological procedures, 8 µm sections were mounted on glass slides and stained with hematoxylin and eosin. Follicle count was performed as described previously (24), using one ovary per animal. In brief, follicles were classified based on

their mean diameter, which was determined by measuring two perpendicular diameters per section. Primordial, nonatretic and atretic growing follicles were counted in every fifth section. The presence of corpora lutea was also determined.

Real-time PCR

Real-time PCR was performed as described previously (12). In short, the qPCR mixture contained 6.25 µl FastStart Universal SYBR Green Master mix (Roche Diagnostics, Almere, The Netherlands), 0.5 µl of forward and reverse primers (1.0 pmol/µl) and 4 µl H_2O per reaction. The qPCR reaction was performed using an ABI 7900 HT apparatus. The expression of the target genes was normalized to the average expression level of three housekeeping genes (β2-microglobulin, hypoxanthine-guanine phosphoribosyl transferase (HPRT), and ribosomal protein S29) using the $2^{-\Delta\Delta Ct}$ method (25). Primer sequences of the used target genes are listed in Table 1.

TABLE 1. Primer sequences

	Gene	Sequence (5′-3′)	Accession number
B2m	B2 microglobulin	F: atccaaagctgaagaacgg R: cagtctcagtgggggtgaat	NM_009735.3
Hprt	Hypocanthine-guanine phosphoribosyl transferase	F: gcagtacagccccaaaatgg R: aacaaagtctggcctgtatccaa	NM_013556.2
Rps29	Ribosomal protein S29	F: tgaaggcaagatgggtcac R: gcacatgttcagcccgtatt	NM_009093.2
Ucp1	Ucoupling protein 1	F: ggcctctacgactcagtcca R: taagccggctgagatcttgt	NM_009463.3
HSL	Hormone Sensitive Lipase	F: cgctacacacaaaggctgctt R: ggatggcaggtgtgaactg	NM_001039507.2
ATGL	Adipose Triglyceride Lipase	F: gagccccggggtggaacaagat R: aaaaggtggtgggcaggagtaagg	NM_001039507.2
LPL	Lipoprotein Lipase	F: gctggtgggaaatgatgtg R: tggacgttgtctaggggta	NM_008509.2
CD36	Fatty Acid Translocase	F: cctccagaatccagacaacc R: cacaggctttccttctttgc	NM_008509.2
AMHRII	Anti-Müllerian hormone type II receptor	F: ggggctttggacactgctt R: gtctcggcatccttgcatctc	NM_144547.2

Intraperitoneal glucose tolerance test (IPGTT)

An IPGTT was performed as described previously (12). Glucose levels were measured by tail vain blood sampling using a Freestyle mini glycometer and test strips (Abbott, Alameda, CA). In brief, mice were fasted overnight (16h) after which a baseline glu-

cose measurement was performed. Then mice were injected intraperitoneally with glucose (2g/kg as a 20% glucose solution) and tail sampling was performed at 15, 30, 60 and 120 min.

Hormone measurements

Total adiponectin serum levels were determined using an Adiponectin (Mouse) ELISA. Leptin serum levels were measured with a Leptin (Mouse/Rat) ELISA. C-peptide serum levels were measured with a Mouse C-peptide ELISA. Plasma insulin levels were measured with an ultrasensitive mouse Insulin ELISA (all Alpco Diagnostics, Salem, NH). Triglycerides were measured with an ABX PENTRA Triglycerides CP reagent with associated calibrators (HORIBA ABX, Montpellier, France). Glycerol and non esterified fatty acids (NEFA) were measured by enzymatic calorimetric procedure using a glycerol or a NEFA-HR kit (both Instruchemie, Delfzijl, The Netherlands).

Measurement of adipocyte size

Adipocyte size distribution in white adipose tissue (WAT) was determined as described previously (12). Four mice per age and per genotype were randomly chosen, and hematoxylin and eosin (HE)-stained sections of 8µm were randomly selected per fat depot and three micrographs were taken with a 100x magnification. The diameter of at least 130 adipocytes per micrograph was measured using ImageJ software (version 2.0; http://imagejdev.org), followed by the calculation of the number of adipocytes per similar size class with intervals of 5 µm.

Immunohistochemistry

Mounted sections of 8µm of both white and brown fat depots were deparaffinized, blocked for endogenous peroxidase activity, rinsed with water, and transferred to PBS. Following antigen retrieval, sections were incubated overnight with an Uncoupling Protein 1 (UCP1) rabbit polyclonal antibody (1:1000) (Abcam, Cambridge MA, USA). Next, sections were incubated with Brightvision poly-HRP-Anti Ms/Rb/Rt IgG kit (Immunologic, Duiven, The Netherlands) and the peroxidase activity was developed with 0.07% 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Finally, all sections were counterstained with hematoxylin.

Statistics

SPSS software (version 20.0 SPSS, Inc., Chicago, IL) was used for statistical analysis. Two-way ANOVA was used to measure effects of genotype (WT vs. AMHKO vs. MRKI) and age (4 vs. 5 months) (genotype x age), followed by post-hoc Tukey's test or Student's t-test when significant differences were observed. Body weight gain was analyzed by two-way repeated measures (RM) ANOVA (genotype x age), followed by Bonferroni correction. Three-way RM ANOVA was used to analyzed IPGTT (genotype x age x sample time point). The adipocyte size distribution was analyzed by three-way ANOVA (genotype x age x size). For analyses at 8 months of age, two-way RM ANOVA (IPGTT), two-way ANOVA (adipocyte size distribution), and Student's t-test were used. Data were expressed as mean ± SEM, and differences were considered significant at $P<0.05$.

RESULTS

Increased follicle numbers in AMHKO and MRKI mice

Female AMHKO and MRKI mice displayed a regular estrous cycle at 4 and 5 months of age. Uterine weight, measured to determine changes in ovarian estrogen production, did not differ between the three genotypes at both ages studied (results not shown). Ovarian weight of AMHKO and MRKI mice was significantly increased compared to WT mice at both 4 and 5 months of age ($P<0.001$, results not shown). Two-way ANOVA revealed that genotype significantly affected the number of primordial follicles ($P=0.002$), number of healthy growing follicles ($P<0.001$), and number of atretic follicles ($P<0.001$). AMHKO and MRKI mice both displayed a 2- to 3-fold increase in healthy growing follicles ($P<0.001$ by post-hoc analysis) and a 2-fold lower number of primordial follicles ($P<0.05$ by post-hoc analysis) compared to WT mice at 4 and 5 months of age (Supplemental Figure 1A). Furthermore, ovaries of AMHKO and MRKI mice contained significantly more atretic follicles compared to WT mice at 4 and 5 months of age ($P<0.001$ by post-hoc analysis) (Supplemental Figure 1A).

Normal body weight but improved glucose tolerance in AMHKO and MRKI mice

To characterize the effect of a loss in AMH signaling and the concomitant change in follicle number on metabolism we compared body weights of WT, AMHKO and MRKI mice up to an age of 5 months. Genotype did not affect body weight (Figure

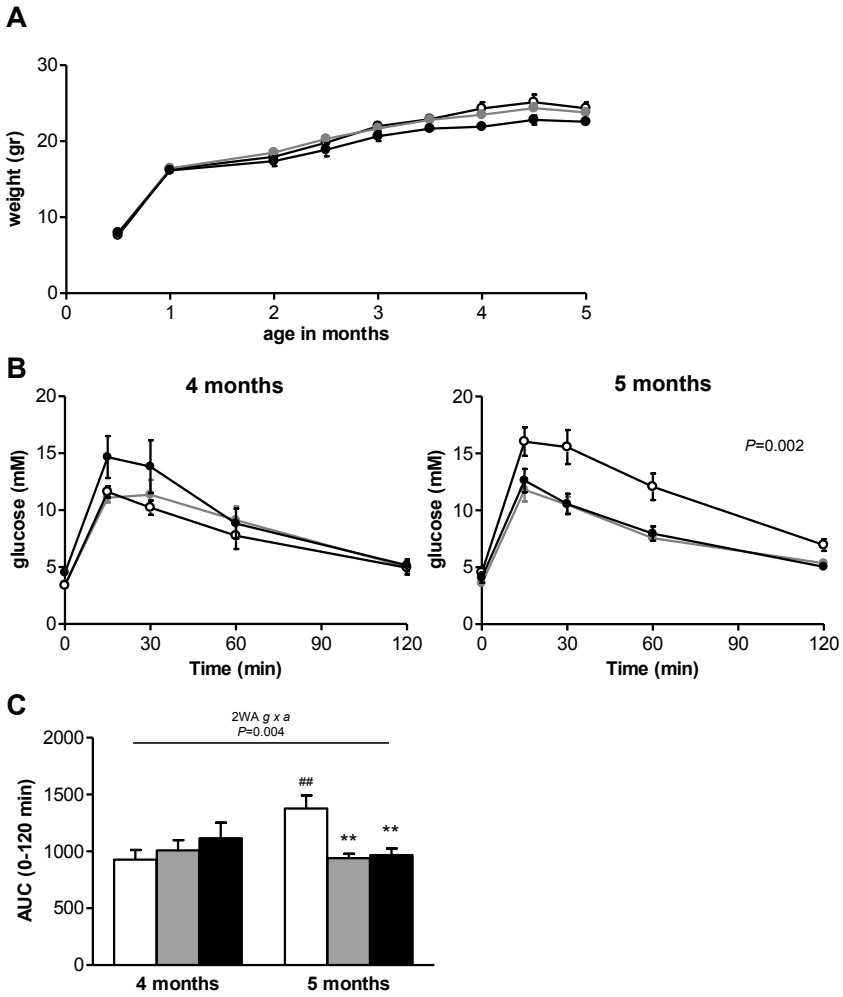


FIGURE 1. Normal body weight but improved glucose tolerance with increasing age in AMHKO and MRKI mice.

A) Body weight was measured for WT mice (open circles), AMHKO mice (closed grey circles) and MRKI mice (closed black circles) every 2 weeks, starting at 2 weeks of age until 5 months of age. Two-way RM ANOVA revealed no significant effect of genotype on body weight. n = 8-17 mice per group. B) The ip glucose tolerance test was performed 5 days before sacrifice at 4 and 5 months of age (WT mice, open circles; AMHKO mice, closed grey circles; MRKI mice, open black circles). For clarity IPGTT graphs are presented per age. Three-way RM ANOVA indicated a significant interaction between genotype, age, and sample time point (P=0.006). RM ANOVA indicated a significant difference between genotypes at 5 months of age (P=0.002), but not at 4 months of age. There was a significant effect of age on glucose tolerance in WT mice (P=0.003). C) Corresponding 0-120 minutes AUC for WT (open bars), AMHKO (grey bars) and MRKI (black bars) mice. Two-way ANOVA indicated a significant interaction between genotype and age (P=0.004). Data represent mean ± SEM (n = 8-9). **, P<0.01, significant differences from WT of same age; $^{\#}$, P<0.05, significant difference between 4 and 5 months, by posthoc tests.

1A). AMHKO and MRKI mice showed a similar gain in body weight with increasing age as WT mice. In addition, we determined the glucose tolerance of these mice. Statistical analysis indicated a significant interaction between genotype, age, and sample time point (P=0.006). Also for the 0-120 min AUC values a significant interaction between genotype and age was observed (P=0.004 by two-way ANOVA). Post-hoc analysis showed that at 4 months of age glucose levels during the IPGTT and the 0-120 min AUC values did not differ between genotypes (Figure 1B and C). In contrast, at 5 months of age AMHKO and MRKI mice had a significantly higher glucose clearance than WT mice (P<0.01 by post-hoc analysis, Figure 1B), reflected by significantly lower 0-120 min AUC values (P<0.01 by post-hoc analysis) (Figure 1C). Post-hoc analysis also indicated that WT mice displayed a significant worsening in glucose tolerance with increasing age (P=0.003), which is reflected by the increased 0-120 min AUC values at 5 months of age compared to 4 months of age (P<0.01 by post-hoc analysis). In contrast, AMHKO and MRKI mice did not show this age-dependent worsening in glucose tolerance (Figure 1B and C). Fasting glucose and insulin levels did not differ between WT, AMHKO, and MRKI mice at both ages. C-peptide levels increased in WT mice with increasing age, while this did not occur in the AMHKO and MRKI mice (Table 2).

TABLE 2. Hormone and lipid levels in WT, AMHKO, and MRKI mice.

	4 months			5 months		
	WT	**AMHKO**	**MRKI**	**WT**	**AMHKO**	**MRKI**
Fasting glucose (mM)	3.38 ± 0.17	3.48 ± 0.31	4.37 ± 0.28	4.54 ± 0.42	3.57 ± 0.27	4.09 ± 0.23
Insulin (ng/ml)	0.67 ± 0.13	0.64 ± 0.08	0.43 ± 0.16	0.56 ± 0.07	0.83 ± 0.13	0.61 ± 0.14
C-Peptide (pM)	532.81 ± 34.40	733.95 ± 78.64*	691.56 ± 50.78	712.30 ± 32.43$^{\#\#}$	684.45 ± 27.80	539.63 ± 56.20*
Leptin (ng/ml)	3.66 ± 0.38	1.48 ± 0.30***	0.74 ± 0.23***	4.69 ± 1.20	2.96 ± 0.45	0.81 ± 0.13**
Adiponectin (µg/ml)	34.34 ± 2.03	36.17 ± 1.79	24.21 ± 2.29**	34.29 ± 1.44	37.88 ± 1.19	25.31 ± 3.02*
Triglycerides (mM)	0.83 ± 0.06	1.01 ± 0.09	0.69 ± 0.03	0.95 ± 0.06	0.70 ± 0.03$^{**,\#\#}$	0.66 ± 0.05**
Glycerol (mmol/L)	0.51 ± 0.03	0.50 ± 0.03	0.43 ± 0.02	0.52 ± 0.03	0.44 ± 0.03	0.39 ± 0.02*
NEFA (mmol/L)	1.02 ± 0.05	0.97 ± 0.06	0.92 ± 0.05	1.18 ± 0.05	1.11 ± 0.07	0.86 ± 0.09*

Serum samples were collected when mice were 4 months or 5 months old. Two-way ANOVA indicated a significant effect of genotype on leptin, adiponectin, glycerol, and NEFA levels (2WA *g*: P<0.001, P<0.001, P=0.003, and P=0.006, respectively). For c-peptide and triglyceride levels there was a significant interaction between genotype and age (2WA *g x a*: P=0.007 and P=0.003, respectively). Values represent mean ± SEM (n = 8-10 mice per group). *, P<0.05; **, P<0.01; ***, P<0.001, significant differences from WT of same age; $^{\#\#}$, P<0.01, significant difference between 4 and 5 months, by posthoc tests.

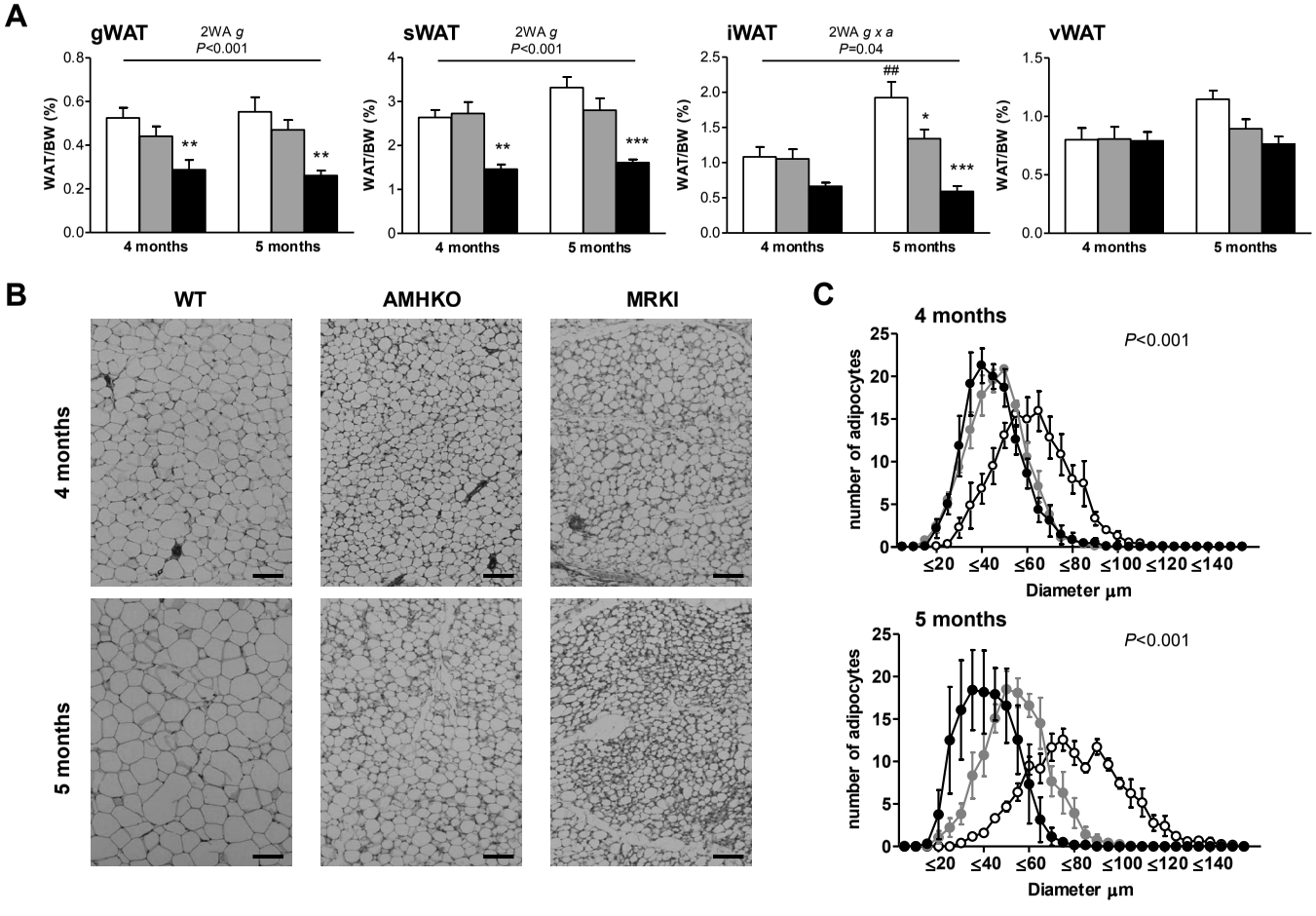
Altered adipokine and lipid profile in mice lacking AMH signaling

To elucidate the improved glucose tolerance in AMHKO and MRKI mice during aging, we measured serum levels of adipokines and lipids. By two-way ANOVA, there was a significant effect of genotype on leptin levels ($P<0.001$) independent of age. Leptin levels were 2.5-fold lower in AMHKO mice and 5-fold lower in MRKI mice compared to WT mice at 4 months of age ($P<0.001$ by post-hoc analysis). Leptin levels remained decreased in 5- month-old AMHKO and MRKI mice by a comparable magnitude, although the difference with WT mice remained significant only for the MRKI mice ($P<0.01$ by post-hoc analysis) (Table 2). Also for adiponectin, a significant effect of genotype was observed. Although adiponectin levels did not differ between WT and AMHKO mice, levels were significantly lower in MRKI mice at both ages studied ($P<0.01$ and $P<0.05$, respectively, by post-hoc analysis) (Table 2). There was a significant interaction between genotype and age on triglyceride levels ($P=0.003$). Levels were markedly decreased in 5-month-old AMHKO and MRKI mice compared to WT mice ($P<0.01$ by post-hoc analysis). There was a significant effect of genotype on serum glycerol and NEFA levels ($P=0.006$), since levels were significantly decreased in 5-month-old MRKI mice ($P<0.05$ by post-hoc analysis) (Table 2).

