The Regulation and Signalling of Anti-Müllerian Hormone (AMH) in human granulosa cells: relevance to Polycystic Ovarian Syndrome (PCOS).

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Keywords: AMH, PCOS, SMAD, androgens, oestradiol, folliculogenesis, FSHR, aromatase.

Abstract

Study question: What is the cause of the high AMH level in PCOS and what are the consequences of this high production for follicle function in these ovaries?

Summary answer: Low doses of androgens inhibited AMH production, but at levels corresponding to hyperandrogenemia this effect was lost. There was dysregulation of the SMAD signalling system in granulosa cells from polycystic ovaries indicating a form of 'AMH-resistance'.

What is known already: In normal ovaries, AMH exerts an inhibitory role on antral follicle development and a fall in levels is prerequisite for ovulation. Levels of AMH are high in PCOS, contributing to the dysregulated follicle growth that is a common cause of anovulatory infertility in these women.

Study design, size and duration: Granulosa cells were cultured with a range of doses of various androgens to determine the effects on AMH production. Cells were also treated with PHTPP (an oestrogen receptor β (ERβ) antagonist) to examine the relationship between AMH expression and the ratio of ERα:ERβ. The differential dose-related effect of AMH on gene expression and SMAD signaling was investigated in human granulosa-luteal cells (hGLC) from women with normal ovaries, polycystic ovarian morphology (PCOM) and PCOS. Cells were also cultured for a prolonged period with AMH at different doses to assess the effect on cell proliferation and viability.

Participants/materials, setting and methods: AMH protein production by cells cultured with androgens was measured by ELISA. The effect of PHTPP on the mRNA expression of AMH, ERα and ERβ was assessed by real-time quantitative PCR (qPCR). The influence of AMH on the relative mRNA expression of aromatase, AMH, AMHRII, FSHR and LHR in control, PCOM and PCOS hGLCs was quantified by qPCR. Western blotting was used to assess the change in levels of SMAD proteins (pSMAD-1/5/8; SMAD-4; SMAD-6 and SMAD-7) after exposure of hGLCs from normal and PCOS women to AMH. The Apotox-Glo Triplex assay was used to evaluate the effect of AMH on cell viability, cytotoxicity and apoptosis.

Main results and the role of chance: Testosterone reduced AMH production at 10^{-9}-10^{-7}M ($p<0.05$; $p<0.005$, multiple uncorrected comparisons Fishers LSD), but equivalent hyperandrogenemic levels favored persistence of AMH levels. 5α-DHT produced a significant dose-related increase in AMH ($p=0.022$, ANOVA). Increasing the ratio of ERα:ERβ produced a corresponding increase in AMH expression ($p=0.015$, two-way ANOVA). AMH increased aromatase ($p<0.05$, one-way ANOVA) and FSHR ($p<0.0001$, one-way ANOVA) in cells from women with PCOM, but not in cells from normal or PCOS (normal n=7, PCOM n=5, PCOS n=4). In contrast to cells from ovulatory ovaries, in PCOS AMH reduced levels of stimulatory pSMAD-1/5/8 and SMAD-4 but increased inhibitory SMAD-6 and -7 ($p<0.05$, normal n=6, PCOS n=3). AMH at 20 and 50ng/ml decreased KGN cell proliferation but not viability after 8 days of treatment ($p<0.005$, two-way ANOVA).

Limitations, reasons for caution: Luteinised GC from women undergoing IVF have a relatively low expression of AMH/AMHRII, but advantageously continue to display responses inherent to the ovarian morphology from which they are collected. To compensate, we also utilized the KGN cell line which has been characterized to be at a developmental stage close to that of immature GC.

Wider implications of the findings: Normal folliculogenesis and ovulation is dependent on the timely reduction in AMH production from GC at the time of follicle selection. Our findings reveal for the first time that theca-derived androgens may play a role in this model but that this inhibitory action is lost at levels of androgens equivalent to those seen in PCOS. The decline may either be direct or indirect via conversion to oestradiol and action through the upregulation of ERα, which is known to stimulate the AMH promoter. Interestingly the ability of GCs to respond to this continually elevated AMH level appears to be reduced in PCOS due to an adaptive alteration in the SMAD signaling pathway and lower expression of AMHRII.

Study funding/competing interest(s): This study was funded by the Thomas Addison Scholarship. The authors report no conflict of interest in this work and have nothing to disclose.

Trial registration number: a trial registration number is only required for clinical trials.

Introduction

Polycystic ovary syndrome (PCOS) is the most prevalent endocrine disorder, affecting 10 to 20% of women of reproductive age worldwide (Homburg, 2008). PCOS is the primary cause of anovulatory infertility and is often accompanied by hyper-androgenism and hyperinsulinemia (Baird *et al.*, 2012). AMH is a product of the granulosa cells (GC) of small antral follicles (AFs), and increased serum levels of AMH in PCOS are due to a combination of an increase in follicle number and excessive AMH production by each follicle (Pellatt *et al.*, 2007; Desforges-Bullet *et al.*, 2010). The consensus is that AMH has an inhibitory or stalling role on antral follicle development (Pellatt *et al.*, 2010; Dewailly *et al.*, 2014, 2016), which in the normal ovary acts to counterbalance over-recruitment of growing follicles (Dewailly *et al.*, 2016). This is supported by the fact that serum AMH levels are two to five times higher in women with PCOS and are considerably higher in women with anovulatory cycles compared to those with ovulatory PCOS (Pigny *et al.*, 2003; Laven *et al.*, 2004; Park *et al.*, 2010).

AMH has been shown to reduce follicle sensitivity to FSH by decreasing FSH-stimulated FSH-receptor (FSHR) expression *in vitro* in human GCs (Pellatt *et al.*, 2011), and aromatase expression *in vivo* in mice (Ma *et al.*, 2016), both of which are needed to drive GC proliferation and follicle growth. Recent studies have shown that gonadotrophins play a role in regulating AMH expression, with FSH shown to be involved in the suppression of AMH expression (Roy *et al*, 2018) but LH in its stimulation, at least in GC from women with PCOS but not control (Pellatt *et al.*, 2007). Conversely LH *downregulated* AMHRII mRNA expression in GC from women with normal ovaries, but not in cells from PCOS (Pierre *et al.*, 2013).

Whilst there appears to be a regulatory relationship between androgens and AMH, the data are conflicting, and it is not always possible to rule out that the observed effects may be mediated by oestradiol (E2), via aromatisation of testosterone (Grynberg *et al.*, 2012; Pierre *et al.*, 2017). Several studies have confirmed a negative correlation between AMH and E2 levels in the follicular

fluid of small antral follicles from human ovaries (Dewailly *et al.*, 2016). The effects of E2 are mediated through oestrogen receptors (ER), which act as ligand-dependent transcription factors in the classic nuclear receptor genomic pathway (Klinge, 2001). There are two forms of ER – Erα and ERß (aka ESR1 and ESR2), which have overlapping and distinct mechanisms of action and are both expressed in various ovarian tissue compartments. Oestrogen signalling is selectively stimulated or inhibited depending upon a balance between ERα and ERβ activities (Lee *et al.*, 2012). In addition, there is an oestrogen response element (ERE) half-site on the AMH promoter

It is clear that GC in follicles of polycystic ovaries are exposed to high levels of AMH; but whether the AMH intracellular signalling pathway continues to operate normally in the face of this continual over-exposure remains unknown. AMH signals by binding to the AMH-specific type II transmembrane serine/threonine kinase receptor (AMHRII), which then forms a complex with and activates a type-1 receptor, phosphorylating SMAD-1/5/8 (pSMAD), leading to the formation of a tetrameric complex consisting of two AMHRIIs and two Type-I receptors (possibly ALK 2, 3 or 6) (Josso *et al.*, 2001). The pSMADs-1/5/8 complex with the common SMAD-4 and translocate to the nucleus to regulate target gene expression via interaction with other transcription factors, co-activators and co-repressors, though to date only a few AMH target genes have been identified (Josso and Di Clemente, 2003). Inhibitory SMADs (I-SMAD) negatively regulate intracellular SMAD signalling: SMAD-6 specifically inhibits activation of BMP pathways by competing with pSMAD-1/5/8 for binding to co-SMAD-4, whereas SMAD-7 inhibits activation by binding to the type I receptor (Attisano, 2002). The complexity of this signalling cascade leave open the prospect of numerous possibilities for pathological changes.

AMH is named for its classic role of causing apoptosis of the cells of the Müllerian duct in the male fetus. It is interesting to speculate therefore whether, in addition to any endocrine or signalling abnormalities, the high AMH concentration found within the follicles in polycystic ovaries could be causing apoptosis of the surrounding GCs also leading to loss of follicle progression (Guibourdenche *et al*., 2003).

In order to elucidate a possible cause for the increased AMH production in PCOS and the consequences for *in-vivo* follicle development, our aims were: (1) to determine whether androgens directly/indirectly altered AMH production from granulosa cells; (2) to clarify the effect of E2 on AMH production by investigating the correlation between levels of AMH expression and that of ERα and ERβ; (3) to determine whether AMH at high (PCOS) concentrations exerts differential effects on the gene expression of the gonadotrophin receptors, aromatase and its own receptor in granulosa-lutein cells (GLCs) from women with normal ovaries, polycystic ovarian morphology (PCOM) or PCOS; (4) whether prolonged exposure to high AMH levels induced apoptosis in granulosa cells; and (5) to provide a mechanistic insight by determining the effect of AMH on the SMAD signalling proteins in granulosa cells from women with or without PCOS.

Material and Methods

All reagents from Sigma, Poole unless stated and all plastic ware from Fisher Scientific, UK.

Subjects and Collection of Human GLC samples

GLCs were isolated from follicular fluid (FF) aspirates obtained from women undergoing IVF. Patients were assigned with normal; asymptomatic polycystic morphology (PCOM) or PCOS status based on the Rotterdam criteria (2004) following ultrasound assessment. The designation of PCOM was based on the presence of polycystic ovaries on transvaginal ultrasound (>12 follicles measuring 2-9mm after spontaneous or progestin-induced menstruation) without accompanying signs of hyper-androgenemia (biochemical/clinical signs of hirsuitism/acne) or oligo-ovulation/anovulation. It is well documented that PCOM is a common age-dependent finding in ovulatory women without any of the accompanying symptoms of PCOS or metabolic significance; allowing it to be considered as a separate defining feature (Balen *et al*, 2009; Jonhstone EB *et al*, 2010). Ethical approval was granted by South West-Frenchay Research Ethics Committee (REC reference: 12/SW/0305), with limited

149 access to patient information beyond age, ovarian status, AFC and basic hormonal profile (see Table
150 1).

