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Androgen signaling pathways driving reproductive and metabolic phenotypes in a PCOS mouse model

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Abstract

As the mechanistic basis of polycystic ovary syndrome (PCOS) remains unknown, current management relies on symptomatic treatment. Hyperandrogenism is a major PCOS characteristic and evidence supports it playing a key role in PCOS pathogenesis. Classically, androgens can act directly through the androgen receptor (AR) or, indirectly, following aromatization, via the estrogen receptor (ER). We investigated the mechanism of androgenic actions driving PCOS by comparing the capacity of non-aromatizable dihydrotestosterone (DHT) and aromatizable testosterone to induce PCOS traits in WT and *Ar*-knockout (ARKO) mice. DHT and testosterone induced the reproductive PCOS-like features of acyclicity and anovulation in WT females. In ARKO mice, DHT did not cause reproductive dysfunction; however, testosterone treatment induced irregular cycles and ovulatory disruption. These findings indicate that direct AR actions and indirect, likely ER, actions of androgens are important mediators of PCOS reproductive traits. DHT, but not testosterone, induced an increase in body weight, body fat, serum cholesterol and adipocyte hypertrophy in WT mice, but neither androgen induced these metabolic features in ARKO mice. These data infer that direct AR-driven mechanisms are key in driving the development of PCOS metabolic traits. Overall, these findings demonstrate that differing PCOS traits can be mediated via different steroid signaling pathways and indicate that a phenotype-based treatment approach would ensure effective targeting of the underlying mechanisms.

Key Words

- ▶ polycystic ovary syndrome
- ▶ hyperandrogenism
- ▶ androgen receptor
- ▶ estrogen receptor

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Introduction

Polycystic ovary syndrome (PCOS) is a significant health problem. It is estimated to have a global prevalence of ~6–20%, depending on the diagnostic criteria used, making it the most common endocrine disorder among women of reproductive age (March *et al.* 2010, Dumesic *et al.* 2015). Unfortunately, as the pathogenesis of PCOS remains unclear, there is no cure, and in the absence of

mechanistic understanding, current medical management relies suboptimally on *ad hoc* empirical treatment of symptoms. PCOS is a complex and heterogeneous condition impacting a wide range of organs reflected in the broad range of clinical manifestations, including endocrine, reproductive, metabolic and psychological disorders (Dumesic *et al.* 2015). Endocrine and reproductive

features include hyperandrogenism, luteinising hormone (LH) hypersecretion, sub-fertility, ovulatory dysfunction, aberrant follicular maturation and an increased risk of miscarriage (Dumesic *et al.* 2015). PCOS is also associated with a significant metabolic impact, as PCOS women often display obesity, metabolic syndrome, hyperinsulinemia, insulin resistance, hepatic steatosis and dyslipidemia, and these traits heighten the risk of type 2 diabetes and cardiovascular disease (Shorakae *et al.* 2014, Dumesic *et al.* 2015, Rubin *et al.* 2017, Glintborg *et al.* 2018).

While there have been several diagnostic criteria for PCOS, including the Rotterdam (Rotterdam Workshop Group 2004), Androgen Excess and PCOS (AE-PCOS) Society (Azziz *et al.* 2006) and the National Institutes of Health (NIH) criteria (Zawadzki & Dunaif 1992), the international evidence-based PCOS guidelines favor the Rotterdam diagnostic criteria (Teede *et al.* 2018). For a diagnosis, there is a requirement for two of the following three PCOS features: clinical and/or biochemical androgen excess, oligo- or anovulation and polycystic ovarian morphology on ultrasound, after exclusion of related disorders (Rotterdam Workshop Group 2004, Teede *et al.* 2018). Hyperandrogenism is the most common and consistent endocrine alteration in PCOS patients (Azziz *et al.* 2006, Livadas *et al.* 2014) and numerous studies have provided strong evidence that androgens and their actions through the androgen receptor (AR) are key mediators in the development of PCOS (Walters *et al.* 2019). Androgen production is up to 20 times greater from theca cells derived from PCOS patients (Gilling-Smith *et al.* 1994), and clinical exposure to endogenous (congenital adrenal hyperplasia (Lucis *et al.* 1966)) or exogenous (female to male transgenders (Pache & Fauser 1993)) androgens can induce the formation of polycystic ovary morphology. Similarly, increased androgen exposure of rodents, sheep and rhesus monkeys, both during prenatal and early postnatal life, induces a wide range of PCOS characteristics (Walters *et al.* 2018a). The AR has been highlighted a key mediator, as treatment of PCOS patients (Paradisi *et al.* 2013) and a PCOS animal model (Ryan *et al.* 2018) with the AR antagonist flutamide restored cycle irregularity and ovulation. Moreover, recent animal studies using long-term blockade of AR signaling (Silva *et al.* 2018) and transgenic mouse models that silence the action of androgens (Caldwell *et al.* 2015) have established that androgen-driven actions, in particular AR-mediated neuroendocrine mechanisms (Caldwell *et al.* 2017), are key mediators in the development of experimental PCOS.