Reduced adiposity in mice lacking AMH signaling

To further understand the improved glucose tolerance in AMHKO mice in the presence of a normal body weight, we analyzed several WAT depots (Figure 2). There was a significant effect of genotype on the relative weight of gonadal WAT (gWAT) and subcutaneous WAT (sWAT) ($P<0.001$). Furthermore, there was a significant interaction

FIGURE 2. Reduced adiposity in mice lacking AMH signaling.
A) Percentage adipose depot weight relative to body weight in WT (open bars), AMHKO (grey bars), and MRKI mice (black bars). Two-way ANOVA indicated a significant interaction between genotype and age for iWAT (2WA *g x a*: $P=0.04$). There was a significant effect of genotype on gWAT and sWAT (2WA *g*: $P<0.001$). Data represent mean ± SEM (n=8-17 except for vWAT: n = 5-8 mice per group). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, significant differences from WT of same age; $^{\#\#}$, $P<0.01$, significant difference between 4 and 5 months, by posthoc tests. B) Representative HE-stained section of sWAT of WT, AMHKO, and MRKI mice of 4 and 5 months of age. Scale bar = 100 μm. C) Adipocyte size distribution in WT (open circles), AMHKO (closed grey circles), and MRKI (closed black circles) mice. In each group the diameter of a total of 130 adipocytes was measured in three randomly selected sections per mouse. Three-way ANOVA indicated a significant interaction between genotype, age and size ($P<0.001$). RM ANOVA indicated a significant difference between genotypes at 4 and 5 months of age ($P<0.001$). There was a significant effect of age on adipocyte size distribution in WT mice ($P<0.001$). For clarity adipocyte size distribution graphs are presented per age. Data represent mean ± SEM (n=4 mice per group).


A
gWAT
2WA g
P<0.001
sWAT
2WA g
P<0.001
iWAT
2WA g x a
P=0.04
vWAT
WAT/BW (%)
4 months
5 months
B
WT
AMHKO
MRKI
4 months
5 months
C
4 months
P<0.001
5 months
P<0.001
number of adipocytes
≤20 ≤40 ≤60 ≤80 ≤100 ≤120 ≤140
Diameter µm

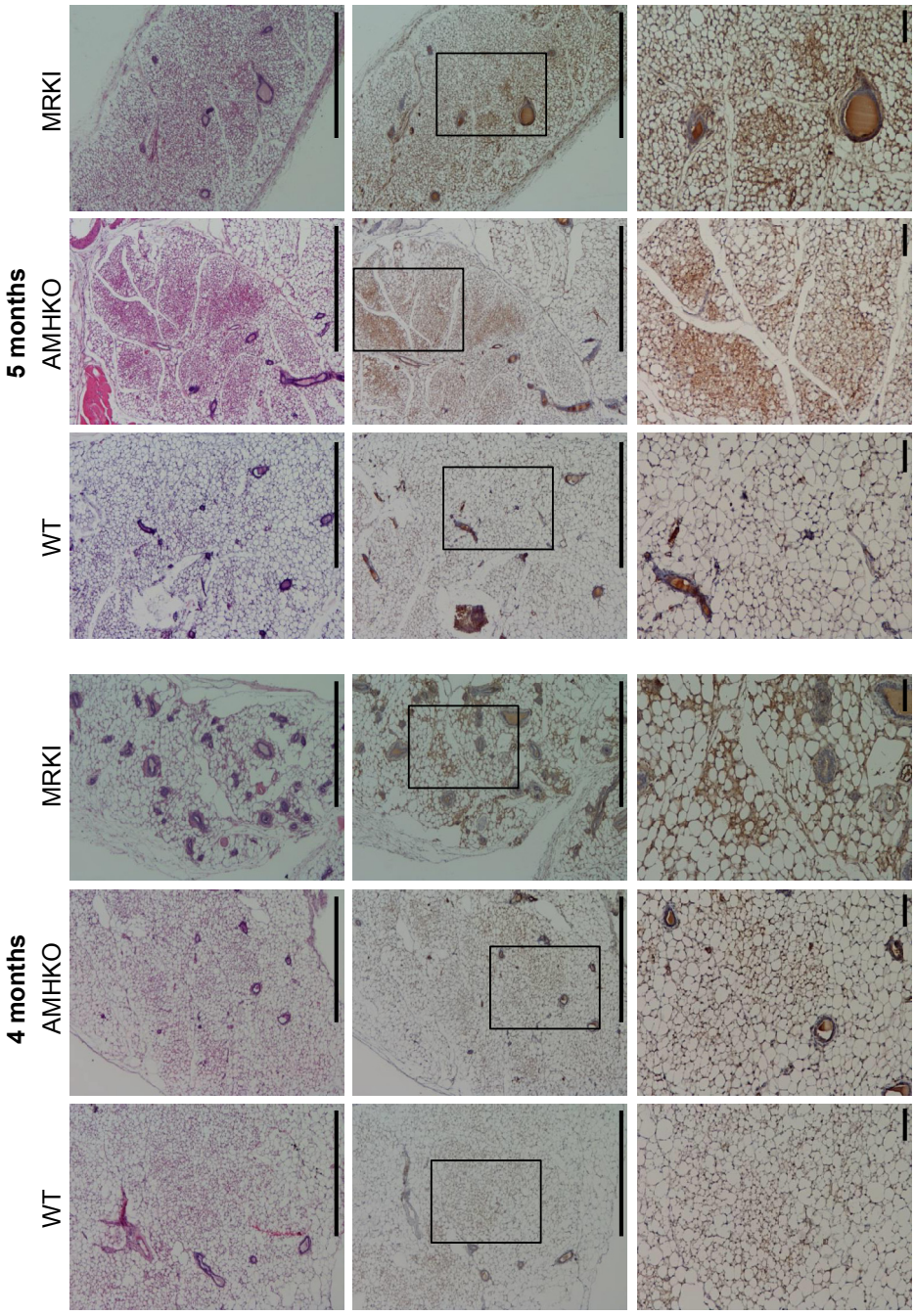
between genotype and age for the relative weight of inguinal WAT (iWAT) (P=0.04). The weight of these WAT depots did not differ between WT and AMHKO mice at 4 months of age, but was significantly lower in MRKI mice (P<0.01 by post-hoc analysis) (Figure 2A). This difference in depot weight, between WT and MRKI mice remained present at 5 months of age (P<0.01 by post-hoc analysis). Also in 5-month-old AMHKO mice the weight of iWAT was significantly lower compared to WT mice (P<0.05 by post-hoc analysis). Interestingly, while iWAT of WT mice showed a significant increase in weight with increasing age (P<0.01 by post-hoc analysis), this increase did not occur for iWAT of AMHKO and MRKI mice (Figure 2A). No significant differences between genotypes were observed for the relative weight of visceral WAT (vWAT).

Histological analysis of sWAT revealed that at 4 months of age AMHKO and MRKI mice had smaller adipocytes compared to WT mice (Figure 2B). This difference in adipocyte size between WT mice and mice lacking AMH signaling was even more pronounced at 5 months of age (Figure 2B). Measurement of the cell diameter of the adipocytes confirmed that sWAT of AMHKO and MRKI mice contained significantly more smaller adipocytes compared to WT mice (P<0.001 by three-way ANOVA, genotype x age x size of the adipocytes), reflected by the left shift in the size distribution at both ages (Figure 2C). The decrease in subcutaneous adipocyte size was also reflected by the average cell diameter, for which there was a significant interaction between genotype and age (P<0.003). The average cell diameter significantly increased with age in WT mice (61.6 ± 2.2 μm vs. 81.8 ± 3.3 μm, at 4 and 5 months of age respectively, P<0.002 by post-hoc analysis). In contrast, the average diameter of subcutaneous adipocytes in AMHKO and MRKI mice did not significantly change with increasing age (AMHKO: 45.6 ± 1.4 μm vs. 53.2 ± 3.1 μm, respectively, P=0.06 by post-hoc analysis; MRKI: 44.0 ± 2.8 μm vs. 40.3 ± 4.2 μm, respectively, P=0.49 by post-hoc analysis). Similar findings were obtained for gWAT (data not shown).

Increased browning of WAT and increased BAT activity in mice lacking AMH signaling

Analysis of the morphology of sWAT revealed that sWAT of AMHKO and MRKI mice contained islets of multilocular adipocytes interspersed within the larger uni-

FIGURE 3. Increased browning of sWAT in mice lacking AMH signaling.
Top panel: HE-stained sections of sWAT showing islets of multilocular beige adipocytes. sWAT of AMHKO and MRKI contained more dense islets compared to WT mice. Middle panel: Immunohistochemical analysis of UCP1 expression in sWAT using adjacent sections. UCP1-positive staining diminished in WT mice while a stronger staining was observed in AMHKO and MRKI mice with increasing age. Bottom panel: 2.5-fold magnification of the inset of the middle panel. Top and middle panel: scale bar = 1000 μm; bottom panel: scale bar = 100 μm.


4 months
5 months
WT
AMHKO
MRKI
WT
AMHKO
MRKI

locular adipocytes (Figure 3), which is indicative for the presence of brown-like or beige adipocytes. Indeed, immunohistochemistry for the BAT-specific marker UCP1 confirmed the presence of UCP1-positive cells in sWAT (Figure 3). UCP1 staining intensity did not differ between genotypes at 4 months of age. However, at 5 months of age, UCP1-positive staining was stronger in sWAT of AMHKO and MRKI mice compared to WT mice. Interestingly, at 5 months of age sWAT of AMHKO and MRKI mice contained more islets of beige adipocytes and more intense staining of UCP1 than at 4 months of age, whereas in WT mice UCP1 staining intensity diminished (Figure 3).

Islets of beige adipocytes were also observed in iWAT. At 4 months of age, iWAT of all three genotypes contained islets of beige adipocytes (Supplemental Figure 2A and B). However, UCP1-positive staining was more intense in iWAT of AMHKO and MRKI mice compared to iWAT of WT mice. With increasing age, the presence of beige adipocyte islets disappeared in iWAT of WT and AMHKO mice but not in iWAT of MRKI mice (Supplemental Figure 2B).

Because AMHKO and MRKI mice have reduced adiposity and increased browning of their WAT depots, we also determined whether the function of the classical BAT depot was affected in AMHKO and MRKI mice. The weight of interscapular BAT was not affected between genotypes (data not shown), however histological analysis of BAT showed that at 4 months of age BAT of AMHKO mice had a more compact structure compared to BAT of WT mice, which was even more pronounced in MRKI mice (Figure 4A). Surprisingly, immunohistochemistry for UCP1 showed a weaker staining of UCP1 in BAT of 4-month-old AMHKO and MRKI mice compared to WT mice (Figure 4B). At 5 months of age, lipids had accumulated in BAT of WT mice resulting in the appearance of unilocular lipid droplets. This accumulation of lipids was reduced in BAT of AMHKO mice and appeared absent in BAT of MRKI mice (Figure 4A). At 5 months of age UCP1-positive staining was stronger in BAT of AMHKO and MRKI mice compared to WT mice (Figure 4B).

Increased lipolysis and decreased lipogenesis in BAT and sWAT of mice lacking AMH signaling

To elucidate whether an altered lipid metabolism could explain the reduced fat mass in AMHKO and MRKI mice, we determined the expression of the lipolytic genes hormone sensitive lipase (HSL) and adipose TG lipase (ATGL), and the lipogenetic genes lipoprotein lipase (LPL) and CD36, also known as fatty acid translocase. In BAT, analysis indicated a significant interaction between genotype and age for HSL (P=0.016) and ATGL (P=0.025). There was a highly significant effect of genotype on the expression of LPL and CD36 independent of age (P=0.001) (Figure 5A).

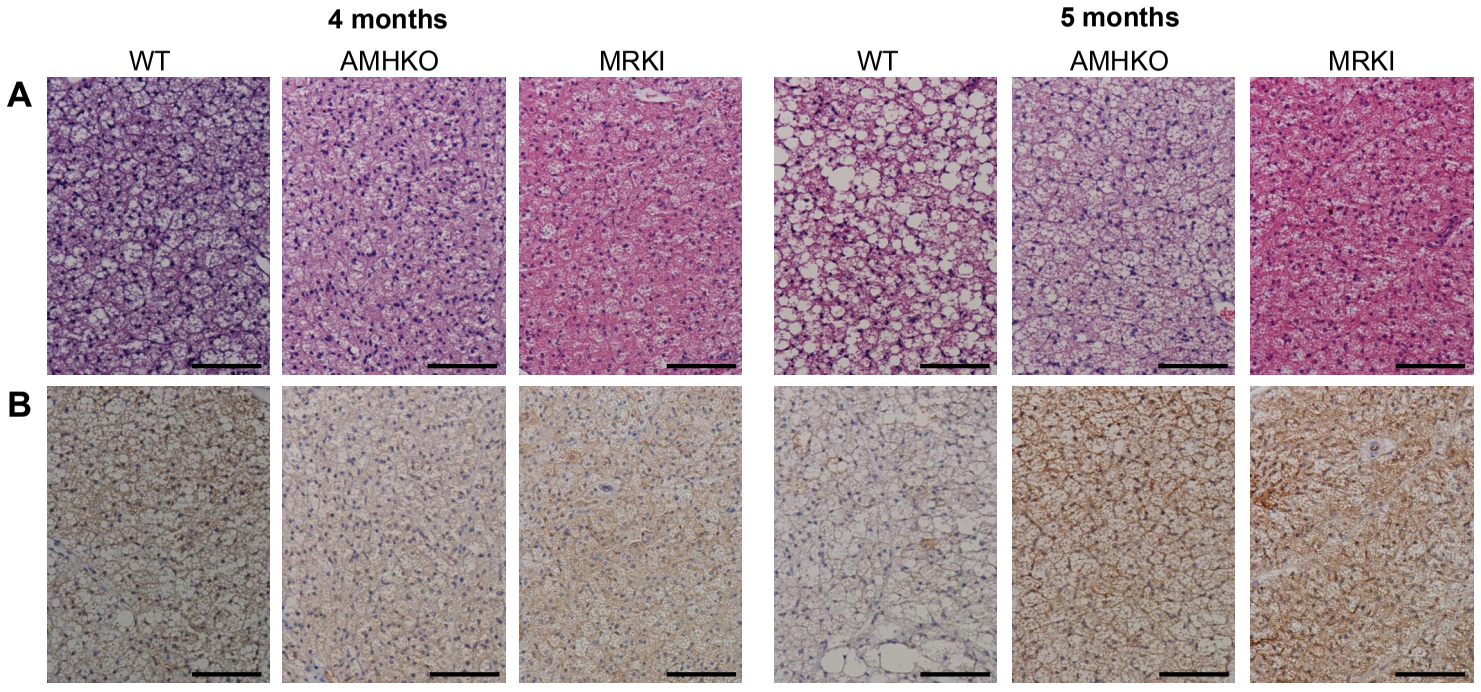


FIGURE 4. AMHKO and MRKI mice have increased BAT activity with aging.
A) HE-stained section of BAT of WT, AMHKO, and MRKI mice at 4 and 5 months of age. B) Adjacent section with immunohistochemical UCP1 expression. A weaker UCP1 staining is present in BAT of 4-month-old AMHKO and MRKI mice compared to BAT of WT mice. UCP1 immunoreactivity decreases in BAT of 5-month-old WT mice, while increased immunoreactivity is observed in 5-month-old AMHKO and MRKI mice. Scale bar = 100 µm.

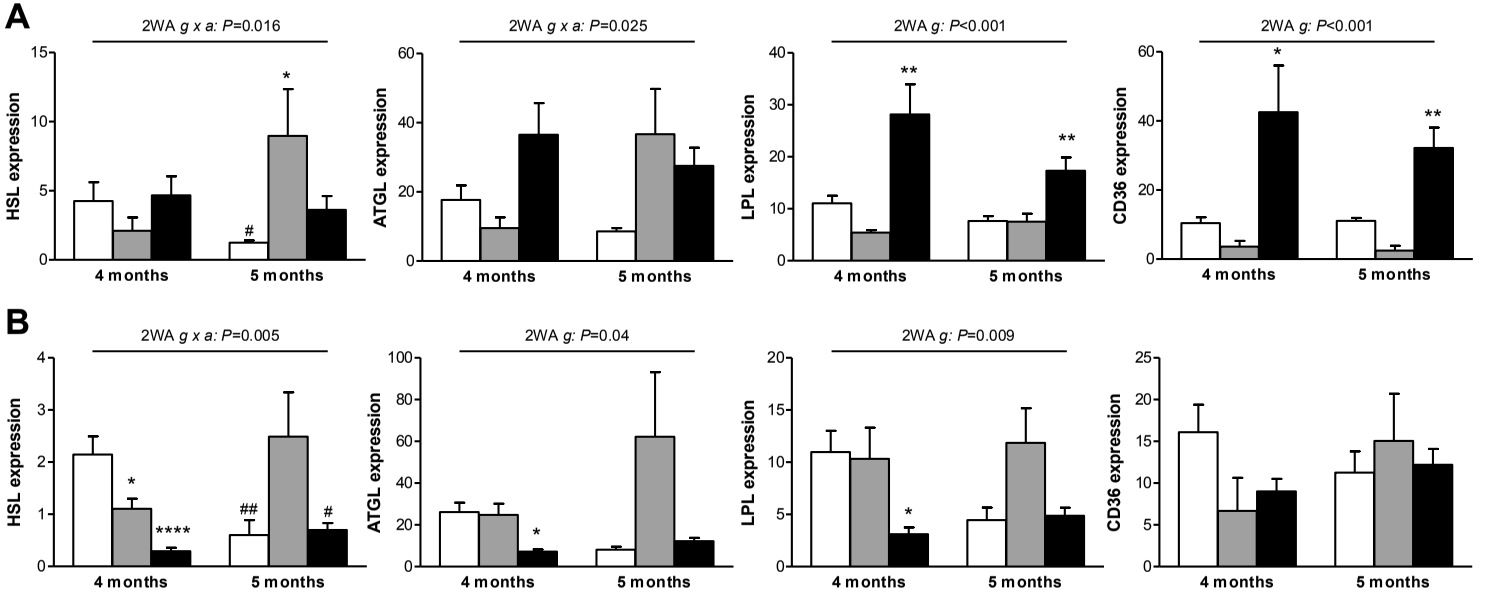


FIGURE 5. Analysis of lipolytic and lipogenic genes in BAT and sWAT of WT, AMHKO and MRKI mice.
A) mRNA expression of the lipolytic genes HSL and ATGL and the lipogenic genes LPL and CD36 in BAT of WT (open bars), AMHKO (grey bars), and MRKI mice (black bars). Two-way ANOVA indicated a significant interaction between genotype and age for HSL (2WA *g x a*: *P*=0.016) and ATGL expression (2WA *g x* a: *P*=0.025). There was a significant effect of genotype on LPL (2WA *g*: *P*<0.001) and CD36 expression (2WA *g*: *P*<0.001). B) mRNA expression of the same genes in sWAT of WT (open bars), AMHKO (grey bars), and MRKI mice (black bars). Two-way ANOVA indicated a significant interaction between genotype and age for HSL (2WA *g x a*: *P*=0.005). Genotype had a significant effect on the expression of ATGL (2WA *g*: *P*=0.04) and LPL (2WA *g*: *P*=0.009). Data represent mean ± SEM (n=6 mice per group). *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001, significant differences from WT of same age; #, *P*<0.05; ##, *P*<0.01, significant difference between 4 and 5 months, by posthoc tests.