151 Experiments that compared effects between normal and polycystic ovaries used GLCs, in
152 spite of their lower expression of AMH/AMHRII, as they retain their PCOS phenotype *in vitro*. GLCs
153 were pooled across follicle sizes and from patients of the same ovarian category i.e. normal, PCOM
154 or PCOS. This allowed for sufficient cellular material to be available for multiple treatments and also
155 downstream analysis, such as Western blotting which require a considerable amount of protein.
156 Further mechanistic insight was provided by the use of the KGN granulosa cell-line, which is well
157 established to correspond to immature GC from smaller antral follicles. (Nishi *et al.*, 2001).

158 **KGN cell culture with androgens and AMH enzyme linked immunosorbent assay (ELISA)**

159 To determine whether androgens alter AMH production, KGN cells were grown and
160 passaged in 10% DMEM-F12 supplemented with L-glutamine and penicillin/streptomycin
161 (Invitrogen), at 37^{o}C in 95% air/CO2. Cells were plated in 12-well plates at $3x10^{3}$ cells/well and
162 cultured in 1% DMEM-F12 (charcoal-stripped) overnight. Testosterone (T), androstenedione (A4),
163 or DHEA (aromatisable androgens) and 5-α-dihydrotestosterone (DHT) (non-aromatisable
164 androgens), at a range of concentrations seen in women with normal and PCOS, were added to the
165 cells for 48h. To distinguish if the effect on AMH by androgens was directly via the androgen receptor
166 (AR) or an indirect effect via aromatisation to E2, flutamide (F) (10^{-6}M), a selective antagonist of the
167 androgen receptor, was added 4 hours before the androgen treatment. AMH secreted into the
168 conditioned medium was measured by ELISA (details of the kit and assay used are described
169 extensively in Pellatt *et al.*, 2007). KGN cells were used for these experiments as their protein
170 production of AMH is greater than in GLCs, hence if levels were inhibited/reduced by androgen
171 treatment they would still be within the readable range of the assay.

KGN cell culture with PHTPP and real-time quantitative PCR (qPCR)

To ascertain whether E2 altered AMH expression via changing the ratio of *ERα:ERβ*, KGN cells were grown and cultured as described above, except that cells were plated in 18-well plates at a density of $3x10^5$ cells/well. After overnight incubation, cells were treated for 48h with varying doses of PHTPP (an ERβ antagonist) (10^{-6} to 10^{-8}M) and testosterone (500nM) as a substrate for E2 conversion. RNA was extracted, reverse transcribed and qPCR performed for *AMH, ERα and ERβ* relative to *L19* as previously described (Rice *et al*, 2006).

MTT and ApoTox assay

To assess the effect of AMH on cell viability and apoptosis, KGN cells at 5,000 cells/well in triplicate wells were cultured for eight days with a range of AMH doses (1 – 50ng/ml) and medium was replenished in all wells every other day. Since these experiments required prolonged culture, the use of KGNs rather than GLCs was more suitable. An MTT assay was performed on day 3, 6 and 8 by adding 25µl of MTT (25mg/ml) for four hours. The medium was then aspirated and 250µl of dimethyl sulfoxide added prior to measuring the absorbance. The ApoTox-Glo Triplex assay (Promega, UK) was used to assess the effect of AMH on cell viability, cytotoxicity and apoptosis within a single assay well as per the manufacturer's protocol.

GLC culture and qPCR for aromatase, AMH, AMHRII, FSHR and LHR

To determine the effect that AMH had on the mRNA expression of aromatase, AMH, AMHRII, FSHR and LHR and whether this was altered in PCOS, GLCs from women with normal, PCOM and PCOS ovarian statue were isolated as previously described (Wright *et al.*, 2002) and cultured with various AMH doses. Briefly, cells were pelleted from FF and layered onto a 45% Percoll (Sigma, Poole, UK) gradient to extract GLCs which were retained at the PBS-Percoll interface. GLCs were washed and plated at 10^5 cells/well in 24-well plates with M199 (5% FBS) for 48 hours, to allow for a return of LH responsiveness, followed by 48 hours in 1% M199 with AMH (0 - 20 ng/mL) (R&D

Systems) prior to RNA extraction. The relative expression of *aromatase, AMH, AMHRII, FSHR* and *LHR* was assessed using qPCR, with normalisation to a reference gene *L19*. This was selected as the most stably-expressed reference gene using a panel of house-keeping genes in the geNorm kit (Primer Design, Southampton, UK). Gene-specific primer sequences are listed in Table 2 (all obtained from Sigma-Genosys apart from AMH, from Primer Design).

GLC culture and SMAD protein Western blot analysis

To ascertain whether there was a differential response to AMH downstream of its binding to the AMHRII in GLCs taken from normal compared to polycystic ovaries, western blotting using various anti-SMAD antibodies was conducted. GLCs were plated at 10^6 cells/well in 6-well plates and treated with AMH (0 - 20 ng/mL) for 30 minutes. Cells were then scraped into ice-cold PBS, centrifuged, and cell pellets re-suspended in RIPA buffer (Cell Signalling Technologies, New England Biolabs, UK) with protease and phosphatase inhibitors and stored at −80°C. Protein levels were measured by Bradford assay and Western blotting performed with equal amounts of protein from each treatment group with the relevant antibodies against pSMAD proteins 1/5/8, -4, -6 & -7 (Table 3). β-actin was used as the reference protein and loading control. Fluorescently-conjugated (infra-red dye) secondary antibodies were used for visualization using the Odyssey Imaging System (Li-Cor Biosciences) (Pellatt *et al.*, 2011).

Statistical Evaluation

All data are represented as the mean ± SEM of triplicate or more observations (detail in legends) from a minimum of 3 or more independent experiments unless otherwise stated. qPCR data were analysed using the ΔΔCt method as described in detail previously (Rice *et al*, 2006), with normalisation to L19 and subsequent normalisation to the Ct value of the control (untreated). In order to use the ΔΔCt method, the amplification efficiency for each GOI and reference gene must be in the recommended range of 90-100%. This was rigorously applied to our study by the inclusion of a

standard curve for every single qPCR assay conducted. Data from Western blots represent the mean densitometry measurements taken from all individual experiments using Image Studio software (Licor™) and normalized to ß-actin and then to the control (untreated) samples. Statistical significance was determined by ANOVA followed by post hoc tests: unpaired Student's or paired t test when 2 groups were compared (depending on the design of the experiment) or a one-sample t test when comparing with normalized control values. Significance was set at $P \leq 0.05$.

Results

Clinical data of the subjects

The main clinical parameters in the control (normal ovaries), PCOM and PCOS women are summarised in Table 1. No difference between the three populations was found for body mass index (BMI) or FSH serum levels. As expected serum AMH levels and antral follicle counts (AFC) were significantly higher in the PCOM and PCOS group compared to normal. PCOS women were younger and had significantly higher LH serum levels than controls, which is agreement with other findings (Pierre *et al,* 2013), and confirms the accuracy of the PCOS categorisation of these women.

The effect of Androgens on AMH protein expression

The effect of various androgens on AMH production by KGN cells was determined in the presence/absence of flutamide (the selective AR antagonist). T reduced levels of AMH protein below that of basal at the lower doses (10^{-9} to 10^{-7}M) (figure 1A). At 10^{-6}M and above, AMH production was the same as basal, with no attenuating effects. The addition of flutamide did not alter the attenuating effects of T on AMH production apart from at 10^{-6}M, where it reduced the basal level of AMH even lower. Treatment with DHEA ± flutamide, had no effect on AMH (figure 1B), and likewise neither did androstenedione (data not shown). Though it would appear that flutamide reduced basal AMH production when added alone to the T experiment (fig 1A), it did not do so in the DHEA (figure 1A & 1B) or the androstenedione experiment, indicating that this it was probably an artefact. This proved

to be the case when all the basal (control) and F+control data from all the androgen experiments were pooled together and analysed (see supplementary figure 1), showing no effect of flutamide on basal AMH production. As with testosterone, treatment with 5α-DHT at 10^{-8}M significantly reduced AMH production, but as the concentration of 5α-DHT increased, levels recovered back to basal values with a small but significant increase in AMH production above control at 10^{-5}M (figure 1C).

The effect of altering the ratio of ERα:ERβ on to determine the oestrogen-mediated effect on AMH expression

Culturing the cells in the presence of PHTPP (the ERβ antagonist) produced a dose-related increase in the expression of ERα to ERβ with a nearly 30-fold increase over basal at the highest dose of PHTPP. There was a commensurate dose-dependent increase in *AMH* expression with a significant 5-fold increase over basal seen at the highest dose of PHTPP (10^{-6}M) used (Fig 2).

The effect of AMH on aromatase, AMH, AMHRII, FSHR and LHR expression in GLCs from women with morphologically normal, PCOM and PCOS ovaries

There was no difference in the basal mRNA expression levels of all genes in GLCs from all three ovarian types, apart from *AMHRII* which had significantly lower mRNA levels in GLCs from PCOS compared to both PCOM and normal (supplementary figure 2). Consequently with respect to the qPCR analysis for *AMHRII*, the second normalisation for both PCOM and PCOS ΔCt values were done to the average ΔCt of the control values for the normal ovaries, rather than their respective untreated ΔCt values.

AMH treatment above 5ng/ml decreased aromatase expression in cells from women with PCOS, but not in cells from normal (figure 3A). In contrast, 10 and 20 ng/mL AMH significantly stimulated aromatase expression in PCOM cells compared to normal (figure 3A). AMH treatment had no effect on its own expression in any ovarian type (figure 3B), nor that of *AMHRII* or *LHR* (figure 3C & E), in spite of the lower basal expression of *AMHRII* in PCOS cells. AMH increased *FSHR*

expression in PCOM cells compared to normal and PCOS, reaching significance at 5, 10 and 20 ng/mL (figure 3D).

The effect of AMH on KGN cell proliferation and apoptosis

Incubation of cells with concentrations of AMH likely to be present in the polycystic ovary (20 & 50 ng/ml), inhibited proliferation after 8 days (figure 4A & B), which reflects the time it would take to alter the balance between cell cycle proliferation and arrest. This was not due to effects on cell viability (figure 4C) and AMH did not appear to be cytotoxic (figure 4D) or to cause increased apoptosis (figure 4E), in fact if anything, it appeared slightly protective of cell death.

The effect of AMH on SMAD signalling pathways in normal and PCOS GLCs

There was no significant difference in the relative levels of SMAD proteins in cells from women with normal or PCOS (supplementary figure 3), though interestingly AMH treatment produced diametrically opposed effects on SMAD protein expression between cells from the two types of ovary. In GLCs from normal ovaries AMH increased levels of pSMAD 1/5/8 by approximately 50% compared to basal, however these results did not reach statistical significance due to the wide variation in levels from individual patients (figure 5A & B). In contrast, AMH significantly *decreased* levels of pSMAD 1/5/8 in PCOS GLCs below basal (figure 5A & 5B). Similarly, AMH (1 and 5ng/mL) increased SMAD-4 protein levels in cells from normal ovaries compared to those from PCOS, with no further increase at the highest AMH dose. In contrast, the highest concentration of AMH significantly down-regulated SMAD-4 levels below that of basal in PCOS cells compared to control (figure 5C). In the normal cells AMH had no effect on SMAD-6 levels, whereas high concentrations (≥5ng/ml) of AMH significantly increased SMAD-6 protein levels in cells from PCO (figure 5D). A similar response was seen for SMAD-7, in that AMH had no effect on SMAD-7 protein in normal cells, but increased protein levels significantly in cells from women with PCOS compared to normal (figure 5E).