While there is strong evidence that AR-mediated actions play an important role in the evolution of PCOS

(Walters 2015, Walters *et al.* 2018b), testosterone can be aromatized into E2, the corresponding estrogens (Hillier *et al.* 1994, Ghayee & Auchus 2007), and act via the estrogen receptor (ER). Notably, testosterone, which is readily aromatized into estradiol, is a major circulating androgen in women and is elevated in the majority of women with PCOS (Davison & Davis 2003, Handelsman *et al.* 2017). This raises the hypothesis that some androgen actions involved in the development of PCOS traits could potentially be indirect and mediated via the estrogenic pathways. In support of this, exposure to elevated levels of estrogens (estradiol valerate) induces anovulation and polycystic ovaries in rats, which resemble reproductive features observed in PCOS patients (McCarthy & Brawer 1990, Stener-Victorin *et al.* 2000). Data from the sheep PCOS model suggests that the programming of adult disease could occur via both androgenic and estrogenic pathways. Prenatal treatment with testosterone increases not only the maternal but also the fetal concentrations of androgens and estradiol (Veiga-Lopez *et al.* 2011, Abi Salloum *et al.* 2015), and treatment with testosterone, but not DHT, induces the PCOS characteristics of polycystic ovaries and abnormal antral follicle morphology (Smith *et al.* 2009). Furthermore, as clomiphene citrate (an ER modulator) and letrozole (an inhibitor of estradiol synthesis) are first-line pharmacological treatment options to restore ovulation in PCOS patients (Legro 2016, Teede *et al.* 2018, Wang *et al.* 2019), this infers a potential role for ER-mediated actions in the signaling pathways underlying the development of PCOS. Therefore, the objective of the present study was to investigate the underlying steroid signaling pathways involved in the development of experimental PCOS by comparing the capacity of non-aromatizable DHT vs aromatizable testosterone to induce PCOS traits in WT mice (functional AR and ER) and androgen receptor knockout mice (non-functional AR but functional ER) (Fig. 1).

Materials and methods

Animals

Mice were maintained under standard housing conditions (*ad libitum* access to food and water in a temperature- and humidity-controlled, 12 h light:12 h darkness cycle environment) at the ANZAC Research Institute. All protocols were approved by the Sydney Local Health District Animal Welfare Committee within National Health and Medical Research Council guidelines for animal experimentation. Mice were housed together

Figure 1

Experimental design. For this study, PCOS was induced in WT and global androgen receptor knockout (ARKO) female mice by s.c. insertion of dihydrotestosterone (DHT) or testosterone (T) implants in peripubertal mice for 3 months. Control mice were implanted with a blank (empty) pellet. Estrous cycling, glucose tolerance test (GTT), body composition (DEXA-scan) and body weights were assessed before collection of serum and tissues 12 weeks after administration of treatments.

based on treatment groups. Female homozygous ARKO mice were generated on a C57Bl/6J background by crossing ARflox mice with Sox2-Cre mice, as previously described (Caldwell *et al.* 2017). Genomic DNA isolated from toe clip or tail biopsy was used as a template for PCR genotyping to detect rearrangements in the mouse *Ar* gene as previously described (Caldwell *et al.* 2017).

Experimental design and induction of PCOS traits in a PCOS mouse model

We examined the ability of the androgens DHT (non-aromatizable) and testosterone (aromatizable) to induce PCOS-like characteristics in WT (control) and global androgen receptor knockout (ARKO, complete global loss of AR signaling (Walters *et al.* 2007)) mice (Fig. 1). Based on our previous development of a PCOS mouse model (Caldwell *et al.* 2014), peripubertal (4–5 week-old) female mice were implanted s.c. with either a DHT or testosterone 1-cm Silastic implant (i.d., 1.47 mm; o.d., 1.95 mm; Dow Corning; 508-006) containing about 10 mg DHT or testosterone or empty (control). Silastic implants are made in-house and provide steady-state DHT release for at least 6 months (Singh *et al.* 1995). At the time of animal collection, all implants were removed and checked to ensure they still had DHT or testosterone powder in them (which they did) and had not ruptured

or leaked. Groups were identified as WT Control ($n=8$), WT+DHT ($n=8$), WT+testosterone ($n=6$), ARKO Control ($n=9$), ARKO+DHT ($n=9$) and ARKO+testosterone ($n=7$). Pre-collection assessments of estrous cycle assessments, glucose tolerance tests (GTTs) and Dual energy x-ray absorptiometry analyzer (DEXA) scans were performed at 10–11 weeks after the initiation exposure to androgens (Fig. 1). DEXA was performed under isoflurane inhalation anesthesia 1–2%, while all terminal collections were carried out under ketamine/xylazine anesthesia. Mice were killed and tissues collected 12 weeks after implants were inserted.

Assessment of estrous cycle

Ten weeks after implant insertion, estrous cycling was assessed for a 12-day period. Estrous cycle stage was determined daily by light microscope analysis of vaginal epithelial cell smears, as previously described (Caldwell *et al.* 2014). Briefly, smears were collected using 15 μ L of 0.9% sterile saline, transferred to glass slides to air dry and stained with 0.5% toluidine blue before examination under a light microscope. The stage of the estrous cycle was determined based on the presence or absence of leukocytes, cornified epithelial and nucleated epithelial cells.