In BAT of AMHKO and MRKI mice the mRNA expression of HSL and ATGL was increased compared to WT mice at 5 months of age ($P<0.05$ and P=0.07, respectively by post-hoc analysis). Interestingly, the expression of these genes tended to decrease in BAT of WT mice with increasing age ($P=0.054$ and $P=0.06$, respectively by post-hoc analysis), whereas a comparable or increased expression was observed in AMHKO and MRKI mice (Figure 5A). The mRNA expression of LPL and CD36 were significantly increased in BAT of MRKI mice compared to WT mice at both ages (LPL: $P<0.01$; CD36: $P<0.05$, by post-hoc analysis) (Figure 5A).

In sWAT, there was a significant interaction between genotype and age by two-way ANOVA for the mRNA expression of HSL ($P=0.005$) (Figure 5B). HSL mRNA expression was decreased in sWAT of AMHKO and MRKI mice at 4 months of age ($P<0.05$ and $P<0.001$, respectively, by post-hoc analysis). Furthermore, while an age-dependent decrease in mRNA expression of HSL mRNA was observed in WT mice ($P=0.009$ by post-hoc analysis), HSL mRNA increased in sWAT of MRKI mice ($P=0.019$ by post-hoc analysis) (Figure 5B). Genotype had a significant effect on the expression of ATGL ($P=0.04$) and LPL ($P=0.009$) independent of age, since ATGL and LPL mRNA expression were decreased in sWAT of 4-month-old MRKI mice compared to WT mice ($P<0.05$ by posthoc analysis) (Figure 5B).

AMHRII mRNA is not expressed in metabolic tissues.

To assess whether the observed metabolic phenotype in AMHKO and MRKI mice could be the result of a direct effect of AMH on metabolic tissue, AMHRII mRNA expression was analyzed by real-time PCR in all adipose depots, muscle, pancreas, liver and hypothalamus. AMHRII mRNA could not be detected in any of the metabolic tissues, while expression could easily be detected in the ovary (results not shown).

Improved metabolic phenotype in AMHKO mice of advanced age

Next we examined whether the improved metabolic phenotype in AMHKO mice persisted at an older age. Unfortunately, MRKI mice were not available for this aging study. It is known that ovaries of AMHKO mice are depleted of their follicles at a younger age than WT mice (22). Indeed, the number of growing follicles was significantly reduced in 8-month-old AMHKO mice compared to WT mice ($P=0.01$). Compared to 4- and 5-months old mice, the number of growing follicles had decreased nearly 4-fold in the aged AMHKO mice, whereas in 8-month-old WT mice the number of growing follicles did not differ from 4- and 5-month old WT mice. Furthermore, ovaries of 8-month-old AMHKO mice were severely depleted

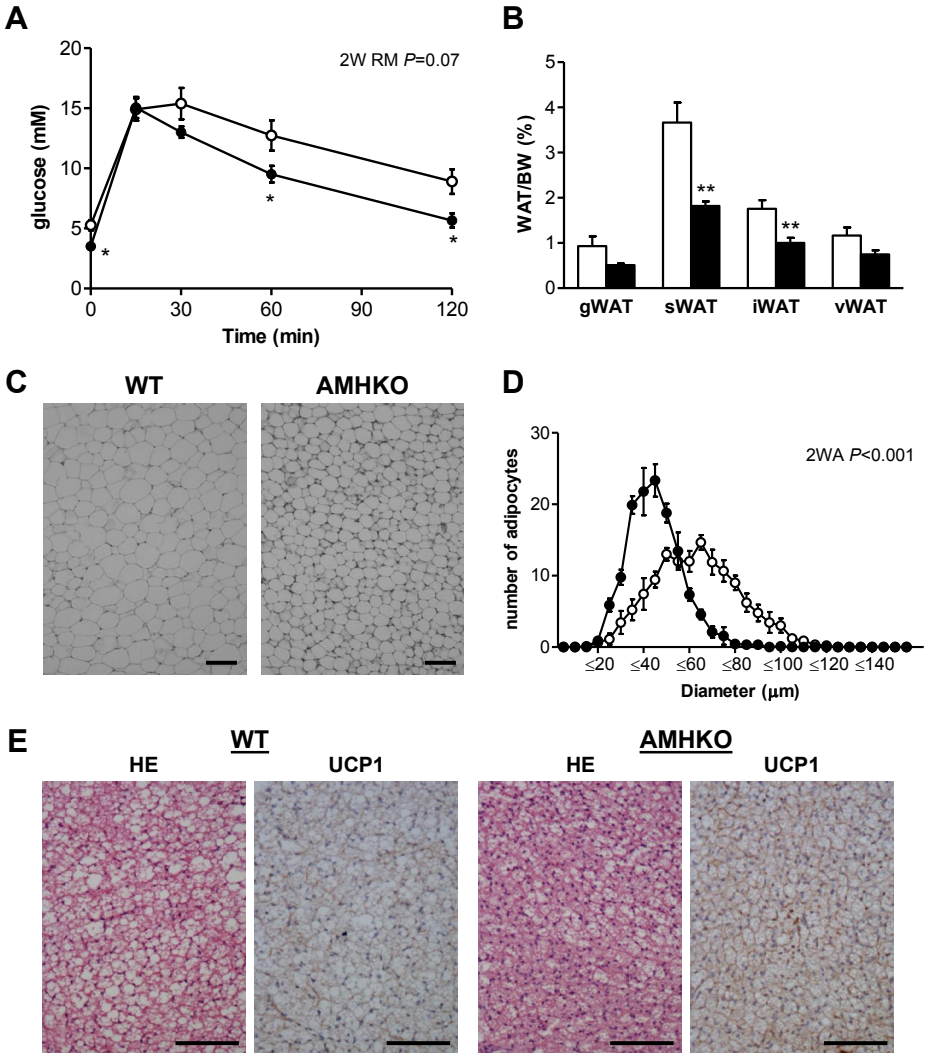


FIGURE 6. Metabolic analyses of 8-month-old WT and AMHKO mice.
A) The ip glucose tolerance test was performed 5 days before sacrifice at 8 months of age (WT mice, open circles; AMHKO mice, closed circles). Two-way RM ANOVA indicated a borderline significant effect of genotype (P=0.07). AMHKO mice have a better glucose clearance compared to WT mice (P<0.05 at 60 and 120 min) (n=6 mice per group). B) Percentage adipose depot weight relative to body weight in WT (open bars) and AMHKO (black bars) mice (n=6 mice per group). C) HE-stained sections of sWAT of 8-month-old WT and AMHKO mice. Scale bar = 100 µm. D) Adipocyte size distribution in WT (open circles) and AMHKO (closed circles) mice. In each group the diameter of a total of 130 adipocytes was measured in three randomly selected sections per mouse (n=3 mice per group). Data represent mean ± SEM. *, P<0.05; **, P<0.01, significant differences from WT mice. E) HE-stained section and section with immunohistochemical UCP1 expression of BAT of WT and AMHKO mice. A stronger UCP1 staining is present in BAT of AMHKO compared to BAT of WT mice. Scale bar = 100 µm.

of primordial follicles, containing nearly 5-fold less primordial follicles compared to WT mice ($P<0.001$) (Supplemental Figure 1B).

At 8 months of age, body weight did not significantly differ between WT and AMHKO mice (27.88 ± 2.24 gr vs. 24.83 ± 0.84 gr, $P=0.23$). However, AMHKO mice tended to have a better glucose tolerance compared to their WT littermates (Figure 6A) (two-way RM ANOVA $P=0.07$). Particularly, AMHKO mice showed a significantly improved recovery phase of glucose levels upon IPGTT ($P<0.05$). In agreement with the observations at the younger ages, 8-month-old AMHKO mice had significantly decreased WAT depot weights compared to WT mice (Figure 6B). Histological analysis of sWAT revealed that 8-months-old AMHKO mice had smaller adipocytes compared to WT mice (Figure 6C). Measurement of adipocyte cell diameter confirmed that sWAT of AMHKO mice contained significantly more small adipocytes compared to WT mice ($P<0.001$ by two-way ANOVA) (Figure 6D). Similar results were observed in gWAT (results not shown). At this age, sWAT of AMHKO mice still contained some beige adipocytes, but islets were less dense than at a younger age (results not shown). Although lipid accumulation had increased in BAT of 8-month-old AMHKO mice compared to 5-month-old AMHKO mice, lipid accumulation remained reduced compared to 8-month-old WT mice. Likewise, UCP1 staining remained stronger in AMHKO mice compared to WT mice (Figure 6E).

Hormone measurements revealed that fasting insulin levels and C-peptide levels were significantly decreased in AMHKO mice compared to WT mice ($P<0.01$) (Table 3). NEFA levels were significantly increased in AMHKO mice compared to WT mice ($P<0.05$). No significant differences were observed in leptin, adiponectin, triglycerides and glycerol levels (Table 3).

TABLE 3. Hormone and lipid levels in 8-months-old WT and AMHKO mice.

	8 months	
	WT	**AMHKO**
Fasting glucose (mM)	5.27 ± 0.43	3.52 ± 0.32**
Insulin (ng/ml)	0.75 ± 0.05	0.45 ± 0.06**
C-Peptide (pM)	625.42 ± 26.01	460.59 ± 31.66**
Leptin (ng/ml)	5.58 ± 1.50	3.39 ± 0.79
Adiponectin (µg/ml)	43.89 ± 3.06	39.50 ± 2.52
Triglycerides (mM)	0.72 ± 0.09	0.86 ± 0.03
Glycerol (mmol/L)	0.48 ± 0.07	0.53 ± 0.03
NEFA (mmol/L)	0.15 ± 0.05	0.31 ± 0.04*

Serum samples were collected when mice were 8 months old. Values represent mean ± SEM (n = 6 mice per group). *, $P<0.05$; **, $P<0.01$, significant differences from WT.

DISCUSSION

In this study, we show that female mice deficient in AMH signaling have an improved metabolic phenotype with increasing age compared to WT mice. AMHKO and MRKI mice had an improved glucose tolerance, reduced adiposity and overall smaller adipocytes. This reduced adiposity was also reflected by decreased leptin levels, and lower reduced glucose and insulin levels in 8-month-old AMHKO mice.

Energy homeostasis is established by a balance between energy intake and energy expenditure. WAT is responsible for energy storage in the form of triglycerides, while BAT is responsible for non-shivering thermogenesis through oxidation of lipids, mediated by its energy burning capacity which is regulated by UCP1 activity (26, 27). Aging is associated with an increase in obesity, reduced insulin sensitivity, and increased serum leptin levels (28, 29). This energy imbalance may, in part, be due to a diminished function of BAT leading to reduced UCP1 expression with increasing age (30-32). Indeed, in the present study, WT mice showed a worsening in glucose tolerance and an increase in WAT depot weight with enlarged adipocytes. These effects of metabolic aging were already evident at 5 months of age and are in agreement with a previous study in female mice in which an increase in body weight and leptin levels was observed between 3 and 6 months of age (33). Interestingly, this worsening in metabolism at a relatively young age did not occur in mice lacking AMH signaling. Furthermore, AMHKO mice remained a healthier metabolic phenotype than WT mice at an older age (8 months). During aging the percentage body fat increases while lean mass decreases (34). AMHKO and MRKI mice showed reduced adiposity with a normal body weight, suggesting that AMHKO and MRKI mice may have an increased lean body mass. Unfortunately, assessment of body composition was not available for this study, and will need to be performed in future studies.

The reduced weight of WAT depots in mice lacking AMH signaling suggests that energy expenditure may be increased in these mice. Increased BAT activity has been associated with increased energy expenditure and reduced adiposity (27). Indeed, we observed that BAT morphology differed between genotypes. BAT of AMHKO and particularly of MRKI mice showed an increased abundance of multilocular adipocytes, giving it a denser appearance. Furthermore, the age-related decline in BAT activity in WT mice, illustrated by lipid accumulation and reduced UCP1 expression, was significantly reduced in mice lacking AMH signaling. The absence of the age-dependent decrease in expression of the lipolytic genes HSL and ATGL in combination with increased UCP1 expression in AMHKO and MRKI mice suggest that these mice have an improved lipid metabolism and mitochondrial function compared to WT mice. Aging and the age-related decline in BAT activity can be delayed by caloric restriction (35, 36). Nevertheless, preliminary results suggest that food intake was not

reduced in 5-month-old mice lacking AMH signaling. Detailed energy expenditure analyses are therefore necessary to confirm that energy expenditure is increased in AMHKO and MRKI mice. However, the finding that the metabolic protection in these mice is accompanied by an increased number of beige adipocytes interspersed within WAT depots strongly supports such a conclusion. An increasing number of studies suggest that these beige adipocytes contribute to energy expenditure, since they phenotypically and functionally resemble classical BAT (37, 38). Particularly sWAT of AMHKO and MRKI mice exhibited an increased brown-like phenotype. Recently, it was shown that in male mice these beige adipocytes in sWAT disappear during aging, independently of adiposity, and that this brown-to-white transition was already initiated before 4 months of age (39). Also in our female WT mice we observed a near complete disappearance of beige adipocytes in sWAT at 5 months of age. In contrast, at this age, AMHKO and MRKI mice even showed increased browning of sWAT. These beige adipocytes in sWAT of AMHKO mice eventually also disappeared, since at 8 months of age only a few brown adipocyte islets could be observed.

AMH is a gonadal-specific member of the TGFβ superfamily, signaling through its specific AMH type II receptor and type I receptors which are shared by BMPs (40). In the ovary, AMH is expressed by granulosa cells of small growing follicles and regulates folliculogenesis. Previously, we showed that in the absence of AMH, primordial follicle recruitment is enhanced resulting in an increased number of small growing follicles, and ultimately cessation of ovarian function at an earlier age (22). Here, we show that MRKI mice are an ovarian phenocopy of AMHKO mice, except that the primordial follicle pool was reduced at a faster rate. Also the metabolic phenotype appeared more pronounced in MRKI mice, with MRKI mice having smaller adipocytes and more intense browning of sWAT than AMHKO mice. This suggests that AMHRII may have an AMH-independent role, and that the loss of this basal receptor activity in the MRKI mice causes a more pronounced phenotype. Alternatively, other ligands may signal through AMHRII since a high degree of crosstalk or promiscuity exists for ligands and receptors of the TGFβ family. For instance, a receptor can interact with multiple ligands, as was illustrated by the different phenotypes in mice deficient for BMP2 and BMP4 or BMPR2 and BMPR1A (41-44). Since AMHRII interacts with BMP type I receptors, BMP ligands are potential candidate ligands for AMHRII. However, similar to AMH, AMHRII is solely expressed in reproductive organs (45), and we were unable to detect expression of AMHRII in metabolic tissues, such as WAT and liver, making a biological action beyond the reproductive system unlikely. This strongly suggests that the observed protection from metabolic aging in AMHKO and MRKI mice is not a direct effect of loss of AMH signaling, but instead may be driven by the altered follicle pool of these mice. The increased number of growing follicles could lead to altered levels of secreted ovarian growth

factors, as illustrated by increased inhibin B levels, which we showed previously (22). Members of the TGFβ family are potential candidates given their ovarian expression and their important role in brown and brown-like adipocyte differentiation. Several BMP ligands have been shown to stimulate brown adipogenesis. Overexpression of BMP7 or treatment with BMP7 induced brown fat mass, BAT activity and browning of WAT in mice (46, 47). BMP8b was reported to increase BAT activity in mice in vitro and in vivo studies (48). Furthermore, in a recent study, BMP6 was shown to induce brown adipocyte differentiation in murine C2C12 myoblast cells and human skeletal muscle precursor cells (49). Interestingly, loss of BMPR1A signaling in brown adipogenic precursor cells resulted in reduced interscapular BAT formation but increased recruitment of brown adipocytes in sWAT upon cold exposure (50), indicating the presence of compensating mechanisms in the regulation of thermogenesis. In contrast, TGFβ and activin signaling have inhibitory effects on brown adipogenesis. Inhibition of TGFβ signaling through genetic deletion of Smad3 (51, 52), genetic deletion of myostatin/GDF8 (53, 54), or inhibition of activin signaling using a soluble decoy receptor ActRIIB-Fc or an ActRIIB neutralizing antibody (55, 56), all led to browning of WAT, reduced obesity, and increased energy expenditure in mice. Given the plethora of effects and interaction with receptors by TGFβ superfamily members, it is currently difficult to identify the responsible factor secreted by ovaries deficient in AMH signaling. In addition, other growth factors such as VEGF and several FGF family members have also been implicated in brown and brown-like adipocyte proliferation and differentiation (57-61), and are also expressed by the ovary. Future studies are aimed at the identification of the ovarian factor(s) that contribute to the maintenance of a healthier metabolic phenotype in mice lacking AMH signaling. Interestingly, the metabolic protection persists at older ages, despite the fact that AMHKO mice cease ovarian function at an earlier age (this study and (22)). During reproductive life, estrogens are known to protect against lipid accumulation in adipose tissues. After menopause in women, this protective effect is lost due to sharp decline in estrogen levels (62). Thus we anticipated that AMHKO mice would show advanced adiposity with increasing age compared to WT mice. In contrast, exposure to the altered ovarian growth factor profile during reproductive life apparently induces a favorable metabolic phenotype that can, at least partly, prevent the negative metabolic effects of estrogen loss.

In conclusion, our results suggest that changes in follicle dynamics affect metabolism. In the presence of an increased pool of growing follicles, as present in mice lacking AMH signaling, this results in protection from metabolic aging through effects on both WAT and BAT. Identification of the ovarian growth factors responsible for the improved metabolic phenotype might provide novel targets in the treatment of obesity.