Discussion

We have revealed new differences in the AMH/AMHRII signalling system in the normal and polycystic ovary, and shown that thecal androgens, in particular T and 5α-DHT, were able to alter AMH production by GC. Levels of androgens seen in hyperandrogenemia (HA) enhanced or maintained AMH protein production, though a proportion of this effect was regulated by its conversion to E2 via alterations in the expression levels of ERα and ERβ, ie. E2 acts to stimulate AMH via ERα but to inhibit it via ERß. As expected, AMH treatment affected the expression of genes involved in follicle growth i.e. aromatase and FSHR (Pellatt *et al.*, 2011), but we have shown for the first time that cells from women with PCOS respond differently to AMH compared to those from normal ovaries or those with solely a polycystic morphology. Most importantly, we have shown that this difference extends from levels of its receptor through to intracellular SMAD signalling, in that cells from women with PCOS have an entirely different response to AMH compared to those from normal ovaries. In spite of this, prolonged exposure to relatively high doses of AMH did not induce apoptosis, which could account for the observation that in spite of AMH stalling AF growth in women with PCOS, the follicles remain viable.

It is well known that androgens have a role to play in folliculogenesis, with a reduction in levels required for normal follicle growth and progression, and increased levels causing follicular dysfunction (Lebbe & Woodruff, 2013). To investigate whether theca-derived androgens were in turn involved in regulating AMH production by GC, cells were cultured with increasing doses of the four major androgens with the inclusion of flutamide to identify whether the actions were occurring indirectly via conversion to oestrogen. In other words if androgens altered AMH production, then this effect would be antagonised by blocking the AR; however if the effect was via the conversion of androgens to E2 then the presence of flutamide would not alter AMH levels further.

Overall, T and 5α-DHT at doses equivalent to normo-androgenaemic levels, inhibited AMH; but doses equating to hyper-androgenaemic levels seen in PCOS, appeared to favour a persistence

of AMH expression. It must be pointed out that whilst the effects were modest, we were measuring AMH protein secreted into media and that the cellular protein levels would in fact have been higher. The fact that the presence of flutamide made no discernible difference to T's actions, indicates that the reduction in AMH expression seen with T could be occurring indirectly through its conversion to E2. There is a growing body of evidence to support the assertion that E2 down-regulates the expression of AMH & AMHRII (Grynberg *et al.*, 2012; Pierrre *et al*., 2017), and that this effect was associated with alterations in the ratio of ERα:ERβ (Grynberg *et al.*, 2012; Pierre *et al.*, 2017). Grynberg *et al* (2012) clearly showed that when cells were transfected with ERα and treated with increasing doses of E2, there was an increase in AMH promoter activity, with a significant attenuation occurring on transfection with ERβ. This supports our findings that blocking ERβ with the antagonist PHTPP, and hence increasing the ratio of ERα:ERβ, produced a significant increase in AMH expression. Interestingly, the non-aromatisable 5α-DHT produced a dose-related increase in AMH protein production at levels associated with HA. This further corroborates the findings of Pierre *et al* (2017), who demonstrated that 5α-DHT increased AMH mRNA expression in GC from women with PCOS overexpressing the AR, but not in normal ovaries.

There have been a limited number of studies investigating the effect of androgens on AMH and the results have been inconsistent, probably due to variability in cell types, species, doses used and methods of expression analysis (mRNA/ELISA kits). High doses of T inhibited AMH production by GC from small bovine AFs (3-4mm) (Nicolás Crisosto *et al.*, 2009), but T stimulated AMH mRNA production in GC from mouse AF (Zhang *et al.*, 2016). In contrast to our findings and that of Pierre *et* al, treatment of a human granulosa cell line (H023) with increasing doses of DHT produced a reduction in AMH mRNA (Lan *et al.*, 2013). Using the androgenised rat model, Chen *et al* (2015) showed that DHT suppressed FSH-stimulated GC proliferation by upregulating PTEN expression. This highlights the need for further investigations into the complex, multi-signalling regulation of AMH production.

One limitation of our study is that we used luteinised GC pooled across follicle sizes for each woman undergoing IVF which have a relatively low expression of AMH/AMHRII, as they have already progressed to form *corpora lutea* due to exogenous gonadotrophin stimulation during IVF. We know however that these cells maintain their phenotype with respect to ovarian morphology *in vitro*, which does mean that they are suitable for investigating the legacy of the impact of PCOS on cellular functions, hence allowing for extrapolation of the changes to the non-IVF cycle. To counter balance this limitation we also used KGN cells that have been characterised as equivalent to immature GC (Nishi *et al.*, 2001) and produce more AMH than GLCs. This was especially important regarding experiments investigating attenuation of AMH expression, since any reduction of the already low expression levels would push the limits of even sensitive laboratory techniques such as qPCR. In addition the robustness of this cell line made them suitable for prolonged culture.

We and others have previously shown that in both un-luteinised and luteinised GCs from normal ovaries, AMH had no effect on unstimulated levels of aromatase, but reduced FSH-stimulated aromatase expression (Pellatt *et al.*, 2011; Sacchi *et al.*, 2016). Likewise, in this study, increasing doses of AMH had no effect on aromatase expression in normal GLCs, but surprisingly reduced aromatase mRNA expression by approximately 25-50% in PCOS GLCs, although these differences did not reach significance due to high inter-patient variability. Interestingly however, AMH (10, 20ng/ml) significantly increased aromatase expression in PCOM compared to PCOS cells, which was probably due to the increased expression of FSHR in PCOM cells compared to PCOS, suggesting that *in vivo* this would have the effect of indirectly increasing aromatase. This highlights the fact that women with anovulatory PCOS are a distinct sub-group, and that the polycystic ovarian morphology *per se* is not associated with diminished FSH responsiveness (Homburg, 2008).

Prolonged treatment of KGN cells with increasing doses (20 & 50 ng/ml) of AMH did not induce apoptosis as measured by caspase activity, though the cells were capable of undergoing apoptosis as shown by addition of the apoptotic agent camptothecin. This is in contrast to the study by Anttonen *et al,* who demonstrated that prolonged culture of KGN cells with AMH reduced cell

numbers and induced apoptosis; though this effect was only at supra-physiological doses of 10 & 25µg/ml AMH which far exceeded the average AMH level of 0.68ng/ml that they measured in culture media (Anttonen *et al,* 2011). This lack of an apoptotic effect of AMH *in vivo* explains the ability to "rescue" stalled follicles with super-ovulation regimes, as shown by Hayes *et al* (Hayes *et al.*, 2016). We speculate that the difference in effects in comparison to the Müllerian ducts may be due to either an indirect action by AMH on the epithelial cells in the latter occurring after initial interaction with mesenchymal cells, which is does not occur in the follicle (Roberts *et al.*, 1999); or a dosing effect as shown by the ability of AMH to promote Sertoli cell proliferation in mice at low doses (10-50ng/ml) and apoptosis at high concentrations (50-800ng/ml) (Rehman ZU *et al,* 2017)

Given the similarity in spectrum of the PCOM cells to normal, we decided to investigate the AMH intra-cellular signalling pathway in normal ovaries and PCOS. Using specific anti-SMAD antibodies, we have demonstrated for the first time that there was no difference in the basal levels of SMAD proteins between normal and PCOS cells. However, AMH treatment at an equivalent concentration to that measured in GC-conditioned media from women with anovulatory PCO (20ng/mL), increased pSMAD-1/5/8 and SMAD-4 protein levels in normal GLCs, but strikingly, significantly *decreased* levels in PCOS GLCs. This indicates that the AMH-mediated activation and nuclear translocation of pSMAD-1/5/8 and SMAD-4 to alter downstream target gene expression is reduced in PCOS cells compared with normal. It must be remembered that other members of the TGF-ß family of signalling proteins also utilise the SMAD signalling pathway, eg BMP-15 secreted from the oocyte activates SMAD-1/5/8 (Knight and Glister, 2006; Liu *et al.*, 2018), adding to the cross-talk and complexity of AMH signalling.

SMADs -6 and -7 act as inhibitors of BMP signalling via a negative feedback loop which leads to cessation of BMP signalling (Ishida *et al.*, 2000). In addition they can inhibit signalling by binding to Type-I receptors and preventing SMAD-1/5/8 phosphorylation (Heldin *et al.*, 1997; Itoh *et al.*, 2001). SMAD-6 also reduces BMP signalling by acting as a SMAD-4 decoy leading to inhibition of SMAD-4 translocation to the nucleus (Hata *et al.*, 1998). Finally, SMAD-6 can bind to DNA within

the nucleus and recruit histone deacetylases leading to repression of gene transcription (Bai and Cao, 2002). SMAD-7 on the other hand suppresses BMP signalling via ubiquitination and degradation of Type-I receptors (Ebisawa *et al.*, 2001). Hence, AMH signalling can be regulated by the levels of SMAD-6 and -7. Interestingly we saw that in GLCs from polycystic ovaries AMH increased the levels of the inhibitory SMAD-6 and -7, an effect not seen in normal cells. Whilst AMH treatment did not affect expression of its own receptor, it is interesting to speculate that the lower basal levels of *AMHRII* in GLCs from PCOS may be linked to the altered SMAD signalling. Detailed analysis of the processing of AMHRII have identified novel mechanisms involved in its negative regulation, through cleavage intracellular retention and oligomerization, which has implications for its signalling output (Hirschhorn *et al*, 2015). The overall effect would appear to dampen AMH signalling in PCOS which may partially negate the effects of the high levels. We need to further understand the downstream effects of AMH and its integration into other signalling pathways to fully interpret this result.

To summarise, there is a complex and finely balanced interaction between AMH, FSH, LH and aromatase to regulate follicle growth and selection via the supply of androgens as a substrate for E2 formation. Crucial to the success of normal folliculogenesis and ovulation is the timely reduction in AMH production from GC and normal signalling events downstream of AMH binding to its receptor, which allow for upregulation of FSHR and progression of AF growth. Though the effect we saw in our study was relatively modest, it appears that the increase in thecal production of testosterone and 5α-DHT contribute to the decline in AMH production from GC seen at the time of follicle selection. Part of this attenuation maybe be attributed to the conversion of testosterone to E2 and its actions via ERβ to reduce AMH production (figure 6a). Excess levels of androgens, equivalent to those seen in PCOS, may prevent this decline which is also mediated by an upregulation of ERα expression. Prolonged exposure to high levels of AMH in PCOS disrupts this balance, as seen by the altered expression patterns of aromatase and FSHR and dysregulated SMAD signalling, with increased levels of the I-SMAD-6 and -7 and reduced activation of SMAD-

1/5/8 and the co-SMAD-4 (figure 6b). We speculate that *in vivo*, these high levels of AMH may cause uncoupling or desensitization of the AMH signalling pathway leading to the dysregulated follicle growth seen in PCOS.