Ovary preparation, morphological analysis and follicle health

Dissected ovaries were weighed, fixed in 4% paraformaldehyde at 4°C overnight and stored in 70% ethanol before histological processing. Ovaries were processed through graded alcohols, embedded into glycol methacrylate resin (Technovit 7100; Heraeus Kulzer), serially sectioned at 20 µm, stained with periodic acid-Schiff and counterstained with hematoxylin. To avoid bias, all ovaries were analyzed without prior knowledge of genotype/treatment group. As previously described (Caldwell *et al.* 2014), total number of corpora lutea (CL) (identified by morphological properties consistent with luteinized follicles and visible throughout several serial sections) were quantified on all serial sections throughout each ovary using an Olympus BX60 light microscope and ImageJ version 1.48 software (open-source, developed by the National Institutes of Health). Large antral follicles were assessed on all ovarian sections and were classified as containing a single large antrum. To avoid assessment repetition, follicles were only assessed in the section where the oocyte's nucleolus was visible. Large antral follicles were categorized unhealthy if they possessed a degenerate oocyte and/or more than 10% of the granulosa cells were pyknotic in appearance, and the percentage of unhealthy follicles per ovary was calculated. All large antral follicles were assessed for granulosa cell-layer thickness using ImageJ version 1.48 software (NIH), as previously described (Caldwell *et al.* 2014).

Steroid analysis

Blood was collected from females by cardiac exsanguination under ketamine/xylazine anesthesia, and serum was stored at -20°C. Serum levels of DHT, testosterone and androstenedione (A4) (extracts of 100 µL of mouse serum) and intra-ovarian estradiol (E2) and estrone (E1) were measured in by LC-MS/MS, as previously described (McNamara *et al.* 2010, Desai *et al.* 2019). The limits of quantitation (defined as the lowest level that can be detected with a CV of <20%) were 100 pg/mL for DHT, 25 pg/mL for testosterone and 50 pg/mL for A4 in serum samples and 5 pg/mL for E2 and 3 pg/mL for E1 in ovarian tissue samples.

Body composition

Body composition measurements were performed on a Lunar PIXImus Densitometer for mice (GE Medical Systems).

Fat-pad weight and adipocyte morphometric analysis

Brown, retroperitoneal, mesenteric and parametrial fat pads were weighed at collection. Parametrial fat pads were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 8 µm and then stained with hematoxylin and eosin. Parametrial adipocyte size was quantified by histomorphometry in images taken at ×40 magnification under a light Olympus BX60 microscope with Stereo Investigator software (DP70 and ImageJ). Five distinct images were taken from each of three sections of the fat pad, with at least 200 µm separating these sections, as described (Caldwell *et al.* 2014).

Adiponectin assay

Total full-length mouse adiponectin concentrations were measured in serum using a Quantikine ELISA Kit from R&D Systems (MRP300) according to manufacturer's instructions. The mean minimum detectable dose of mouse adiponectin was 0.003 ng/mL.

Glucose tolerance test

Glucose tolerance tests were performed by blood glucose measurement after an i.p. glucose injection, as previously reported (Caldwell *et al.* 2014). Mice were fasted for 6 h before a baseline blood glucose reading, followed by an i.p. injection of glucose at 2 g/kg body weight (BW). Blood glucose was then measured at 15-, 30-, 60- and 90-min periods after glucose injection. Blood was obtained from a tail prick, and blood glucose was measured with glucose strips and an Accu-Chek glucometer (Roche).

Cholesterol and triglyceride assays

Serum levels of total cholesterol and triglycerides were assayed enzymatically with commercial kits obtained from Wako (Cholesterol E kit, catalog no. 439-17501; and Triglyceride E kit, catalog no. 342-40201).

Statistics

Statistical analysis was performed using GraphPad Prism 8. Statistical differences were tested by two-way ANOVA (to assess the effect of genotype, treatment and genotype X treatment interaction) with *post hoc* test using Fisher's least significant difference multiple-comparison test. Proportions (% of ovaries with CL) were analyzed by Fisher's exact test. For statistical analysis of steroid levels,

where a significant proportion of serum sex steroids samples were below the limits of detection (testosterone, DHT), a Fisher's exact test was used to compare the proportions of detectable vs nondetectable samples within genotype, as previously described (Cheng *et al.* 2013). GTT was analyzed by a repeated-measures two-way ANOVA. $P \leq 0.05$ was considered statistically significant.

Results

Both DHT and testosterone induce acyclicity in WT mice, but only testosterone causes estrous cycle dysfunction in ARKO mice

To determine the mode of action of androgen excess in the hyperandrogenic PCOS-like mouse model, estrous cycle patterns were assessed for 12 consecutive days, 10 weeks after the insertion of an implant. Acyclicity was evident in 100% of WT females treated with DHT (8/8) or testosterone (6/6) (Fig. 2A, $P < 0.01$; Fig. 2B). In contrast to WT control females that cycled regularly through the four different stages of the estrous cycle, DHT- and testosterone-treated WT females spent most time at the diestrus stage (Fig. 2C; $P < 0.01$). ARKO females treated with a blank or DHT implant maintained normal estrous-cycle patterns

(Fig. 2A, B and C). However, testosterone-treated ARKO females completed significantly fewer cycles (Fig. 2A, $P < 0.01$), exhibited irregular cycles (Fig. 2B) and spent the majority of time at the metestrus stage of the estrous cycle (Fig. 2C, $P < 0.01$). As the AR is non-functional in ARKO mice and testosterone can be aromatized to estradiol, these findings infer that the development of irregular cycles observed in the ARKO+testosterone group may have been mediated via ER-driven mechanisms.