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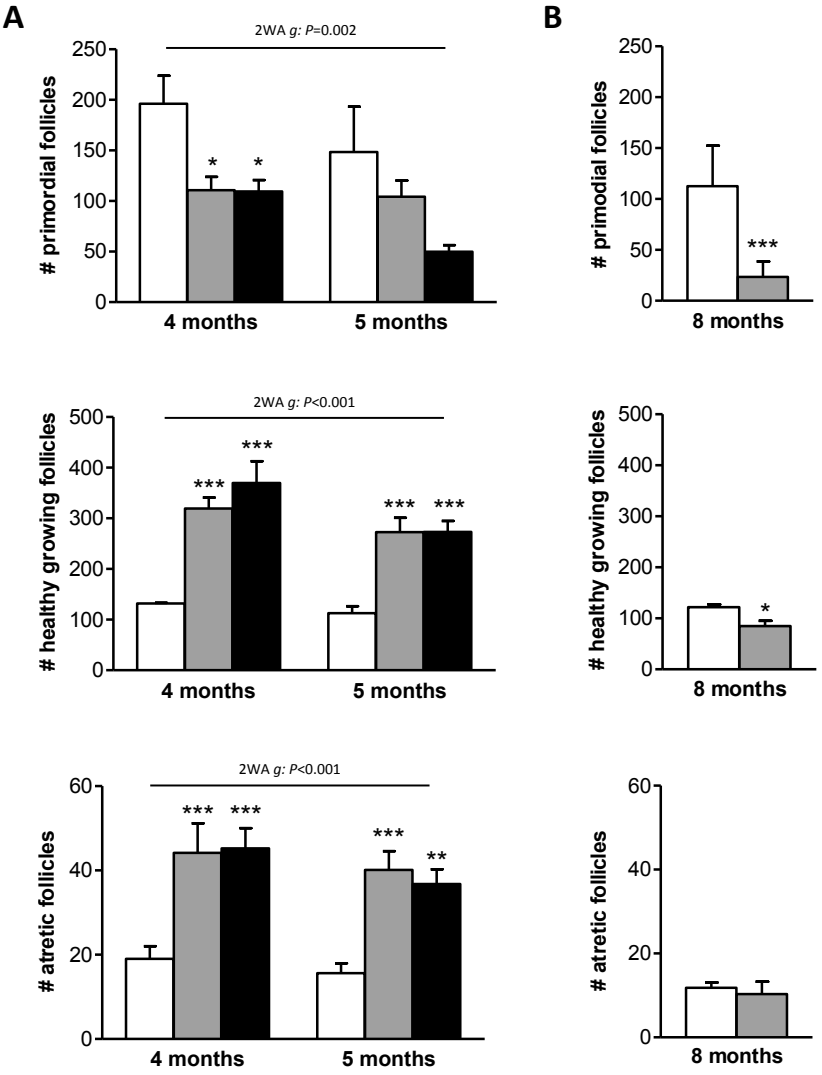
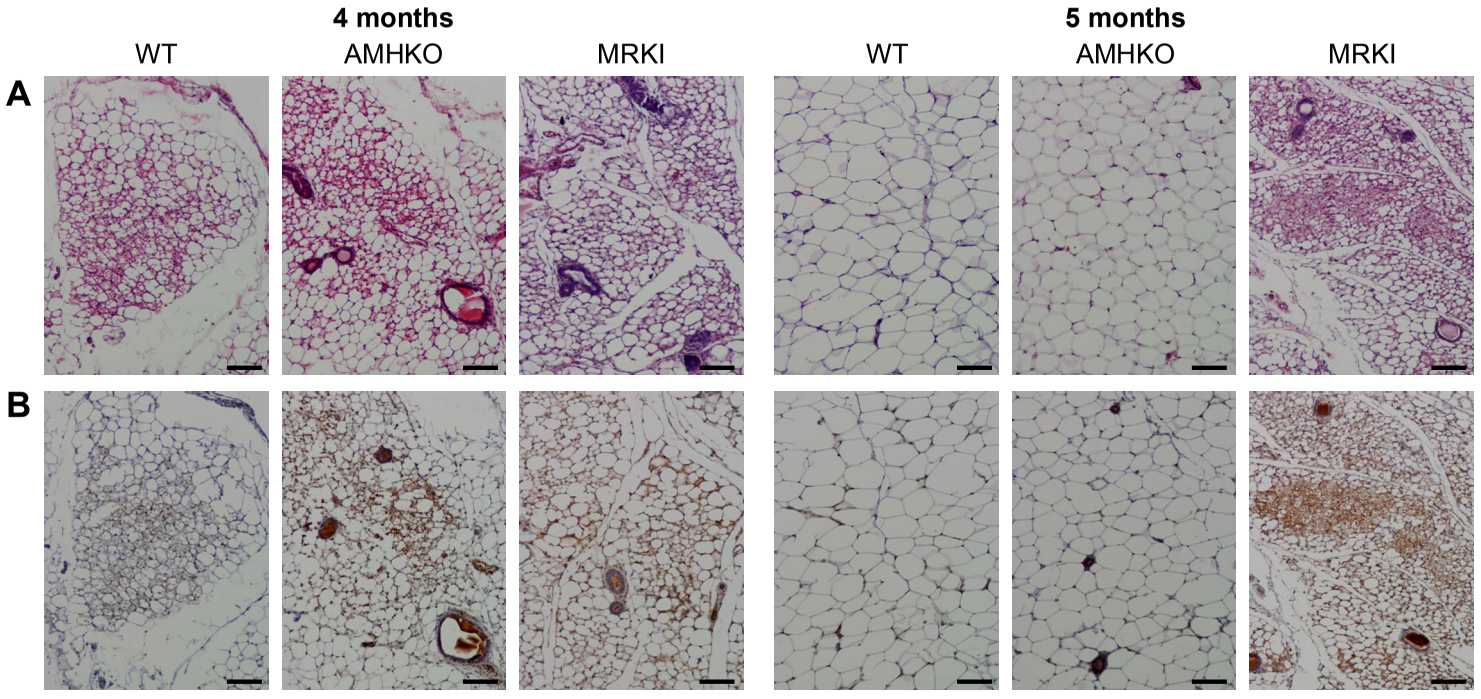
SUPPLEMENTAL DATA



SUPPLEMENTAL FIG. 1. Follicle numbers in mice lacking AMH signaling.
A) Number of primordial follicles, healthy growing follicles and atretic follicles in 4- and 5-month-old mice. AMHKO and MRKI mice display enhanced primordial follicle recruitment leading to a decreased number of primordial follicles and increased number of growing follicles. Two-way ANOVA indicated that genotype significantly affected primordial follicle number, number of healthy growing follicles, and number of atretic follicles independent of age (2WA *g*: P=0.002, P<0.001, and P<0.001, respectively). B) Follicle numbers in 8-month-old WT and AMHKO mice. The number of follicles was determined per ovary in WT (open bars), AMHKO (grey bars), and MRKI mice (black bars). Data represent mean ± SEM (n = 5-10). Significant differences from WT by posthoc tests: *, P<0.05; **, P<0.01; ***, P<0.001.



SUPPLEMENTAL FIG. 2. Islets of beige adipocytes remain present in iWAT of MRKI mice with increasing age.
A) HE-stained section of iWAT of WT, AMHKO, and MRKI mice at 4 and 5 months of age. At 4 months of age iWAT of all three genotypes contained islets of beige adipoctyes. These islets disappeared in iWAT of WT and AMHKO mice, but became denser in iWAT of 5-month-old MRKI mice. B) Immunohistochemical analysis of UCP1 expression in iWAT. UCP1-positive staining disappeared in iWAT of WT and AMHKO mice, while a stronger staining was observed in iWAT of MRKI mice with increasing age.

Chapter 5

Bone Morphogenetic Proteins and the Polycystic Ovary Syndrome

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ABSTRACT

Background

Polycystic Ovary Syndrome (PCOS) is defined by two out of the following three criteria being met: oligo- or anovulation, hyperandrogenism, and polycystic ovaries. Affected women are often obese and insulin resistant. Although the etiology is still unknown, members of the Transforming Growth Factor β (TGFβ) family, including Bone Morphogenetic Proteins (BMPs) and anti-Müllerian hormone (AMH), have been implicated to play a role. In this pilot study we aimed to measure serum BMP levels in PCOS patients.

Methods

Twenty patients, fulfilling the definition of PCOS according to the Rotterdam Criteria, were randomly selected. Serum BMP2, -4, -6 and -7 levels were measured using commercially available BMP2, BMP4, BMP6 and BMP7 immunoassays.

Results

Serum BMP2, serum BMP4 and serum BMP6 levels were undetectable. Three patients had detectable serum BMP7 levels, albeit at the lower limit of the standard curve.

Conclusions

BMP levels were undetectable in almost all patients. This suggests that with the current sensitivity of the BMP assays, measurement of serum BMP levels is not suitable as a diagnostic tool for PCOS.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is one of the most common endocrine disorders in premenopausal women [1]. The diagnosis is based on two of the following three criteria being met: hyperandrogenism, oligo- or anovulation, and polycystic ovaries [2]. PCOS is the most frequent cause of infertility in women in their reproductive years, affecting 6-8% of women in the general population worldwide. Besides the reproductive phenotype, women with PCOS often display a metabolic phenotype: 38-88% of women with PCOS are obese with a characteristic abdominal distribution of fat and up to 70% are insulin resistant and some are found to have type II Diabetes Mellitus [3-4]. Although the etiology is unknown, it is generally agreed that elevated androgens are the main culprit of the syndrome [5]. The increased ovarian androgen production may result from the increased GnRH pulsatility, which leads to increased LH secretion in favor of FSH [6]. Combined with the advanced LH responsiveness of small growing follicles, this causes increased androgen production, which is further enhanced by the elevated insulin levels. This abnormal endocrine environment has been suggested to suppress FSH action and causing follicular arrest [7]. In addition, intrinsic alterations in folliculogenesis have been proposed to contribute to the failure in dominant follicle selection in PCOS [7]. Ovarian growth factors, such as members of the transforming growth factor β (TGFβ) family, play important roles in follicle recruitment, follicle selection, and FSH responsiveness. Studies performed predominantly in rodents showed that the various Bone Morphogenetic Proteins (BMPs) are expressed in a cell-specific manner in the ovary, and display spatial and temporal changes in expression depending on the stage of follicular development [8-10]. BMP15 is specifically expressed by oocytes [11], also in ovine, bovine and human, whereas BMP6 has an oocyte/granulosa cell expression pattern in various species. BMP2 is expressed by granulosa cells in rodents and bovine, while BMP4 and BMP7 are theca cells derived growth factors with mRNA expression detectable from the small preantral stage onwards in rats [8, 12]. However, in mouse, human and bovine ovaries also granulosa cell expression of BMP4 mRNA has been reported [13-15]. Anti-Müllerian hormone (AMH) is specifically expressed by the granulosa cells of small growing follicles in various species including human [16]. BMPs and AMH differently regulate FSH responsiveness and FSH-induced steroidogenesis, hence it has been suggested that these factors may contribute to the pathogenesis of PCOS.

Several studies have shown that serum AMH levels are elevated in women with PCOS. These elevated levels reflect the increased antral follicle count (AFC) in PCOS. In addition, AMH production per granulosa cell appears to be increased [17-19]. Whether serum BMP levels are altered in women with PCOS is unknown.

Given the role of BMPs in FSH-responsiveness and FSH-induced steroidogenesis, we investigated whether serum BMP levels could be used as a diagnostic tool for PCOS.

MATERIALS AND METHODS

Patients

Twenty normogonadotrophic normoestrogenic dysovulatory patients, fulfilling the definition of PCOS according to the Rotterdam Criteria [2], were selected from the Rotterdam PCOS cohort, which comprises of PCOS patients attending our fertility clinic between 1997 and 2011. Standardized initial screening (clinical investigation, transvaginal ultrasound, and fasting blood withdrawal) was performed on a random cycle day between 0900 and 1100 h, irrespective of the interval between blood sampling and the preceding bleeding, as previously described [20]. Biochemically hyperandrogenemia was defined as an elevated (>4.5) free androgen index (testosterone x 100/ SHBG) and clinical hyperandrogenemia was defined as a Ferriman Gallway score > 8. Polycystic ovaries were defined as 12 or more follicles (measuring 2-9 mm) per ovary, and/or an ovarian volume above 10 ml [21]. Endocrine screening included assessment of serum AMH, testosterone and SHBG levels and were determined previously [22-23]. Briefly, blood samples were stored at −20°C until further assessments were made. Serum hormone levels were assessed at the time patients were originally seen. Serum SHBG was measured by luminescence-based immunometric assays (Immulite 2000, Diagnostic Products Corp., Los Angeles, CA). Serum testosterone was measured using a RIA (Diagnostic Products Corp.). AMH levels were measured collectively in samples stored, using an in-house AMH ELISA assay (commercially available through Beckman Coulter, Woerden, The Netherlands). Approval by the local medical ethics committee was obtained and all participants have given informed consent.

BMP measurements

Serum BMP2, -4 and -7 levels were measured using a Quantikine BMP2, BMP4 and BMP7 Immunoassay (R&D Systems, Minneapolis, MN, USA). Serum BMP6 levels were measured using a human BMP6 DuoSet ELISA Development kit (also R&D Systems). All samples were measured in single measurements according to the manufacturer's instructions. For BMP2 the detection limit of the assay was between 62.5 pg/mL and 4000 pg/mL. For BMP4 and -7 the detection limits of the assay were

between 31.2 pg/mL and 2000 pg/mL. For BMP6 the detection limit of the assay was between 78.13 pg/mL and 1000 pg/mL. Controls with low, medium and high concentrations for BMP2 (207-558 pg/mL, 759-1509 pg/mL, 1670-3061 pg/mL), BMP4 (89-140 pg/mL, 512-780 pg/mL, 958-1468 pg/mL) and BMP7 (180-248 pg/mL, 502-698 pg/mL, 1010-1442 pg/mL) were provided by R&D Systems. For BMP6 controls were not available. All samples where measured in one plate per assay.

RESULTS

PCOS patient characteristics are given in Table 1. Serum BMP7 was detectable in only three of the 20 PCOS patients (Table 1), albeit at very low levels. Serum BMP2, -4 and -6 were undetectable in all PCOS patients (Table 1). Extending the standard curve

TABLE 1. Clinical characteristics and BMP levels in PCOS women.

Patient	Age (years)	FAI	BMI (kg/m^2)	AFC	AMH (ng/ml)	BMP2 (pg/mL)	BMP4 (pg/mL)	BMP6 (pg/mL)	BMP7 (pg/mL)
1	28	1.63	21.6	42	13.2	–	–	–	–
2	28	0.92	22	55	28.7	–	–	–	–
3	30	1.68	20	47	15.8	–	–	–	–
4	20	0.62	22.2	50	20	–	–	–	–
5	30	1.03	19.6	59	31.9	–	–	–	57.60
6	30	7.93	24.6	40	30.9	–	–	–	–
7	23	5.53	19.4	73	28.5	–	–	–	–
8	22	5.70	21.1	54	n.d.	–	–	–	–
9	20	5.50	24.7	62	n.d.	–	–	–	43.88
10	25	13.27	24.2	48	26.6	–	–	–	–
11	25	1.50	30.4	32	6.5	–	–	–	–
12	31	1.75	44.1	77	23.5	–	–	–	–
13	31	1.42	30.1	41	8.8	–	–	–	–
14	24	1.21	31.8	44	13.6	–	–	–	–
15	26	13.50	30.1	160	n.d.	–	–	–	–
16	25	15.13	32.3	153	37	–	–	–	42.38
17	27	19.20	32.7	110	n.d.	–	–	–	–
18	27	9.20	32.0	103	17.8	–	–	–	–
19	28	9.50	34.3	101	n.d.	–	–	–	–
20	23	26.57	43.2	91	26.6	–	–	–	–

FAI: Free Androgen Index; BMI: Body Mass Index; AFC: Antral Follicle count.
n.d.: not determined
–: below detection limit of the assay

with an extra dilution step, allowed the detection of serum BMP4 in only one patient, whereas BMP2 and -6 remained undetectable. In contrast, serum AMH levels were easily detectable at an average concentration of 20.9 ng/mL. For four patients AMH levels were unknown, because data and serum of these patients were collected before AMH measurements were routinely determined in the clinic.

DISCUSSION

In this pilot study, BMP2, -4 and -6 were undetectable in all PCOS patients. BMP7 was detectable in only three patients, but with levels close to the lower limit of the standard curve. The addition of an extra lowest point to the standard curve did not improve the measurement of BMPs. The patients with discernible serum BMP7 values did not show a consistent pattern with respect to FAI, BMI or AFC. Since the BMPs studied were undetectable in nearly all of the twenty PCOS patients, and therefore could not be used to further distinguish the heterogeneous PCOS population, we did not attempt to analyze a larger number of PCOS patients nor a cohort of normo-ovulatory women as controls.

In a recent study by Son et al [24], serum BMP4 levels were measured in male and female subjects and shown to be associated with adiposity, insulin resistance and the metabolic syndrome. BMP4 levels ranged between 0.63 ± 0.41 pg/mL and 9.91 ± 4.48 pg/mL and were determined with the same assay as in our study. Since the lowest point of the standard curve of the BMP4 assay is 31.2 pg/mL, these values are well below the standard curve, and therefore it is unclear whether the results of Son et al have any practical implications. Associations of BMP4 values that far below the detection range are weak at best.

Conflicting results have also been reported for BMP2 and BMP7. Using the same assay, one study showed that serum BMP2 levels were undetectable in patients with femoral fractures [25], whereas in another study, serum BMP2 and -7 levels [10, 26-27] could be detected in patients with ankylosing spondylitis, arthritis and healthy subjects [28]. Also, in the latter study BMP levels were near or below the detection range of the assay. In agreement, other studies have also shown that serum BMP7 levels are often below or close to the standard curve of the assay [29-30].

All women used in this study had polycystic ovaries and in accordance with these results increased AMH levels. An explanation for the undetectable serum BMP levels in this study could be that BMPs are not secreted by the human ovary, although it has been reported that BMP2, -4, -6 and 7 are expressed by the human ovary [9]. This explanation may not be likely since this would be in contrast to other ovarian expressed TGFβ family members that are secreted, such as AMH, Inhibin B and

Activin A [18, 31]. Alternatively, BMP immunoreactivity may not have been preserved. Prolonged storage and repeated freeze/thawing of the samples did not affect the immunoreactivity of AMH, a family member of BMPs, but an effect on BMP immunoreactivity cannot be ruled out. However, based on the studies mentioned above and our own study, we prefer to suggest an alternative reason, namely that the current available BMP assays are not sensitive enough to detect BMP ligands in the circulation of human subjects. Therefore, more sensitive assays are necessary to determine whether serum BMP levels could be used as an additional diagnostic tool in PCOS and other metabolic diseases.