Declaration of Interest

The authors report no conflict of interest in this work and have nothing to disclose.

Funding

Thomas Addison Scholarship

Authors Roles

ND, LP & SR performed data acquisition, analysis and contributed to the drafting of the article. EJ and MO performed additional data acquisition and analysis. All the other authors, in particular HM and SR who supervised the experimental work, were involved in critical intellectual input and study design, interpretation of data, and drafting, critical revision and approval of the paper for final submission of the manuscript.

Acknowledgements

We would like to The Fertility Centre, Homerton University Hospital, London for providing GLCs, with particular thanks to Drs William Dooley, Abraham Francis, Gilat Sachs, Shital Sawant for their help with recruiting patients and retrieving the clinical data. Additionally, we would like to thank Mr Michael Lacey for his assistance with the collection of follicular fluid from the Homerton Hospital. Finally, and most importantly we'd like to acknowledge and thank all the patients who donated tissue and cells which allowed us to conduct this study.

References

Anttonen M, Färkkilä A, Tauriala H, Kauppinen M, Maclaughlin DT, Unkila-Kallio L, Bützow R, Heikinheimo M.(2011) Anti-Müllerian hormone inhibits growth of AMH type II receptor-positive human ovarian granulosa cell tumor cells by activating apoptosis. *Laboratory Investigations* **91**:1605-14

Attisano L (2002) Signal Transduction by the TGF-beta Superfamily. *Science* **296** 1646–1647.

Bai S and Cao X (2002) A nuclear antagonistic mechanism of inhibitory Smads in transforming growth factor-β signaling. *Journal of Biological Chemistry* **277** 4176–4182.

Baird DT, Balen A, Escobar-Morreale HF, Evers JLH, Fauser BCJM, Franks S, Glasier A, Homburg R, La Vecchia C, Crosignani PG *et al.* (2012) Health and fertility in world health organization group 2 anovulatory women. *Human Reproduction Update* **18** 586–599.

Balen A, Homburg R, Franks S. (2009) Defining polycystic ovary syndrome. *BMJ* **338**

Chen MJ, Chou CH, Chen SU, Yang WS, Yang YS, Ho HN.(2015) The effect of androgens on ovarian follicle maturation: Dihydrotestosterone suppress FSH-stimulated granulosa cell proliferation by upregulating PPARγ-dependent PTEN expression. *Scietific Reports* **5**:18319.

Desforges-Bullet V, Gallo C, Lefebvre C, Pigny P, Dewailly D and Catteau-Jonard S (2010) Increased anti-Müllerian hormone and decreased FSH levels in follicular fluid obtained in women with polycystic ovaries at the time of follicle puncture for in vitro fertilization. *Fertility and Sterility* **94** 198–204.

Dewailly D, Andersen CY, Balen A, Broekmans F, Dilaver N, Fanchin R, Griesinger G, Kelsey TW, La marca A, Lambalk C *et al.* (2014) The physiology and clinical utility of anti-Müllerian hormone in women. *Human Reproduction Update* **20** 370–385.

Dewailly D, Robin G, Peigne M, Decanter C, Pigny P and Catteau-Jonard S (2016) Interactions between androgens, FSH, anti-Mullerian hormone and estradiol during folliculogenesis in the human normal and polycystic ovary. *Human Reproduction Update* **22** 709–724.

Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T and Miyazono K (2001) Smurf1 Interacts with Transforming Growth Factor-β Type I Receptor through Smad7 and Induces Receptor Degradation. *Journal of Biological Chemistry* **276** 12477–12480.

Grynberg M, Pierre A, Rey R, Leclerc A, Arouche N, Hesters L, Catteau-Jonard S, Frydman R, Picard JY, Fanchin R *et al.* (2012) Differential regulation of ovarian anti-müllerian hormone (AMH) by estradiol through α- and β-estrogen receptors. *Journal of Clinical Endocrinology and Metabolism* **97** E1649-57.

Guibourdenche J, Lucidarme N, Chevenne D, Rigal O, Nicolas M, Luton D, Léger J, Porquet D and Noël M (2003) Anti-Müllerian hormone levels in serum from human foetuses and children: Pattern and clinical interest. In *Molecular and Cellular Endocrinology*, pp 55–63.

Hata A, Lagna G, Massagué J and Hemmati-Brivanlou A (1998) Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes and Development* **12** 186–197.

Hayes E, Kushnir V, Ma X, Biswas A, Prizant H, Gleicher N, Sen A (2016) Intra-cellular mechanism of Anti-Müllerian hormone (AMH) in regulation of follicular development.*Mol.Cell. Endocrinol.* 433:56-65

Heldin CH, Miyazono K and Ten Dijke P (1997) TGF-β signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390** 465–471.

Hirschhorn T, di Clemente N, Amsalem AR, Pepinsky RB, Picard J-Y, Smorodinsky NI, Cate RL and Ehrlich M (2015) Constitutive negative regulation in the processing of anti-Mullerian hormone receptor II. *Journal Cell Science* **126**:1352-1364

Homburg R (2008) Polycystic ovary syndrome. *Best Pract Res Clin Obstet Gynaecol* **22** 261–274.

Ishida W, Hamamoto T, Kusanagi K, Yagi K, Kawabata M, Takehara K, Sampath TK, Kato M and Miyazono K (2000) Smad6 is a Smad1/5-induced Smad inhibitor. Characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter. *Journal of Biological Chemistry* **275** 6075–6079.

Itoh F, Asao H, Sugamura K, Heldin CH, Ten Dijke P and Itoh S (2001) Promoting bone morphogenetic protein signaling through negative regulation of inhibitory Smads. *EMBO Journal* **20** 4132–4142.

Johnstone EB, Rosen MP, Neril R, Trevithick D, Sternfeld B, Murphy R, Addauan-Andersen C, McConnell D, Pera RR, Cedars MI.(2015) The polycystic ovary post-rotterdam: a common, age-dependent finding in ovulatory women without metabolic significance. *J Clin Endocrinol Metab*. 95(11):4965-72.

Josso N and Di Clemente N (2003) Transduction pathway of anti-Müllerian hormone, a sex-specific member of the TGF-β family. *Trends in Endocrinology and Metabolism* **14** 91–97.

Josso N, di Clemente N and Gouédard L (2001) Anti-Müllerian hormone and its receptors. *Molecular and Cellular Endocrinology* **179** 25–32.

Klinge CM (2001) Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Research* **29** 2905–2919.

Knight PG and Glister C (2006) TGF-beta superfamily members and ovarian follicle development. *Reproduction* **132** 191–206.

Lan K-C, Chang SY, Huang F-J, Lin H-J, Lin C-Y, Huang K-E and Kang H-Y (2013) Analysis of androgen receptor and anti-Müllerian hormone pathways in human granulosa cells under luteinizing hormone treatment. *Reproductive Biology and Endocrinology* **11** 11.

Laven JSE, Mulders AGMGJ, Visser JA, Themmen AP, De Jong FH and Fauser BCJM (2004) Anti-Müllerian Hormone Serum Concentrations in Normoovulatory and Anovulatory Women of Reproductive Age. *Journal of Clinical Endocrinology and Metabolism* **89** 318–323.

Lebbe M, Woodruff TK.(2013) Involvement of androgens in ovarian health and disease. *Mol Hum Reprod.* **19(12)**:828-37

Lee H-R, Kim T-H and Choi K-C (2012) Functions and physiological roles of two types of estrogen receptors, ERα and ERβ, identified by estrogen receptor knockout mouse. *Laboratory Animal Research* **28** 71.

Liu C, Yuan B, Chen H, Xu M, Sun X, Xu J, Gao Y, Chen C, Jiang H and Zhang J (2018) Effects of MiR-375-BMPR2 as a Key Factor Downstream of BMP15/GDF9 on the Smad1/5/8 and Smad2/3 Signaling Pathways. *Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology* **46** 213–225.

Nicolás Crisosto, Sir-Petermann T, Greiner M, Maliqueo M, Moreno M, Aedo P and Lara HE (2009) Testosterone-induced downregulation of anti-Mü llerian hormone expression in granulosa cells from small bovine follicles. *Endocrine* **36** 339–345.

Nishi Y, Yanase T, Mu Y-M, Oba K, Ichino I, Saito M, Nomura M, Mukasa C, Okabe T, Goto K *et al.* (2001) Establishment and Characterization of a Steroidogenic Human Granulosa-Like Tumor Cell Line, KGN, That Expresses Functional Follicle-Stimulating Hormone Receptor. *Endocrinology* **142** 437–445.

Park AS, Lawson MA, Chuan SS, Oberfield SE, Hoeger KM, Witchel SF and Chang RJ (2010) Serum anti-Müllerian hormone concentrations are elevated in oligomenorrheic girls without evidence of hyperandrogenism. *Journal of Clinical Endocrinology and Metabolism* **95** 1786–1792.

Pellatt L, Hanna L, Brincat M, Galea R, Brain H, Whitehead S and Mason H (2007) Granulosa cell production of anti-Müllerian hormone is increased in polycystic ovaries. *The Journal of Clinical Endocrinology and Metabolism* **92** 240–245.

Pellatt L, Rice S and Mason HD (2010) Anti-Mullerian hormone and polycystic ovary syndrome: A mountain too high? *Reproduction* **139** 825–833.

Pellatt L, Rice S, Dilaver N, Heshri A, Galea R, Brincat M, Brown K, Simpson ER and Mason HD (2011) Anti-Müllerian hormone reduces follicle sensitivity to follicle-stimulating hormone in human granulosa cells. *Fertility and Sterility* **96** 1246–1251.e1.

Pierre A, Peigné M, Grynberg M, Arouche N, Taieb J, Hesters L, Gonzalès J, Picard J-Y, Dewailly D, Fanchin R *et al.* (2013) Loss of LH-induced down-regulation of anti-Mullerian hormone receptor expression may contribute to anovulation in women with polycystic ovary syndrome. *Human Reproduction* **28** 762–769.

Pierre A, Taieb J, Giton F, Grynberg M, Touleimat S, El Hachem H, Fanchin R, Monniaux D, Cohen-Tannoudji J, di Clemente N *et al.* (2017) Dysregulation of the anti-Müllerian hormone system by steroids in women with polycystic ovary syndrome. *The Journal of Clinical Endocrinology & Metabolism* **102** 3970-3978.

Pigny P, Merlen E, Robert Y, Cortet-Rudelli C, Decanter C, Jonard S and Dewailly D (2003) Elevated serum level of anti-mullerian hormone in patients with polycystic ovary syndrome: relationship to the ovarian follicle excess and to the follicular arrest. *Journal of Clinical Endocrinology and Metabolism* **88** 5957–5962.