Predictions of androgenic and estrogenic activity

Analysis of serum steroid levels by liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed that DHT and testosterone were undetectable in the majority of samples apart from WT and ARKO females implanted with a DHT (Fig. 3A; $P < 0.01$) or a testosterone (Fig. 3B; $P < 0.01$) implant, respectively. Additionally, analysis of circulating A4 levels identified no change in levels in WT or ARKO females (Fig. 3C). In order to predict the presence of androgenic and estrogenic activity present in each treatment group, tissues that act as *in vivo* bioassays for androgenic and estrogenic activity were assessed. Mouse kidneys are highly responsive to androgens and increase in size in response to androgen treatment (Shukla *et al.* 1992),

Figure 2

Both DHT and testosterone (T) induce acyclicity in WT mice, but only testosterone causes estrous cycle dysfunction in ARKO mice. (A) Average number of completed estrous cycles within a 12-day period, showing development of acyclicity in DHT- and testosterone-induced PCOS WT mice and significantly reduced cycles in testosterone-induced PCOS ARKO female mice. $n = 6-9$ per treatment/genotype group. (B) Representative estrous-cycle patterns in female mice over 12 consecutive days assessed 10–12 weeks post implant, showing DHT- and testosterone-induced acyclicity in WT mice, testosterone-induced estrous cycle irregularity in ARKO females, but ARKO mice were protected from DHT-induced acyclicity. P, proestrus; E, estrus; M, metestrus; D, diestrus. (C) Proportion of time spent at each stage of the estrous cycle, showing significantly altered estrous cycles in DHT and testosterone exposed WT mice, and testosterone exposed ARKO mice. For histogram graphs, data are the mean $\pm$ s.e.m.; different letters denote significant statistical differences; two-way ANOVA followed by Fishers's LSD post-hoc test.

Figure 3

Indicators of androgenic and estrogenic activity. (A) Serum DHT concentration: only DHT-treated WT and ARKO mice displayed increased levels of DHT in their sera. (B) Serum testosterone (T) concentration: only testosterone-treated WT and ARKO mice exhibited increased levels of testosterone in their sera. (C) Serum A4 concentration: no change observed between any groups. (D) Kidney weights: confirming an AR-mediated increase in kidney weight only in DHT and testosterone-treated WT female mice and not ARKO female mice. (E) Uterus weight: confirming an increase in uterus weight only in testosterone-treated WT and ARKO female mice, likely via ER-mediated mechanisms. $n = 6-9$ per treatment/genotype group. (F) Intra-ovarian estradiol (E2) concentrations: revealing a trend to higher levels of E2 in testosterone-treated ARKO mice. (G) Intra-ovarian estrone (E1) concentrations: revealing a trend to higher levels of E1 in testosterone-treated ARKO mice. $n = 5-8$ per treatment/genotype group. For all graphs, data are the mean $\pm$ s.e.m.; different letters denote significant statistical differences; Fisher's exact test (A and B) and two-way ANOVA followed by Fisher's LSD post-hoc test (C, D, E, F and G).

while estrogens stimulate growth of the uterus (Armstrong *et al.* 1976). Both DHT and testosterone significantly increased kidney weights in WT mice (Fig. 3D; $P < 0.01$), but no change was observed in the kidney weight of ARKO mice treated with DHT or testosterone (Fig. 3D). Uterine weight significantly increased in both WT (Fig. 3E; $P < 0.01$) and ARKO (Fig. 3E; $P < 0.05$) females in response to testosterone treatment. In addition, as serum E2 levels were undetectable by this method (McNamara *et al.* 2010), we measured intra-ovarian estradiol (E2) and estrone (E1) levels, and ARKO mice treated with testosterone displayed a non-significant trend of elevated intra-ovarian E2 and E1 concentrations (Fig. 3F and G).

Both DHT and testosterone induce anovulation and ovarian characteristics of PCOS in WT mice, but only testosterone causes ovulatory dysfunction in ARKO mice

Oligo/anovulation is a defining reproductive trait of PCOS, and the development of this PCOS-like trait was confirmed by the detection of anovulation in all WT females treated with DHT or testosterone. Histologically, corpora lutea (CL) were absent in all DHT and testosterone-treated WT females (Fig. 4A, B and D; $P < 0.01$). In addition, the absence of CL led to a significant reduction in ovarian weight in

both the WT+DHT and WT+testosterone groups (Fig. 4C; $P < 0.01$). Consistent with previous findings (Caldwell *et al.* 2017), a global loss of AR signaling protected against the DHT-induced ovulatory dysfunction (Fig. 4A, B and D) and associated reduction in ovarian weight (Fig. 4C). However, in line with our finding that testosterone treatment caused irregular estrous cycles in the absence of AR signaling (Fig. 2A, B and C), treatment of ARKO females with testosterone induced the development of ovulatory dysfunction, evident by a significant reduction in the number of CL present in their ovaries (Fig. 4A, $P < 0.05$; Fig. 4B, $P < 0.01$) and a significant decrease in ovarian weight (Fig. 4C; $P \leq 0.05$).