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Chapter 6

Anti-Müllerian hormone serum levels are negatively associated with risk factors of the metabolic syndrome in women with polycystic ovary syndrome

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ABSTRACT

Context: Polycystic ovary syndrome (PCOS) is associated with an increased risk for the metabolic syndrome (MetS). Serum anti-Müllerian hormone (AMH) levels are increased in women with PCOS. Conflicting results are present regarding the relationship between serum AMH levels and metabolic risk factors.
Objective: To determine the association between serum AMH and MetS.
Design and setting: Cross-sectional study at a referral fertility clinic.
Patients: We evaluated 522 Caucasian women with PCOS, diagnosed by the Rotterdam criteria, for whom data on all MetS risk factors were available.
Results: Serum AMH levels were significantly lower in PCOS women with MetS than those without MetS (P=0.003). Multiple regression analyses revealed that, with HOMA-IR and FAI as covariates, serum AMH contributed to the prediction of waist circumference (β= −2.175, P<0.001), triglycerides (β= −0.054, P=0.029), and systolic (β= −1.410, P=0.002) and diastolic (β= −1.695, P=0.002) blood pressure, independently of follicle count. In addition, serum AMH contributed to the prediction of the overall number of metabolic risk factors (β= −0.161, P=0.002). However, serum AMH did not remain an independent predictor for the presence of MetS. Furthermore, the observed associations between serum AMH and MetS risk factors were attenuated when BMI was included as a covariate.
Conclusions: Serum AMH levels are inversely correlated with the severity of MetS in women with PCOS, although this effect is largely driven by BMI. Our results therefore indicate that serum AMH levels should be interpreted with caution in overweight and obese PCOS women.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is one of the most common causes of infertility and affects approximately 6.5-8% of women worldwide (1). PCOS is clinically defined according to the Rotterdam Criteria by the presence of two out of the following three criteria: ovarian dysfunction characterized by oligo- or anovulation, hyperandrogenism, and the presence of polycystic ovaries on ultrasound (2). In addition, PCOS is frequently associated with a metabolic phenotype: 38-88% of PCOS women are obese and 50-70% of women with PCOS are insulin resistant (3, 4). The metabolic derangements present in PCOS patients closely resemble the metabolic syndrome (MetS). MetS is a cluster of multiple risk factors for the development of cardiovascular disease. According to the National Cholesterol Education Program's Adult Treatment Panel III report (ATP III) these risk factors include abdominal adiposity, elevated triglycerides, low levels of high-density lipoprotein cholesterol (HDL-C), elevated blood pressure, and high fasting glucose levels (5). This implies that PCOS not only is a reproductive disorder, but also has severe health implications beyond the reproductive system. Approximately 30-40% of the affected women will develop impaired glucose tolerance and 7.5-10% type 2 diabetes (6). Furthermore, women with PCOS might also have an increased risk for cardiovascular diseases (7).

A relationship between ovulatory function and metabolism in women with PCOS is evident since several studies have shown that even a modest weight reduction is translated into significant improvements in menstrual function, fertility and hyperandrogenic features (8, 9). Furthermore, the use of insulin-sensitizing agents like metformin not only lead to weight reduction but also causes improvements of menstrual frequency and fertility rate (10). These effects suggest that insulin resistance significantly contributes to the severity of the PCOS phenotype (11).

Anti-Müllerian hormone (AMH), a member of the TGFβ family, is expressed by granulosa cells of small growing follicles (12). Serum AMH levels decline gradually with increasing age, and this decline strongly correlates with the decreasing number of growing follicles (13, 14). Also in PCOS, serum AMH levels strongly correlate with follicle count (15, 16). Indeed, in women with PCOS, 2-3 fold increased serum AMH levels are observed (15, 16). Furthermore, analysis of granulosa cells of anovulatory PCOS women indicated that the mRNA expression of AMH is increased by nearly 75-fold compared to control women (17), which is also reflected by increased follicular fluid AMH levels (18). Thus, not only the increased follicle number but also the increased production per granulosa cell contributes to the elevated serum AMH levels in PCOS. Interestingly, serum AMH levels seem to reflect the severity of the ovarian phenotype in PCOS. Hyperandrogenic PCOS women with a polycystic ovary morphology (PCOM) have higher AMH levels compared to normo-androgenic

PCOS women with PCOM (19). Furthermore, serum AMH levels are highest in anovulatory PCOS patients with prominent PCOM compared to those without PCOM but with an equal degree of hyperandrogenism (15, 20).

However, the relationship between serum AMH levels and metabolic parameters in PCOS is less clear. Conflicting results have been reported on the relationship between serum AMH and obesity and insulin resistance. Some studies mention a lack of association between AMH levels and BMI (21), whereas others report a negative association between AMH levels and BMI (20, 22, 23). Likewise, conflicting results were observed for the relationship between serum AMH levels and HOMA-IR in women with PCOS. Studies showed both positive, negative, or even non-existent associations (21-25). Relatively small PCOS cohorts, which varied in severity of PCOS-related characteristics and consisted of different ethnicities, could possibly explain these contradictory results. Because metabolic complications in women with PCOS can result in long term health consequences (26), we investigated in a large cohort of Caucasian PCOS women whether serum AMH levels correlated with MetS risk factors and the presence of MetS.

METHODS

Participants

Patients attending our fertility clinic between 1997 and 2011, who fulfilled the definition of PCOS according to the Rotterdam Criteria (2), were enrolled in this study. Informed consent was obtained from all participants and the study was approved by the Medical Ethics Review board of the Erasmus MC.

Standardized initial screening (clinical investigation, transvaginal ultrasound and fasting blood withdrawal) was performed on a random cycle day between 0900 and 1100 h, irrespective of the interval between blood sampling and the preceding bleeding, as previously described (27). Previously performed laboratory screening included assessment of serum gonadotrophins, estradiol, AMH, testosterone, fasting insulin, fasting glucose and lipid levels. Biochemical hyperandrogenemia was defined as an elevated (>4.5) free androgen index (FAI) (testosterone x 100/ SHBG) and clinical hyperandrogenemia was defined as a Ferriman Gallway score >8. Polycystic ovaries were defined as 12 or more follicles (measuring 2–9 mm) in one or both ovaries, and/or an ovarian volume above 10 ml. Metabolic syndrome was diagnosed according to the ATP III definition (5) and was established when three or more of the following risk factors were present: (i) increased waist circumference (≥88 cm for Caucasian women), (ii) increased triglycerides (≥1.69 mmol/L), (iii) reduced levels of HDL-C

(<1.29 mmol/L), (iv) increased systolic blood pressure ≥130 mmHg or increased diastolic blood pressure ≥85 mmHg, and (v) increased fasting glucose (≥5.6 mmol/L). For the present study, 522 Caucasian women with PCOS, in whom all characteristics of the metabolic syndrome were available, were included.

Assays

Endocrine screening was performed as described previously (28-30). Briefly, blood samples were stored at −20°C until further assessments were made. Serum hormone levels were assessed at the time patients were originally seen. Serum SHBG, LH, FSH and insulin were measured by a luminescence-based immunometric assay (Immulite 2000, Siemens-DPC, Los Angeles, CA) (29, 30). Serum testosterone was measured using a RIA (Siemens-DPC) (29). Serum AMH levels were measured collectively in stored samples, using an in-house AMH ELISA assay (commercially available through DSL, now Beckman Coulter, Woerden, The Netherlands) (28). Total cholesterol and triglycerides were analyzed using commercially available assays (Wako Diagnostics, Osaka, Japan) (30). HDL-C was analyzed using the direct HDL assay from Wako Diagnostics (30).

Statistical analysis

Continuous variables are presented as median and minimum and maximum value rates. Whenever appropriate, hormone levels were log transformed to normalize their distribution (AMH, FAI, glucose, insulin, HOMA-IR (fasting glucose (mmol/L) x fasting insulin mU/L) / 22.5) (31) and triglycerides). Differences between PCOS women with or without MetS were analyzed by one way analysis of variance (ANOVA). Correlations between serum AMH levels and hormonal and metabolic factors were assessed using Pearson's correlation coefficients. Stepwise multiple linear regression analyses were performed to analyze the contribution of serum AMH to the individual risk factors of MetS. The variables AMH, follicle count, age, FAI, and HOMA-IR were considered for selection in the model. Probability-to-enter or to-remove of 0.05 and 0.1, respectively, were used. In addition, stepwise multiple linear regression analysis was performed to determine the contribution of these covariates to the number of MetS risk factors. The number of risk factors of MetS was determined by the sum of the five risk factors (range 0-5), which were coded dichotomously according to above described ATP III definition cutoff values: (i) waist circumference (1 = ≥88 cm; 0 = <88 cm), (ii) triglycerides (1 = ≥1.69 mmol/L, 0 = <1.69 mmol/L), (iii) HDL-C (1 = <1.29 mmol/L, 0= ≥1.29 mmol/L), (iv) systolic blood pressure (1 = ≥130 mmHg or diastolic blood pressure ≥85 mmHg, 0 = <130 mmHg systolic or <85 mmHg diastolic),

and (v) fasting glucose (1 = ≥5.6 mmol/L, 0 = <5.6 mmol/L). Binary multiple logistic regression analysis (enter method) was performed to examine the contribution of serum AMH levels to the presence of MetS. Areas under the receiver operating characteristic (ROC) curve (AUCs) of the covariates for predicting MetS were calculated.

To assess the effect of obesity on the association between AMH levels and MetS, analyses were repeated including BMI as a covariate. For these analyses, waist circumference was excluded from the MetS risk factor summary score.

Analyses were performed using SPSS software (version 21.0, SPSS, Inc., Chicago, IL). *P* values <0.05 were considered significant.

RESULTS

Effect of MetS on baseline characteristics of women with PCOS

The full study cohort consisted of 522 Caucasian women with PCOS, of whom 15% had MetS (n=77) based on the ATPIII definition. Descriptive information for the main characteristics of the full cohort and of those with or without MetS is presented in Table 1. The median age of the study cohort was 28 years. The median body mass index (BMI) of 24.9 kg/m^2 was just within the normal range. However, BMI was significantly increased in women with PCOS with MetS compared to those without MetS (*P*<0.001). As expected, the risk factors of MetS (waist circumference, fasting glucose, triglycerides, systolic and diastolic blood pressure, and HDL-C) were significantly different between PCOS women with or without MetS (fasting glucose *P*=0.014, others *P*<0.001). Furthermore, testosterone levels were significantly higher (*P*<0.001), while SHBG levels were significantly lower in PCOS women with MetS compared to those without MetS (*P*<0.001), explaining the significantly higher FAI in the women with MetS (*P*<0.001). The median serum AMH level was 8.7 ng/ml in the full cohort. Interestingly, serum AMH levels were significantly lower in women with PCOS with MetS compared to those without MetS (7.4 *vs.* 9.0 ng/ml, respectively, *P*=0.003), while no difference in follicle count was observed.

Correlations between serum AMH and MetS risk factors

To gain more insight into the relationship between AMH and MetS, we analyzed whether AMH levels correlated with the risk factors of MetS. In addition, we analyzed the correlation between AMH and parameters of PCOS, such as follicle count and FAI (Table 2). As expected, serum AMH levels were strongly positively correlated with follicle count. A lower but significant positive correlation was observed between

TABLE 1. Main characteristics of Caucasian women with PCOS

		Metabolic Syndrome		
	All PCOS patients	**No**	**Yes**	***P*-value***
n	522	445	77	-
Age (yr)	28 (14-44)	28 (14-40)	27 (19-44)	0.09
BMI (kg/m^2)	24.9 (16.0-45.3)	23.8 (16.0-43.3)	31.6 (22.1-45.3)	**<0.001**
Waist circumference (cm)	80 (55-133)	77 (55-124)	98 (77-133)	**<0.001**
Ferriman-Gallwey score	2 (0-25)	2 (0-25)	3 (0-20)	**0.007**
Systolic BP (mm Hg)	115 (90-175)	114 (90-175)	125 (96-160)	**<0.001**
Diastolic BP (mm Hg)	75 (52-164)	74 (52-96)	85 (66-164)	**<0.001**
Fasting glucose (mmol/L)	4.6 (1.7-10.5)	4.6 (1.7-7.7)	4.8 (3.3-10.5)	**0.014**
Fasting insulin (mU/L)	6.2 (0.3-197.6)	5.5 (0.3-197.6)	14.1 (2.0-59.8)	**<0.001**
HOMA-IR	1.2 (0.6-40.4)	1.1 (0.1-40.4)	2.9 (0.7-14.0)	**<0.001**
Cholesterol (nmol/L)	5.2 (2.2-12.2)	5.2 (2.2-12.2)	5.7 (3.6-9.2)	**0.011**
HDL-C (mmol/L)	1.5 (0.5-4.1)	1.6 (0.5-4.1)	1.1 (0.5-3.1)	**<0.001**
Triglycerides (mmol/L)	1.0 (0.3-5.1)	0.9 (0.3-4.0)	1.7 (0.5-5.1)	**<0.001**
LH (IU/L)	8.3 (0.4-105.0)	8.3 (0.4-105.0)	7.8 (1.2-62.8)	0.987
SHBG (nmol/L)	42.4 (7.6-192.0)	46.2 (7.6-192.0)	23.5 (7.9-148.0)	**<0.001**
Testosterone (nmol/L)	1.7 (0.1-5.3)	1.6 (0.2-5.2)	2.0 (0.1-5.3)	**0.004**
FAI	4.4 (0.3-63.2)	3.9 (0.3-63.2)	8.9 (0.4-32.1)	**<0.001**
Follicle count	37.5 (2-105)	37 (2-97)	38 (4-105)	0.958
AMH (ng/ml)	8.7 (0.1-66.8)	9.0 (0.1-66.8)	7.5 (0.2-52.3)	**0.003**

Values are expressed as median (minimum-maximum). BMI, body mass index; BP, blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; HDL-C, high-density lipoprotein cholesterol; LH, luteinizing hormone; SHBG, sex hormone-binding globulin; FAI, free androgen index; AMH, anti-Müllerian hormone. * *P*-values by ANOVA for the comparison of PCOS patients with or without the metabolic syndrome. Statistically significant *P*-values are shown in bold.

AMH and FAI. A highly significant negative correlation was observed for AMH and age (Table 2). Highly significant negative correlations were observed for serum AMH levels and the metabolic risk factors waist circumference, blood pressure, and triglycerides in women with PCOS. A borderline significant positive correlation was observed between AMH and HDL-C levels, while AMH and fasting glucose levels did not correlate (Table 2). Furthermore, a significant negative correlation was observed between serum AMH and fasting insulin, HOMA-IR, and BMI (Table 2).

Contribution of serum AMH to prediction models for MetS risk factors

Because of the observed correlations between serum AMH and most risk factors of MetS, we performed forward stepwise multiple linear regression analyses to de-

TABLE 2. Correlations between serum AMH and ovarian and metabolic parameters in women with PCOS.

	r	*P*-value
Age (yr)	−0.234	**<0.001**
BMI	−0.219	**<0.001**
Follicle count	0.526	**<0.001**
FAI	0.086	**0.049**
HOMA-IR	−0.119	**0.007**
Fasting insulin (mU/L)	−0.120	**0.006**
Fasting glucose (mmol/L)	−0.024	0.588
Waist circumference (cm)	−0.154	**<0.001**
Systolic BP (mm Hg)	−0.146	**0.001**
Diastolic BP (mm Hg)	−0.143	**0.001**
HDL-C (mmol/L)	0.08	0.066
Triglycerides (mmol/L)	−0.140	**0.001**

Data are given as Pearson's correlation coefficients. BMI, body mass index; FAI, free androgen index; HOMA-IR, homeostasis model assessment of insulin resistance; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol. Statistically significant *P*-values are shown in bold.

termine whether serum AMH contributed in the prediction of the levels of these risk factors in women with PCOS (Table 3). Age and follicle count were included as covariates because of their known correlation with AMH, while FAI and HOMA-IR were included because of their relationship with MetS in PCOS and because of the observed correlations with serum AMH. Serum AMH levels did not significantly contribute to the final model predicting fasting glucose and HDL-C levels. However, serum AMH remained an independent predictor for waist circumference, triglycerides, systolic and diastolic blood pressure, while follicle count did not contribute to the final models (Table 3). HOMA-IR and FAI yielded a positive coefficient for each risk factor, whereas AMH yielded a negative coefficient.

Next, we determined by forward stepwise multiple linear regression whether AMH also contributed to the prediction of the number of metabolic risk factors present (range 0-5). In the final model, 39.7% of the variation in the number of risk factors was explained by HOMA-IR, FAI, AMH and age (Table 4). Again, follicle count did not significantly contribute to the model. HOMA-IR ($\beta = 0.592$; $P<0.001$) and FAI ($\beta = 0.466$; $P<0.001$) were the strongest prediction factors, followed by AMH ($\beta = -0.161$; $P = 0.002$).

TABLE 3. Final stepwise multiple linear regression model of the Metabolic Syndrome risk factors for women with PCOS

MetS risk factor	Variables	b Coefficient	S.E.	*P*-value
Waist circumference	ln(HOMA-IR)	7.664	0.654	<0.001
	ln(FAI)	6.536	0.650	<0.001
	ln(AMH)	−2.175	0.606	<0.001
	Age	0.214	0.104	0.041
	Model R^2=0.473			
Triglycerides	ln(HOMA-IR)	0.162	0.027	<0.001
	Age	0.013	0.004	0.003
	ln(FAI)	0.098	0.026	<0.001
	ln(AMH)	−0.054	0.025	0.029
	Model R^2=0.410			
Systolic BP	ln(HOMA-IR)	3.940	0.636	<0.001
	Age	0.240	0.111	0.001
	ln(AMH)	−1.410	0.659	0.002
	Model R^2=0.092			
Diastolic BP	ln(HOMA-IR)	3.110	0.609	<0.001
	ln(FAI)	1.984	0.590	0.001
	ln(AMH)	−1.695	0.552	0.002
	Model R^2=0.134			
Fasting glucose	ln(HOMA-IR)	0.081	0.009	<0.001
	Age	0.004	0.002	0.012
	Model R^2=0.130			
HDL-C	ln(FAI)	−0.203	0.033	<0.001
	ln(HOMA-IR)	−0.128	0.033	<0.001
	Follicle count	0.004	0.001	0.001
	Model R^2=0.161			

Covariates considered for selection in each model: AMH, age, follicle count, HOMA-IR and FAI.

TABLE 4. Final stepwise multiple linear regression model of the number of Metabolic Syndrome risk factors for women with PCOS

Variables	b coefficient	S.E.	*P*-value
ln(HOMA-IR)	0.592	0.057	<0.001
ln(FAI)	0.466	0.057	<0.001
Age	0.035	0.009	<0.001
ln(AMH)	−0.161	0.053	0.002
Model R^2=0.397			

Covariates considered for selection in each model: AMH, age, follicle count, HOMA-IR and FAI.

Influence of serum AMH on the prediction of MetS

Since our results indicated that serum AMH influences the number of MetS risk factors, we next determined whether AMH also contributed to the prediction of MetS. By binary multiple logistic regression (enter method) serum AMH did not remain an independent predictor of MetS. In the final model, HOMA-IR, FAI and age significantly contributed to the presence of MetS (Table 5). Receiver operating characteristic curve (ROC) analysis showed that a model that included FAI and HOMA-IR had an AUC of 0.86, which was not improved by the addition of AMH to the model. Addition of age to the model resulted in a slightly improved AUC of 0.87 (Figure 1A). The ROC curves of the univariate and corresponding multivariable models are shown in Figure 1B.