Rehman ZU, Worku T, Davis JS, Talpur HS, Bhattarai D, Kadariya I, Hua G, Cao J, Dad R, Farmanullah, Hussain T, Yang L. (2017) Role and mechanism of AMH in the regulation of Sertoli cells in mice. *Journal Steroid Biochemistry Molecular Biology* **174**:133-140

Rice S, Mason HD, Whitehead SA (2006) Phytoestrogens and their low dose combinations inhibit mRNA expression and activity of aromatase in human granulosa-luteal cells. *Journal Steroid Biochemistry Molecular Biology* **101**:216-25.

Roberts LM, Hirokawa Y, Nachtigal MW and Ingraham HA (1999) Paracrine-mediated apoptosis in reproductive tract development. *Developmental Biology* **208** 110–122.

Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop (2004) Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertility & Steriityl* 81:19–25

Roy S, Gandra D, Seger C, Biswas A, Kushnir VA, Gleicher N, Kumar TR, Sen A. (2018) Oocyte-Derived Factors (GDF9 and BMP15) and FSH Regulate AMH Expression Via Modulation of H3K27AC in Granulosa Cells. *Endocrinology* **159(9):**3433-3445

Sacchi S, D'Ippolito G, Sena P, Marsella T, Tagliasacchi D, Maggi E, Argento C, Tirelli A, Giulini S and La Marca A (2016) The anti-Müllerian hormone (AMH) acts as a gatekeeper of ovarian steroidogenesis inhibiting the granulosa cell response to both FSH and LH. *Journal of Assisted Reproduction and Genetics* **33** 95–100.

572 **Wright RJ, Holly JMP, Galea R, Brincat M and Mason HD** (2002) Insulin-like growth factor (IGF)-
573 independent effects of IGF binding protein-4 on human granulosa cell steroidogenesis. *Biology of*
574 *Reproduction* **67** 776–781.
575 **Zhang Y, Wang SF, Zheng JD, Zhao CB, Zhang YN, Liu LL and Huang JH** (2016) Effects of
576 testosterone on the expression levels of AMH, VEGF and HIF-1α in mouse granulosa cells.
577 *Experimental and Therapeutic Medicine* **12** 883–888.
578

Figure & Table Legends

Figure 1. Effect of androgens on AMH production. (A) Treatment of KGN cells with a range of testosterone (T) doses (10^{-9} to 10^{-5}M; black bars) reduced AMH production below basal levels as measured by ELISA (p=0.03; ANOVA), with significance at the lower doses (10^{-9} to 10^{-7}M) (*p<0.05; **p<0.005; multiple uncorrected comparisons Fishers LSD), but there was no attenuation of AMH above 10^{-6}M in comparison to untreated control (white bar). In the presence of the selective AR antagonist flutamide (F; grey bars) (10^{-6}M) there was no further reduction in AMH production in comparison to basal (*p<0.05), apart from at 10^{-6}M (**p<0.05). **(B)** Treatment of cells with DHEA (black bars) ± F (grey bars) had no effect on AMH production. **(C)** Treatment with 5α-DHT (10^{-9} to 10^{-5}M; shaded grey bars) produced a dose-related increase in AMH production compared to control (white bar) (*p=0.022; ANOVA). Low dose 5α-DHT (10^{-8}M) significantly reduced AMH production (*p<0.05), but as the concentration of 5α-DHT increased to 10^{-5}M, there was a small but significant increase in AMH levels (*p<0.05; multiple uncorrected comparisons Fishers LSD) (mean +/- SEM, n = 3).

Fig 2: Effect of altering ERα:ERβ on AMH expression. KGN cells were treated with T (500nM) and a range of PHTPP doses (10^{-8} to 10^{-6}M). The highest dose of PHTPP produced a significant 30-fold increase in ERα:ERβ (black bars) expression compared to basal, with a corresponding 5-fold increase in AMH mRNA expression (white bars) (p=0.014, Two-way ANOVA) (mean±SEM, n=4-6).

Figure 3. Effect of AMH on gene expression in normal, PCOM and PCOS cells. (**A)** AMH had no effect on aromatase in normal cells (solid line) but did inhibit aromatase in PCOS cells (dashed line) (*P* = 0.0014, one-way ANOVA). In contrast, in cells from PCOM

(dotted line) women AMH at high doses *stimulated* aromatase mRNA levels compared to normal cells (*P < 0.05, one-way ANOVA). (**B & E)** AMH treatment had no significant effect on the expression levels of AMH or LHR mRNA in any group. **(C)** Though basal levels of AMHRII were significantly lower in PCOS than normal or PCOM cells, AMH treatment did not affect expression of its receptor in any cell type. (**D)** AMH had no effect on FSHR in GLCs from women with normal (solid line) or PCOS (dashed line), but interestingly, AMH (>5ng/ml) significantly stimulated FSHR mRNA expression in cells from women with PCOM (dotted line) (*P < 0.0001 one-way ANOVA; mean ± SEM, normal = 7 experiments (from 18 women), PCOM = 5 experiments (from 7 women), PCOS = 4 (from 6 women)).

Figure 4. Effect of AMH on KGN cell proliferation and apoptosis. (A & B) AMH at 20 & 50ng/ml (▲) decreased KGN cell proliferation over 8 days of culture compared to non-treated control cells (●). MTT cell proliferation assays were performed on day 3, 6 and 8, absorbance measured, and cell density calculated (mean +/- SEM, n = 6, where 1n is the mean of triplicate wells; *, P < 0.005, Two-way ANOVA). **(C – E)** The ApoTox-Glo triplex assay was performed on day 3, 6 and 8. The fluorescence and luminescence was measured on day 3, 6 and 8 to assess the quantity of viable cells and the level of cytotoxicity and apoptosis induced by 50ng/ml of AMH (mean +/- SEM, n = 4, where 1n is the mean of triplicate wells; **, P < 0.0006, Two-way ANOVA. Control (●); AMH 50ng/ml (▲)).

Figure 5. The effect of AMH on SMAD signalling proteins in normal and PCOS cells. (A) Representative western blot images using anti-pSMAD 1/5/8, -4, -6 & -7 antibodies on total protein lysates extracted from GLCs from women with normal ovaries or PCOS treated with a range of doses of AMH (1-20 ng/ml) for 30 minutes. **(B)** AMH increased

630 pSMAD 1/5/8 protein in cells from normal ovaries (○), while in contrast in PCOS cells (●)
631 there was a dose-dependent inhibition of phosphorylation at 2, 10 and 20ng/ml AMH (* *P*
632 < 0.03; ** *P* < 0.008). **(C)** In normal cells 1 and 5 ng/ml of AMH significantly increased
633 SMAD 4 (** *P* < 0.008, * *P* < 0.05 respectively) but had no effect on SMAD 4 levels in
634 PCOS cells. 20 ng/mL AMH had no effect in normal cells but significantly inhibited SMAD
635 4 in PCOS cells (* *P* < 0.05). **(D)** In the normal cells AMH had no effect on SMAD 6. In
636 PCOS cells the lower doses AMH had no effect on SMAD 6 but above 5ng/mL AMH
637 significantly increased SMAD 6 (** *P* < 0.002; *** *P* < 0.0006). **(E)** AMH had no effect on
638 SMAD 7 in normal cells. At 5 and 10 ng/mL AMH significantly increased SMAD 7 protein
639 in PCOS cells (* *P* < 0.02). (Data represented is mean ± SEM; normal n=6 experiments
640 (17 patients); PCOS n=3 (3 patients); the annotations “a” and “b” are used to denote
641 differences in significance of SMAD levels compared to the control i.e. 0 ng/mL AMH.
642 Asterisks are used to denote significant differences between normal and PCOS at the
643 same dose. All tests done using multiple t test corrected with Holm-Sidak method; P <
644 0.05).
645
646 **Fig 6: Proposed model of AMH regulation and signalling in normal and PCOS. (A)**
647 In the normal ovary it is necessary to get a timely reduction in AMH to allow for AF growth
648 and selection of the dominant follicle. Our results show that T can contribute to this via
649 conversion to E2 and action through ERβ. The non-aromatisable 5α-DHT attenuates AMH
650 production directly. AMH binds and signals exclusively through its Type IIR, and this
651 interaction regulates its actions. The recruitment of the common Type IR opens up
652 interaction of the highly restricted Type IIR with other shared BMP and TGFβ signalling
653 pathways, to allow for the measured growth of follicles. **(B)** In cells from PCOS, hyper-
654 androgenemia prevents the attenuation in AMH directly (5α-DHT) or indirectly via T
655 conversion to E2 and action through the increased expression of ERα. Prolonged

exposure to elevated AMH also reduces aromatase expression which contributes to stalled AF growth. In addition, the normal signalling events downstream of AMH binding to AMHRII are perturbed in PCOS by high levels of AMH that increase protein levels of the inhibitory SMADs, which has implications for progression of follicles. There is also a reduction in the expression of AMHRII which could contribute to the dysregulated signalling events and follicle growth.

Table 1: Main clinical parameters in women with normal ovaries (control), polycystic ovary morphology (PCOM) and polycystic ovary syndrome (PCOS). Comparisons between normal and PCOS showed significant differences with respect to age, AFC and serum levels of AMH and LH. Significant differences were found between women with normal compared to PCOM with respect to AMH serum levels and antral follicle counts (AFC). (Multiple unpaired t-tests,* $p<0.05$, **$p<0.005$, ***$p<0.0005$, ****$p<0.00005$)

Table 2: Primer sequences, concentrations and cycling conditions used in qPCR assays. The cycling conditions were: 95C for 10 minutes, followed by 95C (15sec); annealing temperature (60sec); 72C (60sec) for 50 cycles.

Table 3: Specifications of antibodies used in Western blot analysis, including dilutions of the primary antibodies; the predicted molecular weight of each protein detected and the corresponding fluorescently-labelled secondary antibody.

Supplementary Figure Legends

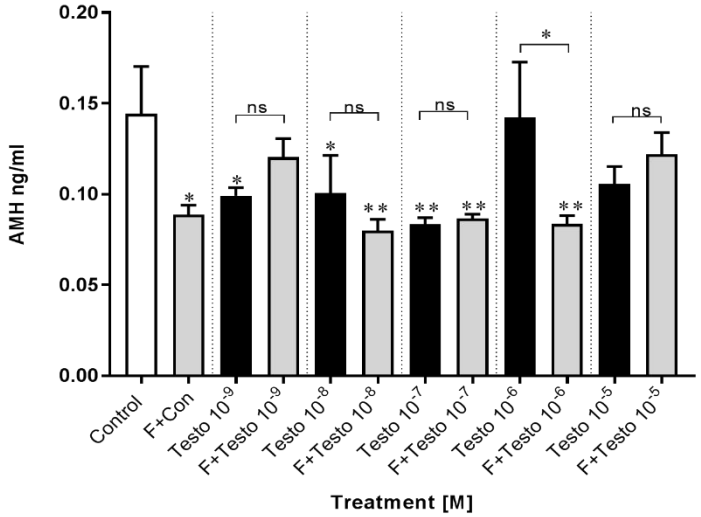
Supplementary Figure 1. The effect of flutamide on basal expression of AMH from all androgen experiments combined. For all the androgen experiments, the average basal (untreated controls, white bar) AMH values (0.14±0.03ng/ml) were compared with the average value of the flutamide-treated cells (grey bar) (0.13±0.05ng/ml) (mean±SEM, n=9) and showed no statistically significant difference.