As expected and previously reported (Caldwell *et al.* 2014), peripubertal DHT treatment induced a characteristic polycystic appearance in WT ovaries (Fig. 5A). Similarly, compared to WT controls, exposure of WT ovaries to testosterone also caused a similar histological appearance of polycystic ovaries (Fig. 5A). Control and DHT-treated ARKO females did not display this phenotype. However, ovaries collected from testosterone-treated ARKO females did resemble the characteristic polycystic appearance (Fig. 5A). In addition, compared with controls, the ovaries of DHT- or testosterone-treated WT females displayed a significant increase in the presence of morphologically unhealthy large antral follicles (Fig. 5B; $P < 0.05$),

Figure 4

Both DHT and testosterone (T) induce anovulation and ovarian characteristics of PCOS in WT mice, but only testosterone causes ovulatory dysfunction in ARKO mice. (A) Average number of corpora lutea (CL) per ovary: showing development of anovulation in DHT- and testosterone-induced PCOS WT mice and significantly reduced CL numbers per ovary in testosterone-induced PCOS ARKO female mice. $n = 4-9$ per treatment/genotype group. (B) Proportion of ovaries exhibiting CL: showing a complete loss of CL in DHT and testosterone-treated WT ovaries and significantly fewer testosterone-treated ARKO ovaries exhibiting CL. (C) Ovary weights: confirming DHT- and testosterone-induced reduction in ovarian weight in PCOS WT mice and testosterone-induced PCOS ARKO female mice. $n = 6-9$ per treatment/genotype group. (D) Histological cross-sections of representative ovaries from each treatment group: showing the presence of CL in WT Control, ARKO Control and ARKO+DHT ovaries only. Star: corpora lutea; magnification: 4 $\times$. For all graphs, data are the mean $\pm$ S.E.M.; different letters denote significant statistical differences; two-way ANOVA followed by Fisher's LSD post-hoc test (A and C) and Fisher's exact test (B).

as large antral follicles exhibited an increase in pyknotic granulosa cells (Fig. 5C). Furthermore, granulosa cell-layer thickness was decreased in testosterone-treated WT females (Fig. 5D; $P < 0.01$) and there was a tendency in WT females (Fig. 5D; $P = 0.07$) in response to DHT treatment. Conversely, these features were completely prevented in the androgen exposed ARKO females, as the proportion of unhealthy large antral follicles (Fig. 5B and C) and granulosa cell-layer thickness (Fig. 5D) did not differ from controls.

DHT, but not testosterone, increased body weight and adiposity in WT mice, and neither androgen impacted body composition in ARKO mice

There is a strong association between PCOS and obesity, with obesity worsening hyperandrogenism in PCOS (Lim *et al.* 2012). We therefore investigated the mechanism of androgen excess in mediating weight gain in the hyperandrogenic PCOS-like mouse model. In WT females, DHT, but not testosterone, treatment induced a significant increase in body weight (Fig. 6A; $P < 0.01$), body fat (Fig. 6B) and retroperitoneal (Fig. 6C; $P < 0.01$), mesenteric (Fig. 6C; $P < 0.01$) and parametrial fat depot weights (Fig. 6C; $P < 0.01$). Brown fat depot weight was significantly increase in WT mice exposed to DHT and testosterone (Fig. 6C; $P < 0.05$). In contrast to WT females, in ARKO mice, neither DHT nor testosterone induced significant increases in body weight or fat depot weights (Fig. 6A, B and C).

In WT mice DHT, but not testosterone, induced the development of adipocyte hypertrophy and reduced adiponectin levels, and neither androgen impacted adipocyte morphology or adiponectin levels in ARKO mice

We discovered that, while WT females treated with DHT exhibited a 67% increase in adipocyte cell size (Fig. 6D and E; $P < 0.01$), adipocyte hypertrophy was not evident in testosterone-treated WT females or any of the ARKO groups (Fig. 6D and E). Additionally, we measured circulating levels of the adipocyte-derived hormone adiponectin, an adipokine reduced in serum of PCOS patients (Toulis *et al.* 2009, Baldani *et al.* 2019) and PCOS animal models (Yuan *et al.* 2016, Caldwell *et al.* 2017). Serum adiponectin concentrations were significantly reduced in DHT ($P < 0.01$) and testosterone ($P < 0.05$) exposed WT females (Fig. 7A), while no difference was observed between any of the ARKO treatment groups (Fig. 7A).

Cholesterol levels were increased in WT mice by DHT, but not testosterone, and neither androgen altered cholesterol levels in ARKO mice

To further assess the mechanism of androgen excess in mediating PCOS-like traits in this hyperandrogenic PCOS-like mouse model, serum cholesterol concentrations were measured, and DHT treatment was observed to induce a significant increase in levels in WT mice (Fig. 7B; $P < 0.01$). In contrast, testosterone-treated WT mice and DHT- and