TABLE 5. Final multiple linear regression model (enter method) for the presence of Metabolic Syndrome in women with PCOS

Variables	b coefficient	S.E.	*P*-value
ln(AMH)	−0.286	0.204	0.161
Follicle count	0.004	0.009	0.628
Age	0.096	0.033	0.004
ln(FAI)	0.985	0.233	<0.001
ln(HOMA-IR)	1.450	0.232	<0.001

A

Variables	AUC	95% CI	P-value
ln(AMH)	0.408	0.338-0.477	0.001
Age	0.534	0.460-0.608	0.342
ln(FAI)	0.779	0.723-0.836	<0.001
ln(HOMA-IR)	0.839	0.795-0.883	<0.001
ln(FAI) + ln(HOMA-IR)	0.858	0.817-0.898	<0.001
ln(AMH) + ln(FAI) + ln(HOMA-IR)	0.863	0.822-0.905	<0.001
Age + ln(FAI) + ln(HOMA-IR)	0.870	0.830-0.910	<0.001

B

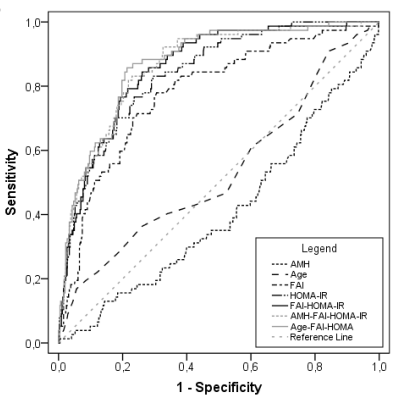

FIGURE 1. Areas under the curve (AUC) and receiver operating characteristics curves (ROCs) for selected variables for the prediction of MetS. A) AUCs of the univariable and multivariable models in the prediction of MetS are shown. In the univariable analysis it is shown that AMH has significant predictive value, while HOMA-IR had the highest predictive value. In the multivariable models the added value of variables to HOMA-IR is shown. Addition of age to a model that included HOMA-IR and FAI resulted in a slightly better accuracy than addition of AMH to this model. B) ROC curves for the single and combined variables for the prediction of MetS.

Effect of BMI on the association between serum AMH and MetS risk factors

Previous studies reported a significant negative relationship between BMI and AMH levels in women with PCOS (20, 22). Also in our cohort of Caucasian women with PCOS we observed a significant negative correlation between serum AMH levels and BMI ($r = -0.219$, $P<0.001$) (Table 2). Hence, we repeated the analyses for the contribution of AMH to the levels of individual MetS risk factors and the number of MetS risk factors present (range 0-4) by including BMI as an independent variable. Inclusion of BMI removed serum AMH as a predictive variable from all models (results not shown). With BMI included, HOMA-IR, FAI and age remained significant predictors of the number of parameters of MetS (all $P<0.001$), although their contribution to the model decreased (HOMA-IR: $\beta = 0.274$, $P<0.001$; FAI: $\beta = 0.166$, $P=0.001$; age: $\beta = 0.024$, $P=0.001$; BMI: $\beta = 0.038$, $P<0.001$).

DISCUSSION

In the present study, we observed that PCOS women with MetS had lower AMH levels than those without MetS. Furthermore, serum AMH was an independent negative predictor of the number of MetS risk factors present, suggesting that lower AMH levels are associated with an increased number of MetS risk factors in women with PCOS. Serum AMH was associated with waist circumference, TG, and hypertension. In agreement with previous studies (15, 16), we observed a strong positive correlation between serum AMH and follicle count. However, in contrast to AMH, follicle count did not differ between PCOS women with or without MetS, and follicle count did not contribute to the prediction models of individual MetS risk factors or the overall number of MetS risk factors. Thus, our results suggest that in the presence of MetS AMH production is reduced while follicle number is not affected. This may suggest that the variance in AMH levels in PCOS is not only explained by the number of follicles, but also by intrinsic properties of the granulosa cell itself. Indeed, several studies have shown that AMH production per granulosa cell is increased in women with PCOS (17, 32). Furthermore, in a prediction model for AMH levels in women with PCOS, follicle number only explained 5.3% of the variance in AMH concentration (20). In the latter study, BMI was shown to be an independent negative predictor of AMH levels (20). A negative correlation between AMH and BMI has also been reported by other studies (22, 23). Our results support these findings, since the observed association between AMH and MetS risk factors was largely driven by BMI. In our cohort nearly 50% of the women had a BMI≥25 and these women had significantly lower AMH levels compared to those with a normal BMI (results not

shown). When BMI was included as a covariate in the regression model serum AMH no longer remained an independent predictor of the number of MetS risk factors. Also, the associations between AMH levels and the individual risk factors were completely attenuated when BMI was added to the model.

The negative association between AMH and BMI may not be limited to women with PCOS. In a large multiethnic cohort of regularly cycling women, low serum AMH was associated with more MetS risk factors (33). In agreement with our findings, this association was driven by BMI. Likewise, in a small cohort of late reproductive regularly cycling women, obese women had lower AMH levels compared to normal weight women, despite a similar follicle count (34, 35). Furthermore, we recently observed in a cohort of female survivors of childhood cancers that obese subjects had lower serum AMH levels (36). Combined these results strongly suggest that obesity may affect granulosa cell function, including AMH production.

Obesity contributes to infertility, leading to reduced fecundity and reduced ovarian response upon ovarian stimulation, and increased cycle cancellation rates in IVF (37-39). Obesity induces insulin resistance, leading to compensatory hyperinsulinemia. Hence insulin has been proposed as a factor causing ovarian dysfunction in obese women (40). Insulin can directly stimulate steroidogenesis (41). Furthermore, in granulosa cells insulin can synergize with LH to induce premature LH receptor expression causing premature granulosa cell differentiation (42). Interestingly, in our study we observed a negative correlation between serum AMH and HOMA-IR. Indeed, serum AMH levels tended to be lower in women with hyperinsulinemia (results not shown). However, whether insulin can directly affect AMH expression levels remains to be determined. A direct effect of AMH on insulin levels seems unlikely given that the specific AMH type II receptor is not expressed beyond the reproductive system.

In addition to insulin resistance, obesity also affects adipocyte function leading to changes in adipokine levels, such as lower adiponectin and elevated leptin levels. Adipokines have been suggested to affect ovarian function (43). The central effect of leptin on GnRH secretion has been well studied (44). However, adipokines also have direct effects on the ovary. In rodents, leptin administration results in reduced follicular growth and reduced ovulation rates (45, 46). In cultured human granulosa and theca cells of regularly cycling women leptin was shown to antagonize the IGF1-induced steriodogenesis (47). Importantly, it was shown recently that in cultured luteinized granulosa cells obtained from non-PCOS infertile women, leptin, but not adiponectin, reduced the expression of AMH (48). Although this finding requires confirmation preferably in non-luteinized granulosa cells, it suggests that obesity may directly affect AMH levels through increased leptin levels.

In our cohort of Caucasian women with PCOS, the prevalence of metabolic syndrome was 15%. This percentage is lower than the 33-47% range that has been

reported previously (49-51). The lower prevalence in our cohort seems mainly due to the lower percentage of women having an increased waist circumference compared to other studies (33.5% vs. 73-79%, respectively) (49, 51). This may suggest that our cohort represents women with PCOS with a relatively mild degree of obesity. Given the observed association of serum AMH with waist circumference, the observed differences in serum AMH levels between women with or without MetS may be even more pronounced in a more obese cohort.

There have been several studies showing that serum AMH reflects the severity of PCOS. Indeed, higher serum AMH levels are observed in hyperandrogenic and anovulatory women (15, 19, 20). In contrast, when referring to the metabolic status, our data suggest that lower AMH levels reflect the worst metabolic profile. A possible direct effect of obesity on AMH expression, may also explain why weight loss did not change AMH levels but did increase ovulatory cycles in those women with a lower baseline AMH levels (52, 53). Although speculative, one might argue that upon weight loss, leptin levels decline leading to improved granulosa cell function and subsequently to increased AMH expression. Since serum AMH reflects the total follicular output, the relative increase in AMH production per follicle may be masked by the normalization of follicle number upon weight loss. Likewise, this may explain why in women with PCOS upon treatment with the insulin sensitizer metformin, serum AMH levels initially do not decline while follicle number does (54). Another plausible explanation might be that upon weight loss the number of non-AMH producing atretic follicles declines, leading to relatively more healthy follicles.

In conclusion, our study shows that serum AMH levels are negatively associated with MetS risk factors in Caucasian women with PCOS, and that these associations were driven by BMI. Obesity therefore may affect serum AMH levels; although a causal relationship remains to be established. Given the importance of serum AMH as a marker of ovarian function in the clinic, understanding of the regulation of AMH in normal and pathological conditions is needed. Our results also suggest that serum AMH levels should be interpreted with caution particularly in overweight and obese PCOS women.

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Chapter 7

General discussion

7.1 INTRODUCTION

Polycystic ovary syndrome (PCOS) is a disorder of fertility but also has metabolic pathophysiologies. The co-morbidities may include obesity and insulin resistance or even type 2 diabetes. As such, PCOS is associated with the metabolic syndrome (MetS), which among other factors includes hypertension and cardiovascular disease (CVD). The exact mechanisms behind this association between PCOS and MetS are unknown, but the consensus in the field is that androgens are the main culprit (1). Indeed, most women with PCOS suffer from hyperandrogenism and high androgen levels correlate with a more severe metabolic phenotype, such as abdominal adiposity, insulin resistance and a disturbed lipid metabolism (2). Androgens are produced both by the ovary and adrenal cortex, although in PCOS mainly the ovary is responsible for the androgen excess. Nevertheless, also normo-androgenic women with PCOS have metabolic pathophysiologies (3), implying that androgens are not the sole cause for the metabolic disturbances. Besides oligo-amenorrhea and hyperandrogenism, a polycystic ovarian morphology, i.e., an increase in the number of growing follicles, is one of the other criteria for the diagnosis of PCOS. This increase could also change the profile of secreted ovarian products. An example is AMH, a member of the TGFβ family of growth and differentiation factors, which is increased in patients with PCOS (4-7). Therefore, we hypothesize that, in addition to androgens, other secreted ovarian products, in particular ovarian growth factors, contribute to the metabolic phenotype of women with PCOS.

The objective of this thesis is to study the role of the ovary in the regulation of metabolism.

In this general discussion we will focus on the metabolic disturbances associated with PCOS. In the next section the role sex steroid hormones play in MetS will be discussed (7.2). Subsequently, we will discuss several mouse models that may aid in deciphering the effect of the ovary on metabolism (7.3). Next, we will focus on the effect of the ovary on metabolism, either directly or indirectly through regulation of the central nervous system (7.4). We will conclude this chapter with some final remarks (7.5).

7.2 SEX STEROID HORMONES AND THE METABOLIC SYNDROME

Men and women in their reproductive years suffer from different metabolic pathologies, especially with respect to the different risk factors of MetS. MetS is a cluster of

multiple risk factors for the development of cardiovascular disease, i.e., 1) abdominal adiposity, 2) elevated triglycerides, 3) low levels of HDL-C, 4) elevated blood pressure and 5) high fasting glucose levels (8). The incidence of MetS is much higher in the male population compared to pre-menopausal women (9). By comparing each of these risk factors in men and women this difference becomes clearer.

1) Abdominal adiposity. Body fat distribution differs between men and pre-menopausal women. In general, women develop peripheral obesity by subcutaneous fat accumulation, while men are more prone to central or android obesity. Central body fat distribution is characterized by fat accumulation mainly in abdominal depots and is a known risk factor for the development of insulin resistance and type 2 diabetes (10).

2 & 3) Elevated triglycerides and low levels of HDL-C. Abdominal obesity is associated with high plasma triglycerides, reduced plasma HDL-C and elevated plasma LDL cholesterol. A study performed in a Dutch cohort between the age of 35 and 60 years, showed that Caucasian men have increased LDL cholesterol and plasma triglyceride concentrations compared to BMI-matched Caucasian women (9). This study also showed that Caucasian Dutch women have reduced levels of HDL-C compared to Caucasian Dutch men. However, the combination of high levels of LDL cholesterol and elevated triglycerides can contribute to the higher prevalence of the MetS in men compared to premenopausal women.

4) Elevated blood pressure. Pre-menopausal women have a lower tendency to develop hypertension than do age-matched men. Furthermore, it was shown that obesity is associated with hypertension and that central obesity is an independent risk factor for the development of hypertension (11).

5) High fasting glucose levels. As mentioned earlier, central obesity is a known risk factor for the development of insulin resistance and type 2 diabetes. As such, elevated fasting blood glucose levels were found more frequently in men compared to women. However, impaired glucose tolerance was found more frequently in women (12).

As a result of the higher incidence of these risk factors men develop CVD at a younger age than women and in addition have a worse prognosis for CVD (13). An obvious explanation for the differences in MetS risk factors between men and premenopausal women may be found in the difference in sex steroid hormone levels in men and women. Whereas both sexes have circulating levels of androgens and estrogens, men have much higher androgen levels, while estrogens dominate in premenopausal women. Based on the levels of sex steroid hormones found in men and women, it may be suggested that either androgens increase the incidence of MetS risk factors, or that estrogens have an ameliorating, protective effect. It appears that the effects of androgens and estrogens are sexually dimorphic, as illustrated by the following findings:

Elevating serum estrogen levels in men, as seen in the treatment of male to female transsexuals, causes insulin resistance, suggesting that high estrogen levels in men are not positive. Interestingly, also in women high estrogens levels are associated with insulin resistance, as often seen during pregnancy (14, 15) and women using oral contraceptives also show an increase in insulin resistance (16-18). Similarly, treatment of males with estrogen led to an increase in the risk of thromboembolisms (19), also seen after oral contraceptive use in women (20). Thus, it appears that high estrogen levels have a negative effect on metabolic parameters in both sexes. The effects of androgens are clearly different between men and women. Testosterone therapy has been demonstrated to reduce total body fat and abdominal adiposity in hypogonadal young men, obese middle-aged and older men with low baseline testosterone levels and also in healthy weight middle-aged men with already normal testosterone levels (21). However, in women and especially in women with PCOS, hyperandrogenism is associated with obesity and hyperinsulinemia.

The effects of sex steroid hormones on different tissues in both sexes, such as visceral or peripheral adipocytes are also not identical. Human adipose tissues express the androgen and estrogen receptors (22). Estrogen sensitivity as indicated by the number of estrogen receptor binding sites is the same for visceral and subcutaneous adipose tissue in women, whereas in men subcutaneous adipose tissue appears to be more sensitive to estrogens than the visceral adipose tissue (23). In addition, higher expression levels of the androgen receptor were detected more often in visceral than in subcutaneous pre-adipocytes of both sexes (24). The differences in sensitivity to estrogens and androgens in combination with the widely different serum concentrations of the steroid hormones between the different sexes may well explain the observed differences in body fat distribution. In addition, the relatively high levels of estrogens in premenopausal women may explain the lower rates of lipolysis found in women, compared to men (25-28). Hence, combining these observations it appears that during their reproductive years, women have a healthier metabolic phenotype compared to men, which could be explained by the difference in sex steroid hormone levels.

However, it is not a specific sex steroid hormone, which has a specific action on different tissues, but rather a shift in ratios between androgens to estrogens (29). One way of interpreting the different effects of androgens and estrogens in men and women is to invoke the ratio between the levels of the two sex steroid hormones. Thus, in men a low estrogen over androgen ratio is seen, whereas in women the opposite is found. Especially during menopause there is a strong drop in estrogen production, whereas the androgen production by the adrenals is less affected, leading to a decrease in the estrogen/androgen ratio and subsequently giving rise to the risk factors of the MetS in menopausal women, as also seen in women with PCOS.

Indeed in women with PCOS, who are normo-estrogenic but have elevated androgen levels and therefore a decreased estrogen/androgen ratio, metabolic pathophysiologies are very common. A large proportion of PCOS women are obese (38-88%, depending on the study (30, 31)), with predominantly abdominal adiposity, which is considered a risk factor for MetS (32, 33). 50-70% of women with PCOS are insulin resistant which may lead to type 2 Diabetes Mellitus (8-10). In addition, their leptin levels are elevated, whereas adiponectin levels are low (34). Indeed, in our PCOS-like mouse model (further referred to as PCOS model) (Chapter II), leptin levels are elevated and adiponectin levels are decreased. Furthermore, these mice had increased adiposity and were glucose intolerant. The more male-like estrogen to androgen ratios found in PCOS women may also contribute to the male-like pattern of adipokine levels in women with PCOS, especially since sex steroid hormones are known to have a large influence on leptin production (35).

An altered estrogen to androgen ratio may also be involved in the observed increased blood pressure in women with PCOS (36). Changing this ratio in female rats by treating them chronically with DHT or testosterone caused elevated blood pressure (37, 38). Although we did not determine blood pressure per se, the PCOS model exhibited decreased endothelium dependent vasorelaxation (39). In this study, mice were also treated with DHT for a shorter period (60 days) which did not induce a metabolic phenotype, but did cause a decrease in vasorelaxation, indicating that elevated blood pressure most likely is a direct effect of androgens and not secondary to obesity (39). As also often seen in women with PCOS, the PCOS model had significantly higher nonesterified fatty acids (NEFA) levels which are associated with the risk of cardiovascular disease (40, 41). Treatment with DHT also caused an adverse change in the expression of genes related to obesity in visceral adipose tissue. Increased mRNA expression of the lipogenic genes aP2 and LPL were found, which is in agreement with previous studies that observed altered expression of both genes in relation to obesity and MetS (42, 43). In addition, a trend for decreased ATGL mRNA expression was observed, consistent with the decrease observed in obese and insulin resistant mice (44).

The incidence of MetS increases with age in both men and women. However, whereas in men this increase is gradual, in women a sharp increase in the incidence of MetS accompanies menopause and therefore most probably is associated with the large alterations in the levels of sex steroid hormones seen at the time of menopause. In perimenopausal women, basal levels of FSH increase throughout the menstrual cycle especially in the early follicular stage as a result of the decreased levels of ovarian inhibin B (45), caused by a decrease in the size of the ovarian follicle pool, accompanied with a shorter follicular phase, shorter overall menstrual cycle length and decreased estrogen levels (46, 47). The remaining estrogens in post-menopausal

women are mainly produced by WAT where the enzyme aromatase converts ovarian and adrenal androgens to estrogens. However, the increase in aromatization by the adipose tissue does not compensate for the decrease in estrogen plasma levels caused by the lack of follicular growth seen in postmenopausal women. The stroma of the post-menopausal ovary continues to express LH receptors and most of the steroidogenic enzymes, but lacks aromatase (48). Thus, in post-menopausal women the ovary produces little estrogens but continues to produce androgens. Combined with the decreased SHBG levels this results in increased free testosterone levels and a completely changed estrogen-androgen ratio (49, 50). This most probably explains the increased incidence of MetS in post-menopausal women (51). Indeed, the increase in the rate of hypertension is higher in post-menopausal women compared to men after 60 years of age (52). Furthermore, the weight gain seen in post-menopausal women is associated with an increase in visceral adipose tissue, leading to the development of an android pattern.