Supplementary Figure 2. A comparison of the basal mRNA expression of aromatase, AMH, AMHRII, LHR and FSHR between all ovarian morphologies. There was no difference in the basal levels of all genes between normal, PCOM & PCOS cells, apart from that of AMHRII, which had statistically significantly reduced expression in PCOS cells compared with either normal or PCOM (*$p<0.05$, unpaired t-test; n=7(normal, white bars), n=5 (PCOM, grey bars), n=4 (PCOS, black bars).

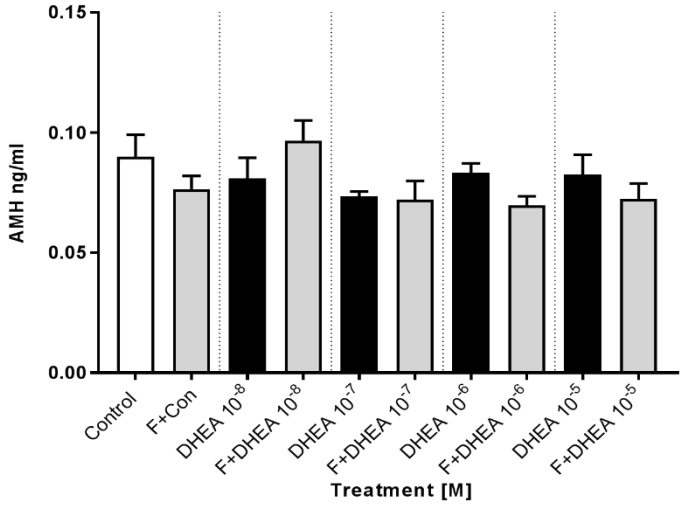
Supplementary Figure 3. A comparison of the basal levels of SMAD proteins between normal and PCOS. The basal levels of all SMAD proteins from the normal (n=6, white bars) and PCOS (n=3, black bars) samples were compared and showed no statistically significant differences between the two groups (unpaired t-test, two-tail)

Figure 1

1A



1B



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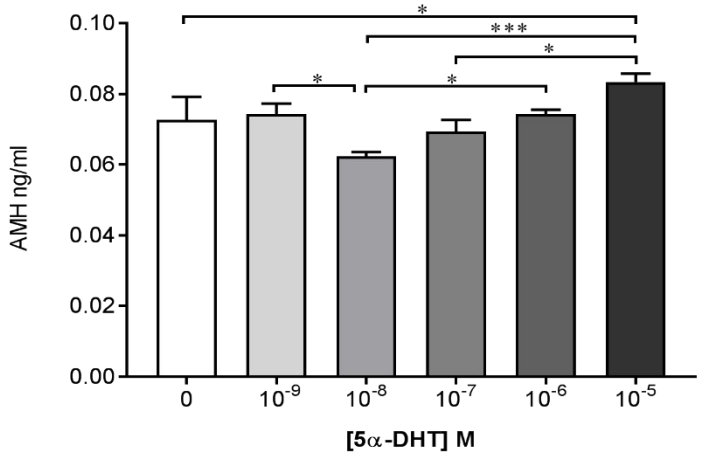


Figure 2

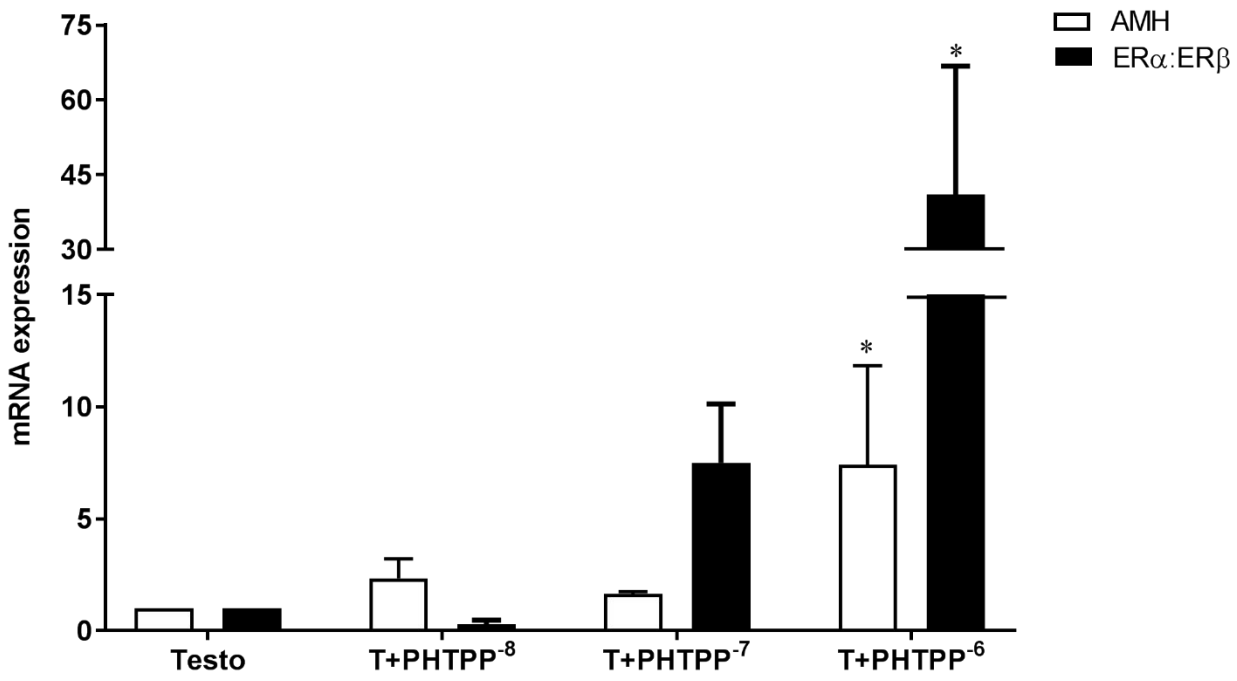

AMH
ERα:ERβ
mRNA expression
75
60
45
30
15
10
5
0
*
*
Testo
T+PHTPP-8
T+PHTPP-7
T+PHTPP-6

Figure 3

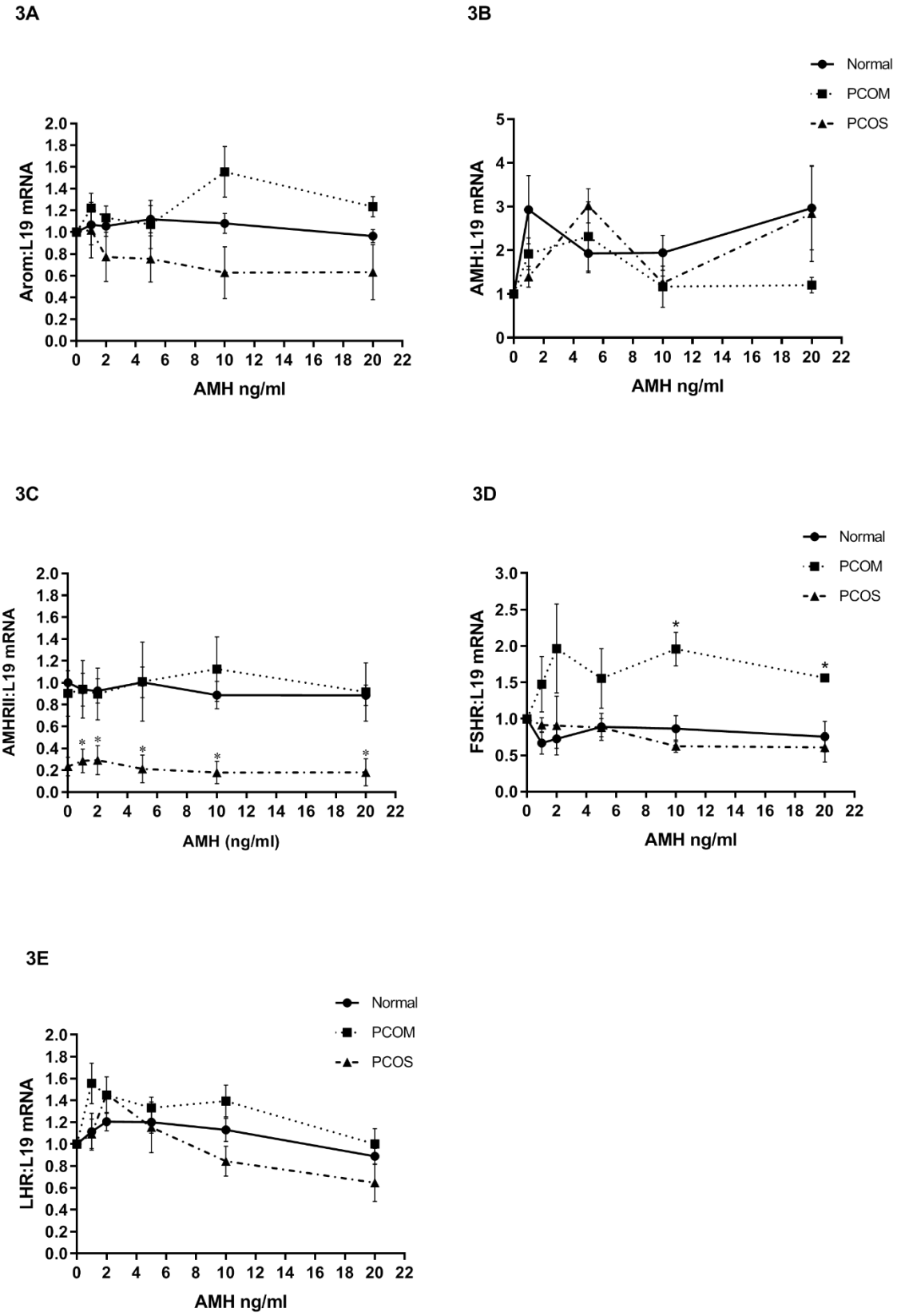
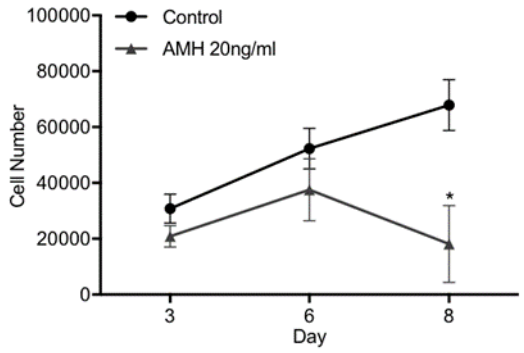
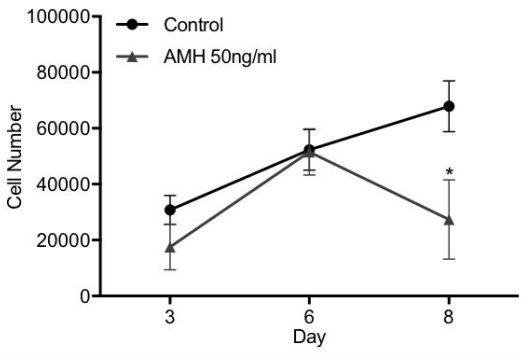

