

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

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

24

25 **Abstract**

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

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

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

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

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

50 **Main results and the role of chance:** Testosterone reduced AMH production at 10^{-9} - 10^{-7} M ($p < 0.05$;
51 $p < 0.005$, multiple uncorrected comparisons Fishers LSD), but equivalent hyperandrogenemic levels
52 favored persistence of AMH levels. 5α -DHT produced a significant dose-related increase in AMH
53 ($p = 0.022$, ANOVA). Increasing the ratio of $ER\alpha:ER\beta$ produced a corresponding increase in AMH
54 expression ($p = 0.015$, two-way ANOVA). AMH increased aromatase ($p < 0.05$, one-way ANOVA) and
55 FSHR ($p < 0.0001$, one-way ANOVA) in cells from women with PCOM, but not in cells from normal
56 or PCOS (normal $n = 7$, PCOM $n = 5$, PCOS $n = 4$). In contrast to cells from ovulatory ovaries, in PCOS
57 AMH reduced levels of stimulatory pSMAD-1/5/8 and SMAD-4 but increased inhibitory SMAD-6 and
58 -7 ($p < 0.05$, normal $n = 6$, PCOS $n = 3$). AMH at 20 and 50ng/ml decreased KGN cell proliferation but
59 not viability after 8 days of treatment ($p < 0.005$, two-way ANOVA).

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

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

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75 Introduction

76 Polycystic ovary syndrome (PCOS) is the most prevalent endocrine disorder, affecting 10 to
77 20% of women of reproductive age worldwide (Homburg, 2008). PCOS is the primary cause of
78 anovulatory infertility and is often accompanied by hyper-androgenism and hyperinsulinemia (Baird
79 *et al.*, 2012). AMH is a product of the granulosa cells (GC) of small antral follicles (AFs), and
80 increased serum levels of AMH in PCOS are due to a combination of an increase in follicle number
81 and excessive AMH production by each follicle (Pellatt *et al.*, 2007; Desforages-Bullet *et al.*, 2010).
82 The consensus is that AMH has an inhibitory or stalling role on antral follicle development (Pellatt *et al.*
83 *et al.*, 2010; Dewailly *et al.*, 2014, 2016), which in the normal ovary acts to counterbalance over-
84 recruitment of growing follicles (Dewailly *et al.*, 2016). This is supported by the fact that serum AMH
85 levels are two to five times higher in women with PCOS and are considerably higher in women with
86 anovulatory cycles compared to those with ovulatory PCOS (Pigny *et al.*, 2003; Laven *et al.*, 2004;
87 Park *et al.*, 2010).

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

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

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

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

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

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

136 Material and Methods

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

138 Subjects and Collection of Human GLC samples

139 GLCs were isolated from follicular fluid (FF) aspirates obtained from women undergoing IVF.
140 Patients were assigned with normal; asymptomatic polycystic morphology (PCOM) or PCOS status
141 based on the Rotterdam criteria (2004) following ultrasound assessment. The designation of PCOM
142 was based on the presence of polycystic ovaries on transvaginal ultrasound (>12 follicles measuring
143 2-9mm after spontaneous or progestin-induced menstruation) without accompanying signs of hyper-
144 androgenemia (biochemical/clinical signs of hirsutism/acne) or oligo-ovulation/anovulation. It is well
145 documented that PCOM is a common age-dependent finding in ovulatory women without any of the
146 accompanying symptoms of PCOS or metabolic significance; allowing it to be considered as a
147 separate defining feature (Balen *et al*, 2009; Jonhstone EB *et al*, 2010). Ethical approval was granted
148 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°C in 95% air/CO₂. Cells were plated in 12-well plates at 3x10³ 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) (10⁻⁶M), 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.