Also in women with PCOS, the estrogen/androgen ratio changes after the menopause. Since ovarian function normalizes when PCOS women approach menopause, the PCOS characteristics, including the increased androgen levels, ameliorate (53, 54). However, postmenopausal PCOS women remain to be exposed to higher androgen levels, of both adrenal and ovarian stroma origin, than postmenopausal non-PCOS women (55). Since PCOS women have already been exposed to increased androgens before menopause, a further gain in body weight and increase in blood pressure does not occur, in contrast to postmenopausal non-PCOS women (56-58). Thus, at 60 years or older the differences in body weight and blood pressure between PCOS and non-PCOS women have disappeared (56-58).

In conclusion, a large part of the differences in the prevalence of MetS between sexes can be explained by a shift in the ratio of estrogens and androgens.

7.3 METABOLISM AND OVARIAN FUNCTION IN MOUSE MODELS

To study the relationship between ovarian function and metabolism we have used AMHKO and MRKI mice, two mouse models without AMH signaling that show a specific ovarian phenotype, i.e., a large increase in the number of growing follicles (chapter IV). We found that this increase in the number of growing follicles is associated with a protection against the adverse metabolic changes that occur in aging mice. AMHKO and MRKI mice have an improved glucose tolerance, display reduced adiposity with WAT depots containing smaller adipocytes and a concomitant reduction in serum leptin and triglyceride levels. A direct effect of the absence

of AMH signaling on metabolic tissues can most probably be excluded since, to our knowledge, AMH and its type 2 receptor are exclusively expressed in ovaries of women and female mice (however, see also section 7.4). In addition, both the AMHKO and MRKI models display a similar metabolic phenotype, excluding AMH as a direct actor, in view of the absence of the ligand in AMHKO mice. Therefore, the observed metabolic phenotype in AMHKO and MRKI mice is probably the result of the increased number of growing follicles in the ovary, which leads to an altered profile of secreted humoral factors. Reduced insulin signaling has been shown to delay metabolic aging and improve longevity (59, 60). Interestingly, aging AMHKO mice have decreased insulin and C-peptide levels (Chapter IV), thus the protection against metabolic aging in these mice may be secondary to an effect of the ovarian factors on insulin signaling, although we cannot rule out that the reduced adiposity in AMHKO and MRKI mice drives the reduced insulin levels. Also the results from our studies of the PCOS mouse model (Chapter 3) indicate a pivotal role of the ovary, since ovariectomy prevented some of the metabolic effects resulting from the DHT treatment. The next important question is the identification of these factors and their further development into biomarkers and targets for intervention in patients with PCOS or with other metabolic disturbances.

Of course, many products are secreted by the ovarian follicles, many of which are unknown. Interestingly, members of the TGFβ family, most notably BMP subfamily members play essential roles in the function of the ovarian follicles and may very well be secreted into the circulation. Moreover, several BMP family members have been demonstrated to be involved in the regulation of metabolism. In rat L6 myotube cells, BMP9 signaling through Smad5 improves insulin sensitivity by increasing Akt2 expression, a step-limiting effector involved in insulin-induced GLUT4 translocation and glucose uptake (61). BMP2 and BMP4 stimulate the differentiation of mesenchymal stem cells into lipid-storing white adipocytes, although these effects are dependent on the stem cell model and also appear to be affected by the BMP-dosage used (62). Interestingly, another BMP family member, BMP7 changes the differentiation of these cells into a brown adipocyte direction, resulting in cells that express UPC1 and can oxidize lipids to produce heat (62). BMP4 and its receptor BMPR1A are expressed in pancreatic β cells. When BMPR1A function is attenuated in a transgenic mouse model, the mice were found to be glucose-intolerant. Many β cell genes involved in glucose-induced insulin secretion were expressed at lower levels, showing that BMPR1A signaling in β cells is critical for glucose homeostasis (63). In addition, systemic administration of BMP4 to intact mice improved their glucose tolerance (63). These observations suggest a possible involvement of BMPs in the link between ovarian function and metabolic state and, therefore, identification of the BMP family members involved may be a worthy target for more detailed investigation.

A less candidate protein-driven approach would be to identify secreted proteins by using proteomics to compare follicular fluids from control and PCOS women in parallel with a similar comparison in the PCOS mouse model. Although technically challenging, this approach may be feasible if the right material can be obtained (especially the human follicular fluid may be a challenge). Alternatively, whole transcriptome RNA sequencing could be performed. One of the challenges that hampers this approach is the heterogeneity of the ovarian follicle population, i.e., at the same time follicles of all sizes are present, including healthy and atretic follicles and recent and older corpora lutea. Therefore, analysis of carefully microdissected ovarian tissue should not be omitted.

Another possibility would be to use mouse models in which follicle development is arrested at a specific stage. Female FSHβ knockout mice are infertile due to a block in folliculogenesis at the pre-antral secondary stage, rendering ovaries isolated from these mice an excellent source of products of follicles at an early stage of growth and differentiation (64). A mouse model with an even earlier arrest of follicle growth and differentiation are GDF9 null mice (65). Since follicles larger than the primary stage with one layer of granulosa cells are not observed, ovaries from these mice will only contain products from the earliest growing follicles. Obviously, these models harbor the risk of an altered expression pattern of the follicles present due to the absence of FSH or GDF9 itself. Nevertheless, these mouse models, including the AMHKO mice, could be used to identify the factors present in the altered profile of secreted ovarian factors. By comparing the profiles of several mouse models, one may identify the most important factors, which could then be validated in studies in which levels of these factors are correlated with metabolic parameters. Such studies may include cohorts of women with PCOS, but also obese women without cycle disturbances. Interestingly, our studies showed that AMHKO mice, despite having an early cessation of ovarian function, are protected against metabolic aging (Chapter IV). This would suggest that exposure to ovarian growth factors early in life has a protective effect on the age-related metabolic decline. Several studies have shown that a large variation in number of growing follicles, reflected by AMH levels, is present in the human general female population of reproductive age (66, 67). The identified factors therefore may prove to be markers to identify women of reproductive age at risk for MetS after menopause. Furthermore, these factors might be used as drug targets for the treatment of MetS.

7.4 A POSSIBLE LINK BETWEEN THE OVARY, METABOLIC TISSUES, AND THE CENTRAL NERVOUS SYSTEM?

The classical feedback loop of the hypothalamic-pituitary-gonadal (ovarian) (HPG) axis is established by gonadotropins, estradiol and the inhibins A and B. However, it is well known that the metabolic status has a major impact on the HPG axis. Both under- and overnutrition lead to ovarian dysfunction. The adipokine leptin has been identified as a major player integrating metabolic and reproductive function (reviewed by (68-70)). Serum leptin levels are positively correlated with fat mass (71). Leptin has an anorectic effect via the arcuate nucleus of the hypothalamus, which contains NPY/agouti related peptide (AgRP) and POMC neurons. These neurons are important in the regulation of appetite, i.e., NPY and AgRP stimulate, whereas melanocortins, products of the POMC gene, inhibit appetite. Leptin activates POMC neurons and inhibits NPY/AgRP neurons, thereby inhibiting food intake (72). Indeed, leptin deficient ob/ob mice are severely obese (73) but strikingly are also infertile. Ovarian lipotoxicity leading to advanced follicular atresia contributes to this infertility, but importantly, ob/ob mice also are defective in hypothalamic GnRH release, resulting in significantly reduced gonadotrophin secretion (74). Pituitary gonadotropin secretion is regulated by the pulsatile secretion of hypothalamic GnRH, under control of the GnRH pulse generator, which is driving sexual development. Before puberty, the GnRH pulse generator is in a quiescent state. At the onset of puberty, the GnRH pulse generator is activated leading to enhanced gonadotropin secretion (75). Although leptin is not the trigger for puberty, its levels need to reach a certain threshold for pubertal development to proceed (76). Humans with congenital leptin deficiency show pubertal failure and infertility, while women suffering from anorexia nervosa, leading to reduced circulating leptin levels, show a suppression of gonadotropin release (77). Thus, leptin is a critical factor stimulating GnRH release.

The effects of increased leptin levels as occurs in obesity on GnRH release have been less well studied. Interestingly, although obese subjects have elevated leptin levels, food intake is not suppressed (72). The development of hypothalamic leptin resistance, similar to insulin resistance, has been suggested as an explanation for this contradictory finding (78, 79). Thus, both under- and overnutrition may lead to similar effects on GnRH release. Since leptin receptors are not present in GnRH neurons (80), the effects of leptin on GnRH secretion are indirect. Several studies have nicely shown that Kiss1 neurons form the afferent pathway by which leptin mediates its effect (81, 82).

Kiss1 neurons in the arcuate nucleus produce kisspeptins, which can directly stimulate GnRH neurons to secrete GnRH (83-85). Interestingly, a recent study

showed that kisspeptin levels are elevated in both obese and normal weight women with PCOS compared to control women (86). Therefore, it would be interesting to explore the role of kisspeptin in the connection between metabolism and ovarian function in the various mouse models described in this thesis to further dissect the relationship between the ovary, metabolic tissues, and the neuroendocrine system.

Although it is evident that the hypothalamic-pituitary axis plays a crucial role in the regulation of endocrine glands, recent studies using retrograde viral tracing techniques have clearly demonstrated that also pituita a role through neural pathways (87-89). For instance, adrenal innervation controls catecholamine release, the circadian rhythm of cortisol secretion, and plays a role in the compensatory adrenal hypertrophy after unilateral adrenalectomy (90-92). Furthermore, several studies have indicated that bidirectional communication between brain and WAT exists through the sympathetic nervous system (reviewed by (93, 94)). WAT is innervated by sympathetic endings of the autonomic nervous system which stimulate lipolysis, leading to the release of glycerol and free fatty acids (reviewed by (93, 95)). Furthermore, WAT innervation also controls adipocyte proliferation since the number of adipocytes increased upon surgical denervation of rat WAT (96).

Studies, originally described by Gerendai et al (97), have shown that also the ovary is innervated by sympathetic and parasympathetic fibers. The nerves to and from the ovary, i.e., the superior ovarian nerve, the ovarian plexus, and the vagus nerve, play a role in the regulation of several ovarian functions. Denervation of the ovary in rats delayed puberty onset, reduced ovulations, decreased steroid secretion, and resulted in acyclicity (98). Similar to the adrenal, innervation of the ovary is asymmetrical since the left ovary is denser innervated (89, 99). Interestingly, women with left-sided epileptic seizures more frequently develop PCOS, while women with right-sided seizures may develop hypogonadotropic hypogonadism (100). These findings suggest that alterations of the sympathetic tone may contribute to ovarian pathologies.

The association of an altered ovarian sympathetic input and development of PCOS has been suggested by several studies (101-104). In ovaries of PCOS patients an increased density of catecholaminergic nerves has been reported and nerve growth factor (NGF) levels are elevated in follicular fluid of PCOS patients (102, 103, 105). NGF belongs to the neurotrophin family, which also includes brain-derived neurotrophic factor (BDNF) and neurotrophin 3 and 4 (NT3 and NT4) (106). Neurotrophins are important for the survival and differentiation of neuronal cells, and indeed recovery of ovarian sympathetic innervation after transplantation is driven by increased expression of NGF in the ovary (107, 108). In the ovary, neurotrophins are expressed by oocytes and granulosa cells of growing follicles (109, 110). Analysis of knockout mice and in vitro studies demonstrated that, in addition to neuronal control, neurotrophins contribute to the formation of the primordial follicle pool and the regulation

of its recruitment (110, 111). Stimulation of neonatal rat ovaries with NT4, and high levels of BDNF, enhanced recruitment of primordial follicles (110). NGF has been shown to stimulate ovulation (112). However, overexpression of NGF in mice has an opposite effect. In transgenic mice in which NGF expression is driven by the 17-hydroxylase promoter, allowing selective overexpression of NGF in steroidogenic cells, ovaries become hyperinnervated. These mice also display antral follicle accumulation and increased apoptosis. Furthermore, androgen production is increased upon PMSG stimulation and LH exposure leads to cyst formation (105). These findings suggest that increased sympathetic innervation of the ovary might be involved in the development of a polycystic ovarian (PCO) morphology. In agreement, in a rat model, in which PCO was induced by a single injection of estradiol valerate, ovaries displayed an increased sympathetic tone reflected by increased immunoreactivity of tyrosine hydroxylase, increased norepinephrine (NE) production, and increased NGF production (113-115). Interestingly, denervation of the ovary, through transection of the superior ovarian nerve, restores cyclicity in this model (113). Similarly, inhibition of NGF action by neutralizing antibodies and antisense oligonucleotides against its receptor also restored ovarian function (114), implicating an important role for NGF in the development of the phenotype. Whether hyperinnervation contributes to both the reproductive and metabolic phenotype in PCOS remains to be established. Tracing studies of dual labeled viruses showed that overlap exists in the innervations of different organs. For instance, when both the adrenal and the ovary were injected, double labeled neurons were detected in brain regions, such as the hypothalamic paraventricular nucleus and the lateral hypothalamus, suggestion a common nervous regulation of the ovary and the adrenal (116). Given that PCOS has often been suggested to be driven by both ovarian and adrenal disturbances, a role of hyperinnervation in the etiology of PCOS is tantalizing. Whether overlap exists in the nervous regulation of ovarian and adipose tissue remains to be established. However, in the DHT-induced rat PCOS model, which displays both the cystic ovarian phenotype and a metabolic phenotype, NGF expression is increased in WAT (117, 118). Furthermore, exercise and electro-acupuncture, which have a beneficial effect on sympathetic innervation, reduce NGF expression in WAT and improve insulin sensitivity without affecting obesity (119).

Based on these findings, it would be interesting to investigate the contribution of the sympathetic nervous system in our mouse models. We would expect that also in our DHT-induced mouse PCOS model ovarian and adipose tissue innervation is increased. Although we suggest that ovarian growth factors directly modulate metabolic function, we cannot rule out that these effects are in part mediated through the nervous system. Given that ovariectomy not only results in loss of ovarian growth factors but also in ablation of the nervous communication between the ovaries and

the brain, we cannot rule out an effect of an improved sympathetic tone of metabolic tissues in the partial improvement of the metabolic phenotype in DHT-treated mice upon OVX. In an elegant study by Lazzarini et al (120), in which the retroperitoneal white adipose tissue was unilaterally denervated in OVX mice, it was shown that intact fat pads lost more weight than the denervated fat pads upon subcutaneous estrogen replacement. These results demonstrate that the sympathetic nervous system plays a role in the protective effects of estrogens on adiposity. A similar link may exist between the sympathetic nervous system and ovarian growth factors. Therefore, it would be interesting to study the expression of neurotrophins and immunoreactivity in ovaries and adipose tissues of our mouse models.

7.5 CONCLUSION AND FINAL REMARKS

In this thesis we have used several perspectives to investigate the relationship between ovarian function and metabolism in women. The known association of several characteristics of the MetS with PCOS prompted us to develop a mouse PCOS model enabling the study of metabolic features of the model in mice that would allow experimental interventions in the future. The model, DHT treatment, indeed shows many adverse metabolic changes that can be partially ameliorated by removing the ovaries from the DHT-treated animals; thus showing that the ovary is directly involved in the physiological regulation of lipid and glucose metabolism. Similarly, manipulating the follicular makeup of the ovaries by introducing *null* alleles of AMH (AMHKO) and its AMH type 2 receptor (AMHR2) (MRKI) again elegantly shows that changes in ovarian function have direct effects on the metabolic phenotype. Finally, returning to the PCOS patients we could show that the levels of the ovarian-specific hormone AMH are inversely associated with the severity of MetS, although this association is largely driven by BMI.

Overall the observations as outlined in this thesis clearly show an important role of the ovary in the regulation of metabolic homeostasis in women. Our results warrant the identification of the mechanisms by which this relationship is established and such studies should include a more thorough investigation of the involvement of the CNS in addition to the identification of ovarian secreted factors. The results of such studies will identify targets amenable to pharmacological intervention in women with severe aberrations of their metabolic state.

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Chapter 8

Summary / Samenvatting

SUMMARY

Obesity, insulin resistance and hypertension are risk factors which are part of the metabolic syndrome (MetS). Unfortunately, these risk factors comprise a major health problem of epidemic proportions. During the reproductive years, the incidence of the MetS is higher in the male population compared to women. However after menopause, the incidence of the MetS is significantly increased in women. In women with the polycystic ovary syndrome (PCOS), metabolic disturbances are very common. PCOS is the most common endocrine disorder in women in their reproductive age, with a prevalence of up to 15% in the general population. The clinical expression includes oligo- or anovulation, hyperandrogenism and the presence of polycystic ovaries.

In **Chapter I**, the general introduction, the background of the reproductive and the metabolic phenotype observed in women with PCOS is provided. In addition, the currently used androgen-induced and transgenic mouse models for PCOS are described. Furthermore, the role of the transforming growth factor β family members, particularly anti-Müllerian hormone (AMH), in the development of follicles in the ovary (folliculogenesis) and in PCOS is discussed. Finally, the aim and scope of this thesis are introduced: to study the role of ovarian growth factors in the regulation of metabolism.

To learn more about the etiology of PCOS, the use of animal models is inevitable. **Chapter II** describes the development of a mouse model of PCOS. By treating prepubertal female mice for 90 days with a continuous release pellet containing dihydrotestosterone (DHT), we developed a mouse model that displays both the reproductive and the metabolic phenotype as observed in women with PCOS. DHT-treated mice were acyclic and had an increased number of atretic follicles which had a cyst-like structure. Furthermore, these mice were glucose intolerant and showed an increase in adiposity. This model allows us to understand more of the factors involved in the pathophysiology of PCOS.

In **Chapter III** we investigated whether, besides androgens, also other factors secreted by the ovary contribute to the metabolic dysregulation in PCOS. We showed that removal of the ovaries of our DHT-induced PCOS mice normalized the glucose tolerance and reduced lipid accumulation in brown adipose tissue (BAT). This indicates that indeed, in addition to androgens, ovarian factors may contribute to the metabolic phenotype of PCOS.

Next we investigated the relationship between ovarian function and metabolism in a mouse model with an increased number of growing follicles. In **Chapter IV** we studied the metabolic phenotype of mice lacking AMH signaling. AMH is secreted by small growing follicles in the ovary and inhibits the recruitment of follicles from the

primordial follicle pool. Therefore, female mice deficient in AMH signaling display an enhanced recruitment of primordial follicles resulting in an increased number of growing follicles and therefore an increase in ovarian growth factors. Interestingly, we showed that with increasing age female mice with an increase in number of growing follicles had a better metabolic phenotype compared to wild type mice, such as reduced adiposity, improved glucose tolerance and increased browning of white adipose depots. This indicates that an increased number of growing follicles, and therefore altered profile in secreted ovarian growth factors, affects metabolic aging.

Bone Morphogenetic Proteins (BMPs), members of the TGFβ family, are crucial for follicular development and also play an important role in metabolism. Therefore, BMPs are promising candidates to study in women with PCOS and their serum levels might be used as a possible diagnostic tool, which we investigated in **Chapter V**. Twenty PCOS patients with a normal or elevated BMI and normal or elevated free androgen index levels were randomly selected, providing a heterogeneous patient group. Unfortunately, BMP2, 4, 6, and 7 levels were undetectable in almost all patients. Since it is known that BMPs are secreted by the human ovary, we concluded that the currently available BMP ELISAs are not sensitive enough to detect BMPs in the circulation.