Figure 5

Both DHT and testosterone (T) induce the development of polycystic ovary morphology in WT mice, but only testosterone causes polycystic ovaries in ARKO mice. (A) Histological cross-sections of representative ovaries: showing the development of the classic PCOS ovarian phenotype, defined by the presence of multiple arrested large antral follicles, in DHT- and testosterone-induced PCOS WT mice and testosterone-induced PCOS ARKO female mice. Triangles: arrested large antral follicles; magnification 4 $\times$. (B) Proportion of unhealthy large antral follicles per ovary: showing a significant decrease in large antral follicles health in DHT and testosterone-treated WT mice. $n = 4$ per treatment/genotype group. (C) Histological cross-section of representative antral follicles: showing presence of pyknotic cells (white arrows) within granulosa cell layer. Magnification: 40 $\times$. (D) Average thickness of granulosa cell layer per follicle: showing a significant reduction in granulosa cell-layer thickness in testosterone-induced PCOS WT mice. Average of 19 follicles assessed per treatment/genotype group. For all graphs, data are the mean $\pm$ S.E.M.; different letters denote significant statistical differences; two-way ANOVA followed by Fisher's LSD post-hoc test.

testosterone-treated ARKO females displayed comparable cholesterol levels to control mice (Fig. 7B). Androgen excess caused no significant alteration in serum triglyceride levels (Fig. 7C). Compared with controls, testosterone-treated WT females had a significantly altered response 15 min and 30 min after glucose exposure and DHT-treated WT females displayed a similar but non-significant trend ($P=0.08$) 15 min after glucose exposure. No effect of androgen excess was observed in ARKO females (Fig. 7D) and overall glucose tolerance did not change in WT or ARKO mice (Fig. 7E).

Discussion

Although hyperandrogenism is a key defining feature of PCOS, the specific mechanism(s) through which androgens mediate the development of the PCOS phenotype remain unclear. The present study provides evidence to support the notion that androgens acting directly through the AR and indirectly, likely via the ER, both drive important mechanisms involved in mediating the development of reproductive dysfunction in PCOS. By contrast, only direct AR-mediated actions were observed to play a key role in establishing the PCOS-like metabolic

traits (Table 1). Our data provide experimental evidence to support that the heterogeneity in the spectrum of PCOS symptoms observed may be due to difference in the underlying steroid signaling pathways involved.

We show that both testosterone and DHT were able to induce the reproductive PCOS traits of acyclicity, anovulation and polycystic ovarian morphology in WT mice, confirming the importance of androgen-driven mechanisms in PCOS pathogenesis (Walters *et al.* 2019). However, several androgens elevated in the serum of women with PCOS (testosterone, dehydroepiandrosterone (DHEA) and androstenedione (A_4) (Handelsman *et al.* 2017)) are aromatizable and can be further converted into corresponding estrogens which can mediate their effects via the ER (Hillier *et al.* 1994, Ghayee & Auchus 2007). We show here that, while DHT-treated ARKO mice were protected against reproductive dysfunction, in the absence of a functional AR, reproductive features of PCOS can still be induced by excess exposure to testosterone. Testosterone-treated ARKO females exhibited irregular cycles, ovaries with a classic polycystic appearance and ovulatory dysfunction, which was confirmed by the reduction in CL numbers. AR activity is completely ablated globally in ARKO mice and while DHT can be converted into 3 β -diol (5 α -androstane-3 β , 17 β -diol), which has the

Figure 6

DHT, but not testosterone (T), increased body weight, adiposity and adipocyte hypertrophy in WT mice but neither androgen impacted body composition in ARKO mice. (A) Body weight: demonstrating increased body weight only in DHT-induced PCOS WT mice. $n = 6-9$ per treatment/genotype group. (B) Representative dual-energy X-ray absorptiometry (DEXA) scan images: showing increased body fat in DHT-induced PCOS WT mice. (C) Brown, retroperitoneal, mesenteric and parametrial fat-pad weights: demonstrating an overall consistent significant increase in adiposity only in DHT-induced PCOS WT mice. (D) Parametrial adipocyte size: demonstrating that only DHT-induced PCOS WT mice displayed adipocyte hypertrophy. $n = 4-5$ per treatment/genotype group. (E) Histological sections of representative parametrial adipose tissue from each treatment group: showing development of adipocyte hypertrophy in DHT-induced PCOS WT mice. Magnification: 40 $\times$. For all graphs data, are the mean $\pm$ s.e.m.; different letters denote significant statistical differences; two-way ANOVA followed by Fishers's LSD post-hoc test.

potential to elicit ER β -mediated effects (Kuiper *et al.* 1997, Miller & Auchus 2011), all ARKO females treated with DHT were protected against the development of reproductive features of PCOS. Taken together, the observed effects in the current study imply that androgen excess acting indirectly plays a role in mediating the development of reproductive dysfunction in PCOS, which we propose is primarily mediated via the ER. This proposal is consistent with current clinical recommendations that the first-line treatment options to induce ovulation in PCOS patients are the anti-estrogens letrozole and clomiphene (Kar 2012, Legro 2016, Teede *et al.* 2018), both of which modulate ER actions. Current evidence points to a key role for aberrant neuroendocrine actions in the origins of PCOS (Walters *et al.* 2018b), with AR signaling in the brain

highlighted as a key mechanism involved (Caldwell *et al.* 2017, Silva *et al.* 2018). However, new data indicate that dysregulation of hypothalamic ER α signaling in ER α KO mice caused irregular LH secretion and the development of a PCOS-like cystic ovarian phenotype (Arao *et al.* 2019). Hence, it may be speculated that neuroendocrine estrogen actions also play an important intermediate role in the androgen-mediated origins of reproductive traits of PCOS.