3A
Arom:L19 mRNA
AMH ng/ml
3B
Normal
PCOM
PCOS
AMH:L19 mRNA
AMH ng/ml
3C
AMHRII:L19 mRNA
AMH (ng/ml)
3D
Normal
PCOM
PCOS
FSHR:L19 mRNA
AMH ng/ml
3E
Normal
PCOM
PCOS
LHR:L19 mRNA
AMH ng/ml

Figure 4

4A



4B



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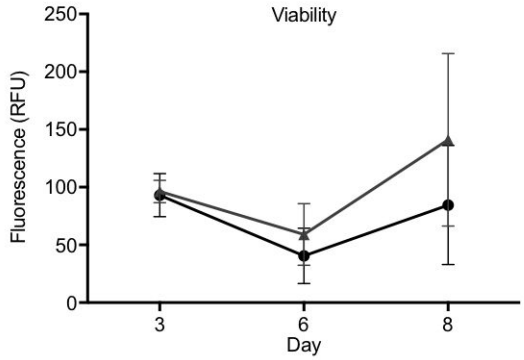

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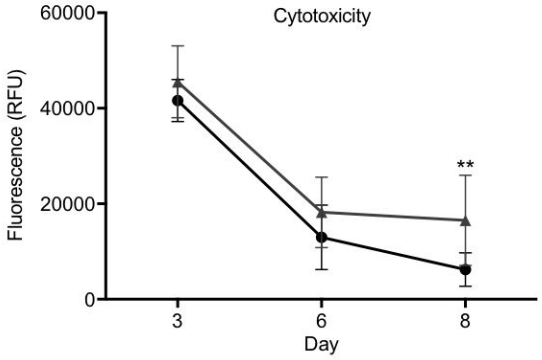

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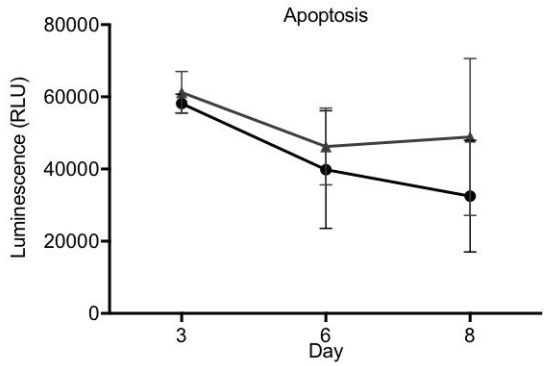
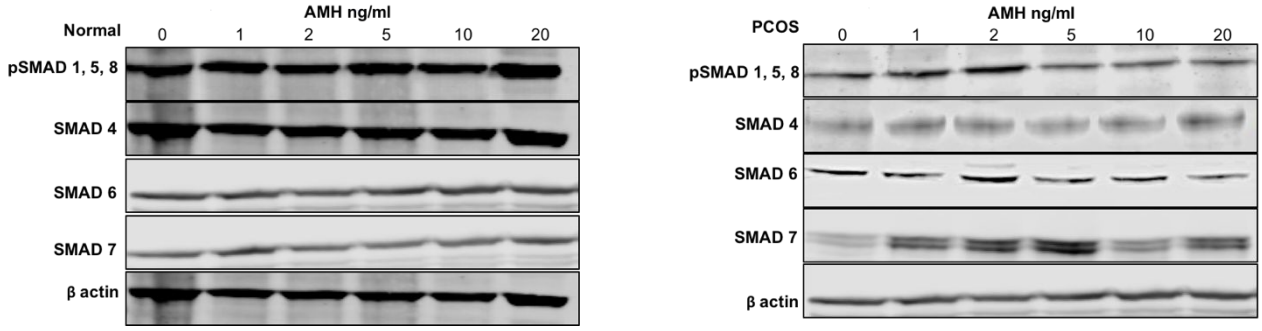
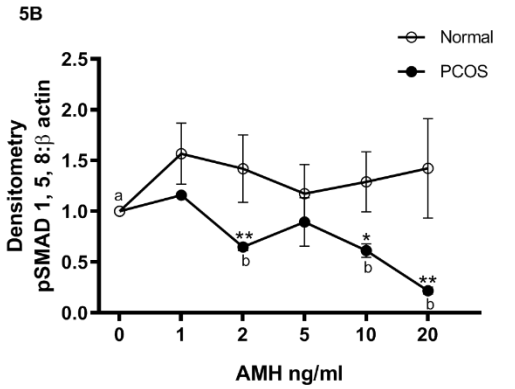
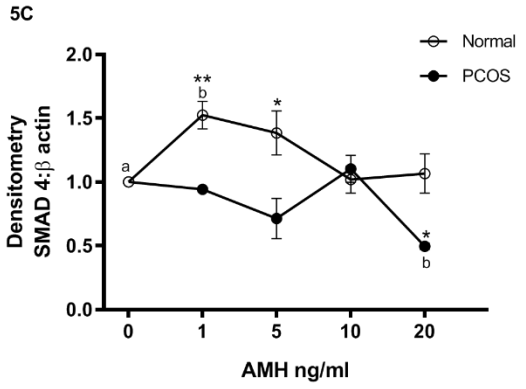


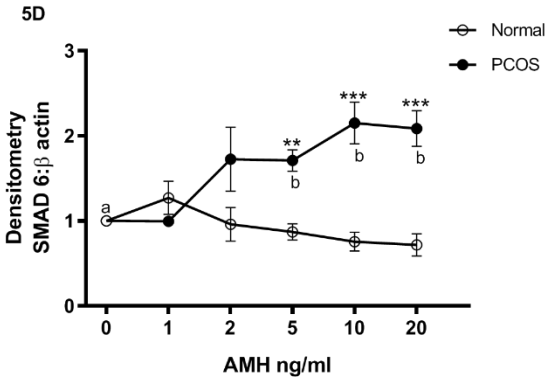
Figure 5

5A


Normal
AMH ng/ml
0 1 2 5 10 20
pSMAD 1, 5, 8
SMAD 4
SMAD 6
SMAD 7
β actin
PCOS
AMH ng/ml
0 1 2 5 10 20
pSMAD 1, 5, 8
SMAD 4
SMAD 6
SMAD 7
β actin


5B
Normal
PCOS
Densitometry pSMAD 1, 5, 8:β actin
AMH ng/ml


5C
Normal
PCOS
Densitometry SMAD 4:β actin
AMH ng/ml


5D
Normal
PCOS
Densitometry SMAD 6:β actin
AMH ng/ml

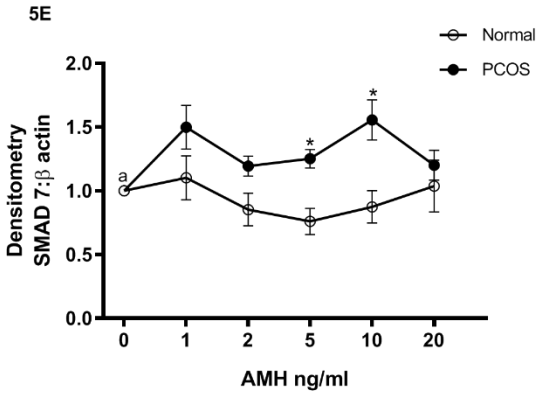

5E
Normal
PCOS
Densitometry SMAD 7:β actin
AMH ng/ml

Figure 6A (Normal)

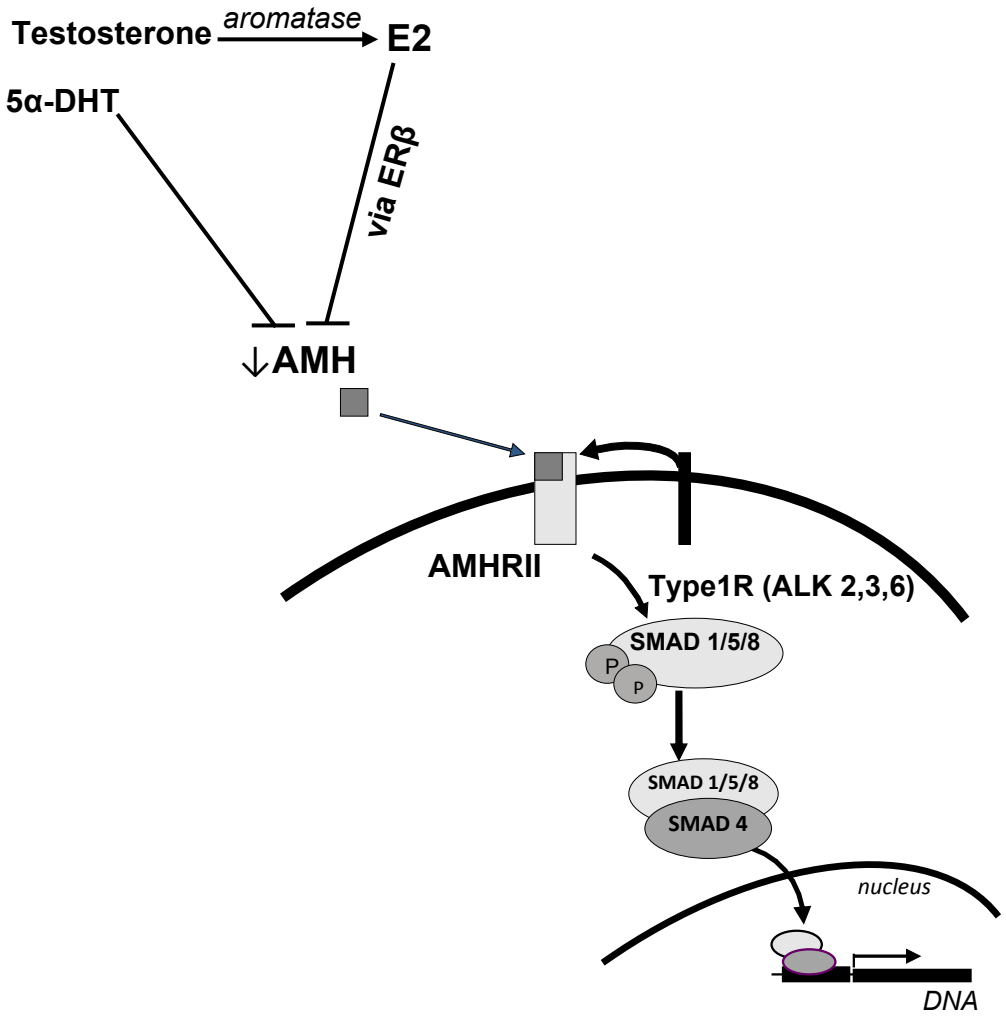


Figure 6B (PCOS)