In **Chapter VI** we investigated the association of serum AMH levels with risk factors of the MetS in women with PCOS. Serum AMH levels are increased in women with PCOS, however conflicting results regarding the relationship between AMH levels and metabolic risk factors have been reported. We found that PCOS women with MetS had lower AMH levels than those without MetS. In addition, lower AMH levels are associated with an increased number of metabolic risk factors in women with PCOS. However, serum AMH was not an independent predictor for the presence of MetS. Interestingly, the negative association between serum AMH and MetS risk factors was largely driven by BMI. This may indicate that obesity affects AMH production and therefore AMH levels should be interpreted with caution in overweight and obese PCOS women.

In the general discussion, **Chapter VII**, the role of sex steroid hormones in the development of the MetS in men, women and women with PCOS throughout their adult years is discussed. In addition, the effect of an altered ovarian function, as observed in our and other mouse models, on metabolism is evaluated and suggestions for further research are made. Finally, a potential role of the central nervous system linking ovarian and metabolic function is proposed.

SAMENVATTING

Overgewicht, insulineresistentie en hoge bloeddruk zijn belangrijke risicofactoren van het metabool syndroom (MetS). Helaas vormen deze risicofactoren een gezondheidsprobleem van epidemische omvang. Tijdens de vruchtbare jaren is de incidentie van MetS hoger in mannen dan in vrouwen. Maar na de menopauze neemt de incidentie van MetS significant toe bij vrouwen. Metabole verstoringen komen vaak voor bij vrouwen met het polycysteus ovarium syndroom (PCOS). PCOS is de meest voorkomende hormonale ziekte bij vrouwen in de vruchtbare levensfase, met een prevalentie in de algemene bevolking oplopend tot 15%. De ziekte wordt gekenmerkt door oligo- of anovulatie, hyperandrogenisme en de aanwezigheid van polycysteuze ovaria.

In **Hoofdstuk I**, de algemene inleiding, wordt de achtergrond van het reproductieve en metabole fenotype van vrouwen met PCOS besproken. Daarnaast worden ook de huidige androgeen geïnduceerde en transgene muismodellen voor PCOS beschreven. Ook de rol van transforming growth factor β familieleden, vooral het anti-Müllers hormoon (AMH), in de regulatie van follikelgroei in het ovarium (folliculogenese) en in PCOS wordt bediscussieerd. Het doel van dit proefschrift was het bestuderen van de rol van ovariële groeifactoren in de regulatie van de stofwisseling.

Om meer inzicht te krijgen in de oorzaak van PCOS is het gebruik van diermodellen onvermijdelijk. **Hoofdstuk II** beschrijft de ontwikkeling van een muismodel voor PCOS. Door prepuberale vrouwelijke muizen te behandelen met een pellet dat 90 dagen lang dihydrotestosteron (DHT) afgeeft, hebben we een muismodel ontwikkeld dat zowel het reproductieve als het metabole fenotype laat zien dat ook voorkomt bij vrouwen met PCOS. DHT behandelde muizen waren acyclisch en hadden een toename van atretische follikels met een cyste-achtige structuur. Daarnaast waren deze muizen glucose-intolerant en hadden zij meer vet (obesitas). Dit model geeft ons inzicht in de factoren die een rol kunnen spelen in de pathofysiologie van PCOS.

In **Hoofdstuk III** wordt onderzocht of er naast androgenen ook andere factoren door het ovarium worden uitgescheiden die bijdragen aan de metabole veranderingen in PCOS. Het verwijderen van de ovaria in ons DHT geïnduceerde PCOS muismodel leidde tot een genormaliseerde glucosetolerantie en een afname van de ophoping van vetdruppels in bruin vetweefsel. Dit houdt in dat, naast androgenen, ovariële factoren inderdaad bijdragen aan het metabole fenotype van PCOS.

Vervolgens hebben wij de relatie tussen de ovariële functie en metabolisme onderzocht in een muismodel met een toename in het aantal groeiende follikels. **Hoofdstuk IV** beschrijft het metabole fenotype van vrouwelijke muizen waar AMH werking ontbreekt. AMH wordt uitgescheiden door de kleine groeiende follikels in het ovarium en het remt de rekrutering van follikels uit de primordiale follikel voor-

raad. Daardoor hebben muizen zonder AMH werking een toename in de rekrutering van primordiale follikels, wat leidt tot meer groeiende follikels en daarom tot een toename in ovariële groeifactoren. Opmerkelijk was dat met veroudering deze muizen met meer groeiende follikels een betere stofwisseling hadden dan wild type muizen, namelijk een afname in vetzucht, een verbeterde glucosetolerantie en een toename in de "bruining" van de witte vetweefsels. Dit suggereert dat een toename van het aantal groeiende follikels en daarmee samengaand een veranderd profiel van uitgescheiden ovariële groeifactoren een effect heeft op metabole veroudering.

De bot-morfogenetische proteïnen (BMPs) zijn leden van de TGFβ familie. Zij zijn cruciaal voor de ontwikkeling van follikels en spelen ook een belangrijke rol in de stofwisseling. Daarom zijn BMPs veelbelovende kandidaten om te bestuderen in vrouwen met PCOS en kunnen hun serumspiegels mogelijk gebruikt worden als een diagnostisch hulpmiddel. Dit hebben wij onderzocht in **Hoofdstuk V**. Twintig PCOS patiënten met een normale of verhoogde BMI en normale of verhoogde vrije androgeenspiegels werden willekeurig geselecteerd, wat leidde tot een heterogene studiegroep. Helaas waren BMP2, -4, -6 en -7 spiegels niet meetbaar in bijna alle patiënten. Aangezien het bekend is dat BMPs uitgescheiden worden door het ovarium, concludeerden wij dat de huidige bepalingsmethoden voor BMPs niet gevoelig genoeg waren om BMPs in de circulatie te meten.

In **Hoofdstuk VI** hebben wij de associatie van serum AMH spiegels met risicofactoren van MetS bij vrouwen met PCOS onderzocht. Serum AMH spiegels zijn verhoogd bij vrouwen met PCOS, maar tegenstrijdige resultaten zijn gemeld over de verhouding tussen AMH spiegels en metabole risicofactoren. In onze studie bleek dat PCOS vrouwen met MetS lagere AMH spiegels hadden dan PCOS vrouwen zonder MetS. Daarnaast waren lagere AMH spiegels geassocieerd met een toename in het aantal metabole risicofactoren bij vrouwen met PCOS. De AMH spiegels waren echter geen onafhankelijke voorspeller voor de aanwezigheid van MetS. Opmerkelijk was dat de negatieve associatie tussen serum AMH spiegels en de risicofactoren voor MetS grotendeels gedreven werd door de BMI. Dit kan betekenen dat obesitas een effect heeft op de AMH productie. Daarom dient men voorzichtig te zijn met de interpretatie van AMH spiegels bij PCOS vrouwen met overgewicht en obesitas.

In de algemene discussie, **Hoofdstuk VII**, wordt de rol van de geslachtshormonen in de ontwikkeling van MetS bij mannen en vrouwen met en zonder PCOS bediscussieerd. Daarnaast wordt het effect van een veranderde ovariële functie, zoals gezien in onze maar ook in andere muismodellen, op de stofwisseling geëvalueerd en worden er suggesties voor toekomstig onderzoek gedaan. Ten slotte wordt een mogelijke rol van het centrale zenuwstelsel als schakel tussen ovariële en metabole functie voorgesteld.

DANKWOORD

En dan nu waarschijnlijk het meest populaire deel van dit proefschrift. Mijn dankwoord.

In de afgelopen 5 jaar hebben veel mensen mij met raad en daad terzijde gestaan en een steentje of meer bijgedragen aan de totstandkoming van dit proefschrift. Zonder die ondersteuning zou dit proefschrift niet tot stand zijn gekomen. Ik waardeer hun bijdragen zeer.

In het bijzonder en met name gaat mijn dank uit naar mijn co-promotor Dr. Ir. J.A Visser, beste Jenny. Jij verdient je eigen hoofdstuk. Je hebt mij uitstekend begeleid. Jouw adviezen en raadgevingen verstrekte je zowel binnen het EMC met een kop koffie als daar buiten met een goed glas wijn. Zie hier het resultaat. Je enthousiasme bij bijzondere onverwachte resultaten die zich tijdens het onderzoek manifesteerden was altijd aanstekelijk en motiverend. Uiteraard zat het soms ook tegen, maar dan was je de eerste om even "rond de tafel" te gaan zitten om samen te brainstormen. Ik wil je bedanken voor je geduld en daarnaast je vertrouwen in mij. Ik kwam binnen na een afstudeeronderzoek en jij wilde dit pad met mij bewandelen.

Uiteraard Prof. Dr. Ir. A.P.N Themmen, beste Axel. Ik wil jou bedanken voor je kritische vragen en je heldere kijk vanuit verschillende invalshoeken. Jij dwong mij tijdens besprekingen om weer even terug te gaan naar het begin, afstand te nemen van die ene grafiek waar het resultaat toch echt significant was en weer een logische opbouw te creëren. Ondanks dat het onderwijs, junior medschool en decentrale selectie veel van je aandacht op eisen, kon ik altijd bij je binnenlopen. Vooral in de afrondende fase van dit proefschrift heb je me erg geholpen.

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Uiteraard de leden van de grote commissie: Prof. Dr. E.A.P. Steegers, Dr. E.F.C. van Rossum, Prof. Dr. A. Kalsbeek en Prof. Dr. B.C.J.M Fauser, bedankt voor het plaatsnemen in mijn commissie.

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al ben je al een tijd weg van het lab, je hoort er nog wel bij. Bedankt voor al je hulp met SPSS in onze klinische studie. Ik heb veel van je geleerd.

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Lieve Roy, mijn alles. Geen woorden kunnen beschrijven wat je voor mij betekent, ik hou van je.

Rotterdam, september 2014

CURRICULUM VITAE

Leonie van Houten werd op 15 juni 1982 geboren in Leiden. Zij volgde het gymnasium aan het Comenius College te Capelle aan den IJssel, alwaar zij in 2001 haar diploma haalde. In 2001 volgde zij de studie Kunstgeschiedenis aan de Rijksuniversiteit in Utrecht. In 2002 werd zij decentraal toegelaten voor de studie geneeskunde aan de Erasmus Universiteit in Rotterdam. In 2005 volgde zij een 8-weekse keuze stage op de afdeling chirurgie in het Horatio E. Oduber ziekenhuis op Aruba. In 2008 startte Leonie met haar afstudeeronderzoek, onder begeleiding van Dr. A. Umar en Prof. Dr. P.M.J.J. Berns, waarbij ze 6 maanden onderzoek deed op het oncologisch laboratorium van de afdeling Interne Oncologie, Erasmus MC, Rotterdam. Dit resulteerde in het verslag "Kinase activity profiling in breast cancer" en het behalen van haar doctoraal Geneeskunde. In 2009 haalde ze haar arts-diploma en startte Leonie vervolgens met haar promotie onderzoek op de afdeling Endocrinologie, Inwendige Geneeskunde, Erasmus MC, onder supervisie van Dr. Ir. J.A. Visser, Prof. Dr. Ir. A.P.N. Themmen en Prof. Dr. J.S.E. Laven, waarvan het onderzoek beschreven in dit boek het resultaat is. Gedurende haar promotietraject was zij ambassadeur van Nederland voor de YARE (Young Active Researchers in Endocrinology), commissie lid van de EYES (European Young Endocrine Scientists), was zij bestuurslid van de redactie van ESE news (European Society of Endocrinology) en commissie lid van de Wetenschapsdagen. Daarnaast heeft zij deelgenomen aan de organisatie van de eerste EYES meeting in Rotterdam. In juni 2013 startte zij als arts-assistent gynaecologie en obstetrie in het Ikazia Ziekenhuis in Rotterdam.

PHD PORTFOLIO

Name PhD student:	Leonie van Houten
Erasmus MC Department:	Internal Medicine
Research School:	Molecular Medicine
PhD period:	april 2009 – May 2013
Promotor:	Prof. Dr. Ir. A.P.N. Themmen, Prof. Dr. J.S.E. Laven
Co-promotor:	Dr. Ir. J.A. Visser

	Year	**Workload** Hours/ ECTS
General courses		
Omgaan met groepen, Desiderius School, Workshop Tutoren Erasmus	2009	1
Cursus Proefdierkunde (Artikel 9)	2009	2
Course Biomedical Research Techniques	2009	1
Cursus Management voor Promovendi en Postdocs, NIBI, Utrecht	2010	2
Research Management for PhD students & Postdocs	2010	1
Statistiek cursus Classical Methods for Data Analysis, Netherlands Institute for Health Sciences (NIHES)	2010	2
Course on Basic and Translational Endocrinology	2011	1
English Biomedical Writing and Communication	2011	2
Endocrine Trainee Day, Boston, Verenigde Staten	2011	1
Endocrine Trainee Day, Houston, Verenigde Staten	2011	1
Oral presentations		
Wetenschapsdagen Inwendige Geneeskunde, Antwerpen, België – *Development of a PCOS-like mouse model*	2011	1
13th European Congress of Endocrinology, Rotterdam, Nederland – *Development of a PCOS-like mouse model*	2011	2
Endo Retreat, Parijs, Frankrijk – *Metabolic function in a mouse model for PCOS*	2011	2
13th Annual YARE meeting, Stockholm, Zweden – *Development of a PCOS-like mouse model*	2011	1
Dutch Endocrine Meeting, Noordwijkerhout, Nederland – *DHT treatment in mice induces a persistent PCOS phenotype*	2012	1
14th European Congress of Endocrinology, Florence, Italië – *DHT treatment in mice induces a persistent PCOS phenotype*	2012	2
94th Annual Meeting of the Endocrine society, Houston, Verenigde Staten – *Mice lacking Anti-Müllerian hormone are protected against increased adiposity and glucose intolerance with increasing age*	2012	2
14th Annual YARE meeting, Dresden, Duitsland – *Mice lacking Anti-Müllerian hormone are protected against increased adiposity and glucose intolerance with increasing age*	2012	1

Wetenschapsdagen Inwendige geneeskunde, Antwerpen, België – *Mice lacking anti-Müllerian hormone are protected against the age-related decline in metabolism*	2013	1
Molmed dag, Rotterdam, Nederland – *Mice lacking anti-Müllerian hormone are protected against the age-related decline in metabolism*	2013	1

Poster Presentations

Summer School on Endocrinology, Bregenz, Oostenrijk – *Development of a mouse PCOS model*	2009	1
Molmed dag, Rotterdam, Nederland – *Development of a mouse PCOS model*	2011	1
13th European Congress of Endocrinology, Rotterdam, Nederland – *Development of a mouse PCOS model*	2011	1
93th Annual Meeting of the Endocrine Society, Boston, Verenigde Staten – *Development of a mouse PCOS model*	2011	1
Wetenschapsdagen, Inwendige Geneeskunde, Antwerpen, België – *DHT treatment in mice induces a persistent PCOS phenotype*	2012	1
Molmed Dag, Rotterdam, Nederland *DHT treatment in mice induces a persistent PCOS phenotype*	2012	1
94th Annual Meeting of the Endocrine society, Houston, Verenigde Staten – *DHT treatment in mice induces a persistent PCOS phenotype*	2012	1

Other (inter)national conferences

Endo Retreat, Rotterdam	2009	2
Novel Insights in Adipose Cell Functions, Fondation Ipsen colloquium, Parijs	2009	2
Nederlandse Endocrinologie Dagen, Noordwijkerhout	2010	2
Wetenschapsdagen Inwendige Geneeskunde, Antwerpen, België	2010	1
Rotterdamse Internistendag, Rotterdam, Nederland	2010	1
Nederlandse Endocrinologie Dagen, Noordwijkerhout, Nederland	2010	1

Supervising practical and excursion, tutoring

Tutoring first year medical students	2009-2011	2
Endocrinology practical lectures for medical students	2010-2011	2

Supervising Master's Theses

Mirjam 't Mannetje, Medical student	2010-2011	2
Rianne Rodenburg, Medical student	2011	2
Christian von Kriegenbergh, Medical student	2012	2

Awards

Women in endocrinology young investigator award, Boston, USA	2011
Goodlife Healthcare travel award of the Dutch Endocrine Society of Endocrinology, Rotterdam, the Netherlands	2011
Endocrine Society Outstanding abstract award, Houston, USA	2012
Endrocrine Society Trainee Day travel award, Houston, USA	2012

Science Meeting Grant of the European Society of Endocrinology, Florence, Italy	2012
Basic Science Meeting Grant of the European Society of Endocrinology, Florence, Italy	2012

LIST OF PUBLICATIONS

van Houten EL, Themmen AP, Visser JA. Anti-Müllerian hormone (AMH): regulator and marker of ovarian function. Annales d'Endocrinologie 3:191-7, 2010

van Houten EL, Kramer P, McLuskey A, Karels B, Themmen AP, Visser JA. Reproductive and metabolic phenotype of a mouse model of PCOS. Endocrinology153(6): 2861-9, 2012

van Houten EL, Laven JS, Louwers YV, McLuskey A, Themmen AP, Visser JA. Bone morphogenetic proteins and the polycystic ovary syndrome. Journal of ovarian research 6: 32, 2013

Labruijere S, **van Houten EL**, de Vries R, Musterd-Bagghoe UM, Garrelds IM, Kramer P, Danser AH, Villalón CM, Visser JA, Van Den Brink AM. Analysis of the vascular responses in a murine model of polycystic ovary syndrome. Journal of Endocrinology 218: 205-213, 2013

Dowman JK, Hopkins LJ, Reynolds GM, Armstrong MJ, Nasiri M, Nikolaou N, **van Houten EL**, Visser JA, Morgan SA, Lavery GG, Oprescu A, Hübscher SG, Newsome PN, Tomlinson JW. Loss of 5α-reductase type 1 accelerates the development of hepatic steatosis but protects against hepatocellular carcinoma in male mice. Endocrinology 154(12): 4536-47, 2013

van Houten EL, Visser JA. Mouse models to study polycystic ovary syndrome: a possible link between metabolism and ovarian function? Reproductive Biology 14: 32-43, 2014

Grefhorst A, van den Beukel JC, **van Houten EL**, Steenbergen J, Visser JA, Themmen APN. Estrogens regulate expression of bone morphogenetic protein 8b in brown adipose tissue. Journal of Endocrinology, accepted

van Houten EL, Kevenaar ME, Louwers YV, van Dorp W, Themmen APN, Laven JSE, Visser JA. Anti-Müllerian hormone serum levels are negatively associated with risk factors of the metabolic syndrome in women with polycystic ovary syndrome, submitted for publication

van Houten EL, Kramer P, McLuskey A, Karels B, Themmen APN, Visser JA. Ovarian growth factors contribute to the metabolic dysregulation in a mouse model of Polycystic Ovary Syndrome, To be submitted

van Houten EL, Hoek J, Kramer P, McLuskey A, Karels B, Themmen APN, Visser JA. Improved metabolic phenotype with increasing age in female mice lacking anti-Müllerian hormone signaling, To be submitted