Women affected by obesity have a higher prevalence of PCOS, and women with PCOS have an increased risk of developing obesity and adverse metabolic traits (Lim *et al.* 2012, Moran *et al.* 2015). Hyperandrogenism and insulin resistance are both clinically present in women with PCOS and thought to play a role in PCOS pathogenesis, but the underlying mechanisms driving the development

Figure 7

Circulating adiponectin and cholesterol levels are influenced by androgen excess in WT mice, but neither androgen altered serum levels in ARKO mice. (A) Serum adiponectin concentrations: showing DHT and testosterone (T) treatments in WT mice only induced decreased levels of adiponectin. (B) Serum cholesterol levels: demonstrating that only DHT-induced PCOS WT mice displayed elevated cholesterol levels. (C) Serum triglyceride levels: showing no elevation in triglyceride concentrations due to exposure to androgen excess. (D) Glucose tolerance test (GTT): glucose levels following intra-peritoneal injection of glucose, showing an altered response after glucose exposure in testosterone-treated PCOS WT mice only. (E) Area under the curve (AUC) analysis of the glucose tolerance test (GTT): showing no overall effect of androgen treatment. $n = 6-9$ per treatment/genotype group. For all graphs, data are the mean $\pm$ S.E.M.; different letters denote significant statistical differences; two-way ANOVA followed by Fishers's LSD post-hoc test (A, B, C and E) and repeated-measures two-way ANOVA followed by Fishers's LSD post-hoc test (D).

of adverse metabolic traits associated with PCOS remain to be fully elucidated. In the current study, we provide further support of a direct role for hyperandrogenism, acting directly via the AR, in driving the high prevalence

of obesity in women with PCOS. Treatment of WT females with non-aromatizable DHT induced a significant increase in body fat and fat-pad weights but, importantly, aromatizable testosterone had no effect in ARKO mice.

Table 1 Summary of the PCOS-like traits induced in WT and ARKO female mice after exposure to excess levels of an aromatizable (testosterone) and a non-aromatizable (DHT) androgen.

	DHT		Testosterone	
	WT	ARKO	WT	ARKO
Functional AR	✓	✗	✓	✗
Functional ER	✓	✓	✓	✓
Clinical PCOS trait	Development of PCOS-like trait?			
Reproductive features				
Absent/irregular cycling	✓	✗	✓	✓
Oligo-anovulation	✓	✗	✓	✓
PCOM	✓	✗	✓	✓
Change in ovary weight ^a	✓	✗	✓	✓
↑ Unhealthy large antral follicles	✓	✗	✓	✗
↓ Granulosa cell-layer thickness	✗	✗	✓	✗
Metabolic features				
↑ Body weight	✓	✗	✗	✗
↑ Parametrial fat-pad weight	✓	✗	✗	✗
↑ Retroperitoneal fat-pad weight	✓	✗	✗	✗
↑ Mesenteric fat-pad weight	✓	✗	✗	✗
↑ Brown fat-pad weight	✓	✗	✓	✗
Adipocyte hypertrophy	✓	✗	✗	✗
↓ Serum adiponectin	✓	✗	✓	✗
↑ Serum cholesterol	✓	✗	✗	✗
↑ Serum triglyceride	✗	✗	✗	✗
Glucose intolerance	✗	✗	✗	✗

✓, clinical PCOS trait present; ✗, clinical PCOS trait not present; ^a, ↑ in humans; ↓, in mice.

These findings infer that androgen excess acting via AR, and not ER, signaling was important in the establishment of the PCOS-like metabolic trait of increased adiposity. Androgen excess is known to induce metabolic features of PCOS in several animal models (Abbott *et al.* 2013, Padmanabhan & Veiga-Lopez 2013, Walters *et al.* 2019) and a global loss of AR signaling fully protects female mice from the development of PCOS metabolic traits (Caldwell *et al.* 2017), inferring direct androgen signaling through the AR is key in the development of adverse metabolic traits. Clinical evidence supporting this comes from the findings that treatment of PCOS patients with the AR blocker flutamide improved lipid profile independent of changes in weight (Diamanti-Kandarakis *et al.* 1998), and in combination with a hypocaloric diet in overweight-obese PCOS women, flutamide had a beneficial effect on glucose-stimulated glucose levels, insulin sensitivity and low-density lipoprotein and cholesterol levels (Gambineri *et al.* 2006). Moreover, mechanistic support of a role for AR comes from a study using a PCOS-prone metabolic syndrome rodent model (generated without androgen excess treatments), where it was shown that flutamide improved the dyslipidemia observed in this model (Kupreeva *et al.* 2019).