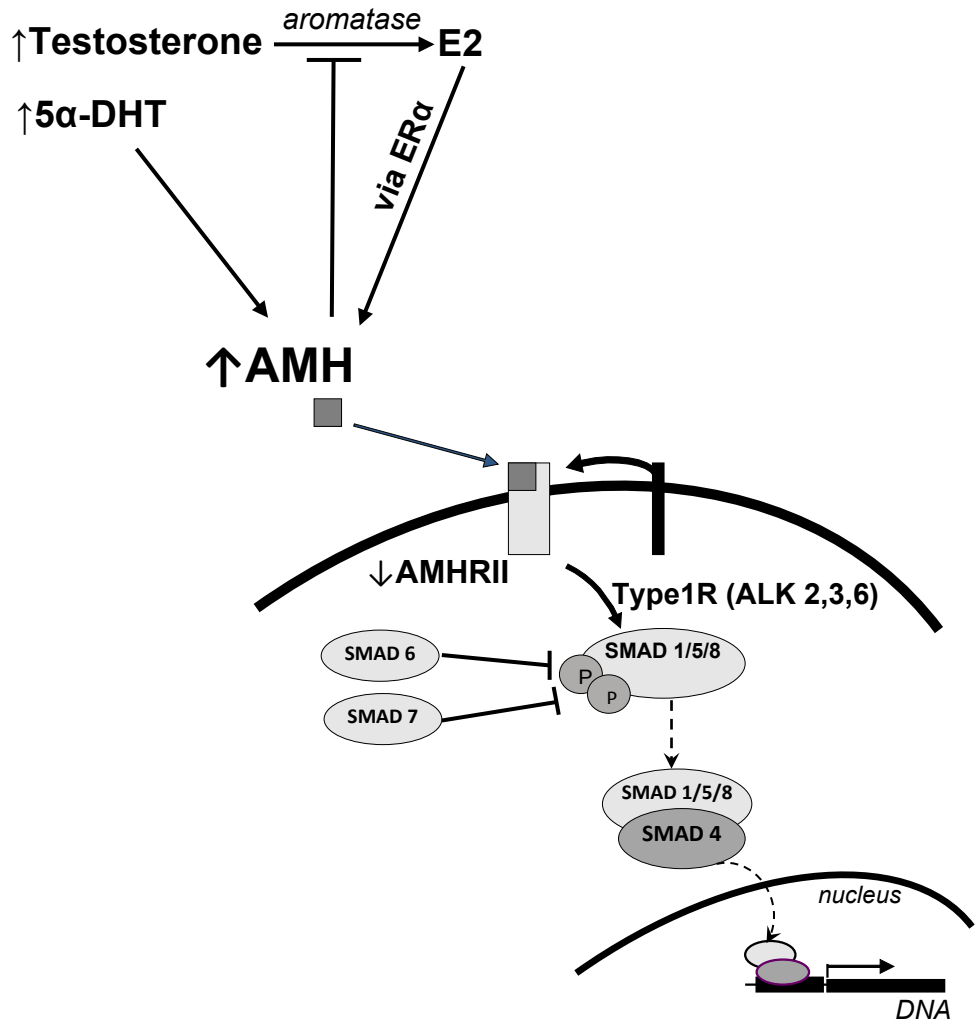


Table 1

Ovarian Status	Age (yrs)	BMI (kg/m^2)	AMH (pmol/L)	FSH (IU/L)	LH (IU/L)	AFC
Normal (n=26)	35.2±0.76	25.9±0.70	12.4±1.68	6.4±0.5	4.0±0.34	11.75±0.81
PCOM (n=7)	33±2.02	25.2±1.19	36.1±11.57 **p=0.001	5.0±0.73	3.2±0.37	22.86±2.38 ****p=00004
PCOS (n=9)	30.3±1.49 **p=0.003	24.8±1.10	33.7±6.98 ***p=0.0001	4.5±0.49	6.3±1.49 *p=0.03	37.3±8.32 *****p=000002

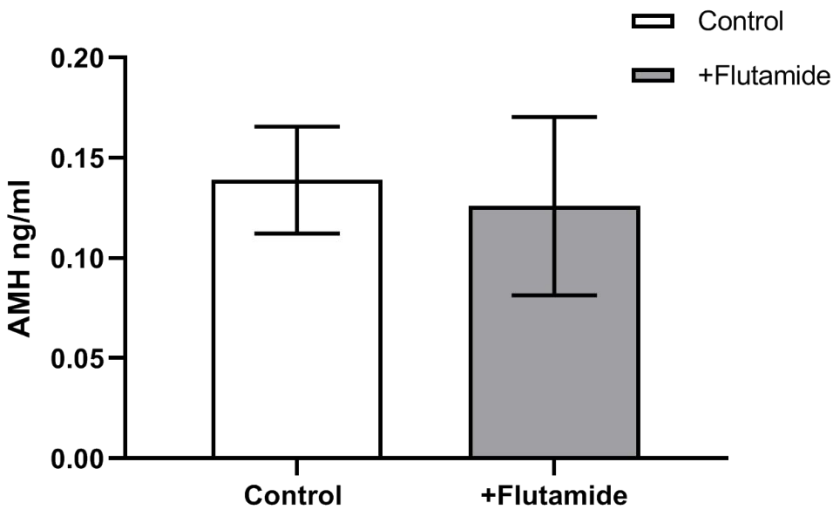
Table 2

Gene (Accession Number)	Primer Sequence	Primer Concentration (nM)	Annealing Temperature (°C)
AMH (NM_000479)	F – GCATGTTGACACATCAGGC R – GAGTGGCCTTCTCAAAGAGC	100	60
AMHRII (NM_020547)	F – CCCTGCTACAGCGAAAGAAC R – ATGGCAACCAGTTTTCCTTG	150	60
Aromatase (NM_000103)	F – GACTCTAAATTGCCCCCTCTG R – CAGAGATCCAGACTCGCATG	100	60
FSHR (NM_000145)	F – AAAAGCTTGTCGCCCTCATG R – ACCATATCAGGACTCTGAGG	200	50
LHR (NM_000233)	F – TCCTTTCCAGGGAATCAATC R – GGCCGGTCTCACTCGAC	200	60
L19 (NM_000981)	F – GCGGAAGGGTACAGCCAAT R - GCAGCCGGCGCAAA	100	60
ERα (NM_001328100)	F – CCACCAACCAGTGCACCATT R – GGTCTTTTCGTATCCCACCTTTC	150	60
ERβ (NM_001271877)	F – AGAGTCCCTGGTGTGAAGCAAG R – GACAGCGCAGAAGTGAGCATC	150	60

Table 3

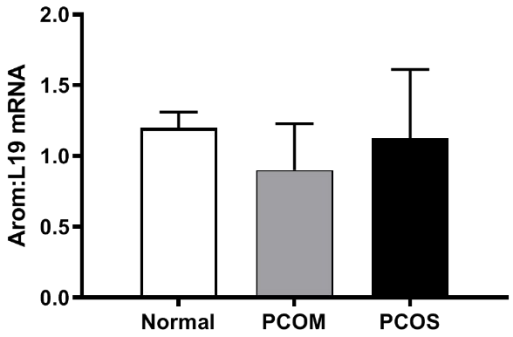
Antibody	Molecular Weight (kDa)	Antibody dilution	Supplier of Primary antibody	Poly- or Monoclonal	Secondary Antibody (Licor IRDye 800/680)
AMHRII	72	1:500	Abcam	Polyclonal	Goat Anti-Rabbit
pSMAD 1/5/8	52 – 60	1:500	Cell Signalling	Polyclonal	Goat Anti-Rabbit
SMAD 4	70	1:500	Cell Signalling	Polyclonal	Goat Anti-Rabbit
SMAD 6	52 – 53	1:200	Abcam	Polyclonal	Goat Anti-Rabbit
SMAD 7	40 – 45	1:500	Merck Millipore	Monoclonal	Goat Anti-Mouse
Beta Actin	42 – 45	1:1000	Abcam	Monoclonal	Goat Anti-Mouse

Supplementary Figure 1

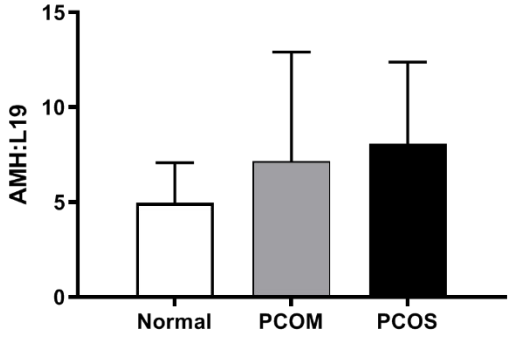


Supplementary Figure 2

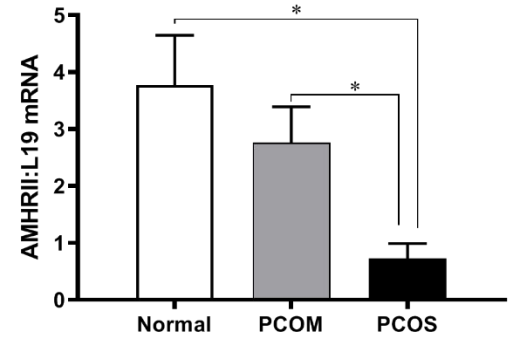
S2a



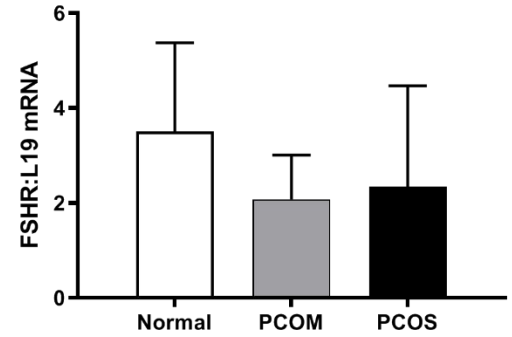
S2b



S2c



S2d



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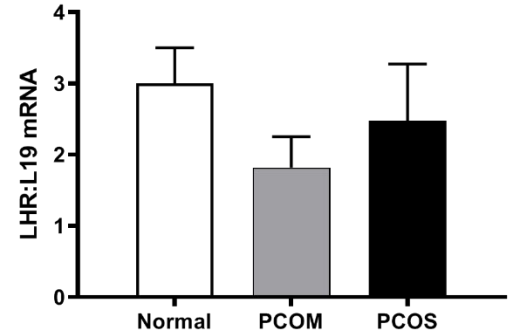

Supplementary Figure 3