Several clinical studies demonstrate a positive correlation between circulating androgen concentrations and the prevalence of adverse metabolic traits in patients with PCOS (Christakou & Diamanti-Kandarakis 2008, O'Reilly *et al.* 2014, Zhang *et al.* 2018). However, in the current study, testosterone was not able to induce the adverse metabolic traits observed in the DHT-treated WT females. One reason for this could be that the testosterone dose used was not potent enough to induce metabolic traits, or another possibility is that testosterone was being aromatized into estradiol opposing some androgen actions, further indicating that ER signaling is not crucial in the development of PCOS metabolic traits. This lack of effect of testosterone differs from hyperandrogenic PCOS women who have an adverse metabolic phenotype (reduced insulin sensitivity and dysglycemia) which is associated with increased testosterone and androstenedione (A4) serum levels (O'Reilly *et al.* 2014). This may reflect that women with PCOS may display reduced aromatization of testosterone to E2, an interpretation supported by finding that PCOS patients exhibit significantly lower E2/testosterone ratios (Chen *et al.* 2015) and consistent with the reported reduced expression of CYP19A1 in ovaries from PCOS patients (Yu *et al.* 2013). A key role for direct androgen signaling via AR, and not via ER, in the establishment

of adverse metabolic traits was further supported by the findings that, in WT mice, DHT, but not testosterone, also induced the PCOS-like metabolic features of adipocyte hypertrophy, a decrease in serum adiponectin levels and an increase in circulating cholesterol levels. Consistent with these findings, hyperandrogenized rodent, sheep and primate PCOS models all display altered adipocyte morphology (Keller *et al.* 2014, Cardoso *et al.* 2016, Caldwell *et al.* 2017) and intra-abdominal fat mass is significantly increased in women with PCOS, and this increase is positively correlated with serum androgen levels and fasting insulin levels (Dumesic *et al.* 2016). In addition, females rats exposed to elevated levels of estradiol valerate exhibit several reproductive PCOS-like traits (Brawer *et al.* 1986, McCarthy & Brawer 1990), but lack metabolic features associated with human PCOS (Stener-Victorin *et al.* 2000).

PCOS patients can be divided into four phenotypes (A–D) based on the presence of the Rotterdam diagnostic criteria (Lizneva *et al.* 2016). Interestingly, non-hyperandrogenic PCOS patients (phenotype D) are reported to display a lower risk of developing metabolic PCOS traits than the other three hyperandrogenic PCOS phenotypes (Zhang *et al.* 2009, Kar 2013, Clark *et al.* 2014, Altintas *et al.* 2017, Tripathy *et al.* 2018). Therefore, this raises the question of whether non-hyperandrogenic PCOS patients are etiologically distinct from women with PCOS who display hyperandrogenism (Azziz *et al.* 2006, 2009). In our current study, direct AR-mediated androgen actions were required for the manifestation of metabolic PCOS-like features in WT mice. This suggests that, in PCOS phenotypes displaying hyperandrogenism, AR- but not ER-mediated actions are primarily responsible for the higher prevalence of metabolic traits in these patients and supports treatments based on AR antagonism. There is also a striking similarity between the PCOS characteristics displayed in non-hyperandrogenic PCOS patients and those we propose are induced via ER-mediated action in our mice, as both of these groups exhibit disrupted ovulation and polycystic ovarian morphology. Based on our data, we hypothesize that, ER, and not AR, signaling may be the key mediator in the development of the reproductive abnormalities observed in PCOS women with phenotype D, and treatments that modulate ER actions may be more beneficial, hence this area warrants further research.

While species differences need to be considered when interpreting rodent models of PCOS, carefully designed experiments using animal models that display characteristics of the human condition of PCOS with

Figure 8

Schematic diagram of hypothesized pathophysiological mechanisms underlying the development of different PCOS traits. Evidence from clinical and preclinical animal model studies strongly suggests that androgen excess plays an important causative role in the pathogenesis of PCOS. Data derived from molecular modeling in a preclinical model of PCOS reveal that the development of reproductive and metabolic PCOS traits can be driven by direct androgenic actions mediated via the AR. Additionally, indirect androgen actions, likely mediated via the ER, are capable of inducing reproductive PCOS traits in androgenized PCOS-like mice. Androgens AR: androgen receptor; ER: estrogen receptor.

high fidelity are valuable tools as they afford insights into fundamental biological mechanisms impacting on the development of PCOS, noting that reproductive functions are highly conserved in mammals. This study used a rodent model of PCOS to provide insight into the androgen signaling pathways involved in the genesis of both reproductive and metabolic abnormalities that are characteristic of many women with PCOS. We have provided evidence to support that hyperandrogenism acting directly via the AR, and likely also indirectly via the ER, can induce key PCOS-like reproductive traits in a preclinical PCOS mouse model. We also highlight the important role for AR-mediated androgen actions, and lack of evidence for estrogenic actions, in the pathogenesis of adverse metabolic traits associated with experimental PCOS. Future studies which incorporate ER antagonists and which undertake a detailed characterisation of intracrine steroid biosynthesis in PCOS animal models could further define the precise underlying mechanisms and pathways involved. Overall, these data provide evidence to put forward a hypothesis that the manifestation of the different clinical PCOS sub-phenotypes arise through variations in the underlying signaling pathways involved, with both direct and indirect androgen signaling pathways playing key and divergent roles (Fig. 8). The clinical implications of these findings are that potentially different PCOS phenotypes may have distinct pathogenesis and prognosis, which require a phenotype-driven therapeutic approach to ensure the underlying mechanisms are effectively targeted.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A A, D J H and K A W Walters designed research. A A, M C E, V R P and R D conducted research and acquired data. A A, R B G, D J H and K A W analyzed and interpreted data. A A, M C E, M J B, R D, W L L, R B G, D J H and K A W wrote, reviewed and revised the manuscript.

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