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

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

179 **MTT and ApoTox assay**

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

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

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

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

201 **GLC culture and SMAD protein Western blot analysis**

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

213 **Statistical Evaluation**

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

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

226 Results

227 Clinical data of the subjects

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

234

235 The effect of Androgens on AMH protein expression

236 The effect of various androgens on AMH production by KGN cells was determined in the
237 presence/absence of flutamide (the selective AR antagonist). T reduced levels of AMH protein below
238 that of basal at the lower doses (10^{-9} to 10^{-7} M) (figure 1A). At 10^{-6} M and above, AMH production was
239 the same as basal, with no attenuating effects. The addition of flutamide did not alter the attenuating
240 effects of T on AMH production apart from at 10^{-6} M, where it reduced the basal level of AMH even
241 lower. Treatment with DHEA \pm flutamide, had no effect on AMH (figure 1B), and likewise neither did
242 androstenedione (data not shown). Though it would appear that flutamide reduced basal AMH
243 production when added alone to the T experiment (fig 1A), it did not do so in the DHEA (figure 1A &
244 1B) or the androstenedione experiment, indicating that this it was probably an artefact. This proved

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

250 **The effect of altering the ratio of ER α :ER β on to determine the oestrogen-mediated effect on** 251 **AMH expression**

252 Culturing the cells in the presence of PHTPP (the ER β antagonist) produced a dose-related
253 increase in the expression of ER α to ER β with a nearly 30-fold increase over basal at the highest
254 dose of PHTPP. There was a commensurate dose-dependent increase in *AMH* expression with a
255 significant 5-fold increase over basal seen at the highest dose of PHTPP (10⁻⁶M) used (Fig 2).

256 **The effect of AMH on aromatase, AMH, AMHRII, FSHR and LHR expression in GLCs from** 257 **women with morphologically normal, PCOM and PCOS ovaries**

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

264 AMH treatment above 5ng/ml decreased aromatase expression in cells from women with
265 PCOS, but not in cells from normal (figure 3A). In contrast, 10 and 20 ng/mL AMH significantly
266 stimulated aromatase expression in PCOM cells compared to normal (figure 3A). AMH treatment
267 had no effect on its own expression in any ovarian type (figure 3B), nor that of *AMHRII* or *LHR* (figure
268 3C & E), in spite of the lower basal expression of *AMHRII* in PCOS cells. AMH increased *FSHR*

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

271
272 **The effect of AMH on KGN cell proliferation and apoptosis**
273 Incubation of cells with concentrations of AMH likely to be present in the polycystic ovary (20
274 & 50 ng/ml), inhibited proliferation after 8 days (figure 4A & B), which reflects the time it would take
275 to alter the balance between cell cycle proliferation and arrest. This was not due to effects on cell
276 viability (figure 4C) and AMH did not appear to be cytotoxic (figure 4D) or to cause increased
277 apoptosis (figure 4E), in fact if anything, it appeared slightly protective of cell death.

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

279 There was no significant difference in the relative levels of SMAD proteins in cells from
280 women with normal or PCOS (supplementary figure 3), though interestingly AMH treatment
281 produced diametrically opposed effects on SMAD protein expression between cells from the two
282 types of ovary. In GLCs from normal ovaries AMH increased levels of pSMAD 1/5/8 by approximately
283 50% compared to basal, however these results did not reach statistical significance due to the wide
284 variation in levels from individual patients (figure 5A & B). In contrast, AMH significantly *decreased*
285 levels of pSMAD 1/5/8 in PCOS GLCs below basal (figure 5A & 5B). Similarly, AMH (1 and 5ng/mL)
286 increased SMAD-4 protein levels in cells from normal ovaries compared to those from PCOS, with
287 no further increase at the highest AMH dose. In contrast, the highest concentration of AMH
288 significantly down-regulated SMAD-4 levels below that of basal in PCOS cells compared to control
289 (figure 5C). In the normal cells AMH had no effect on SMAD-6 levels, whereas high concentrations
290 (≥ 5 ng/ml) of AMH significantly increased SMAD-6 protein levels in cells from PCO (figure 5D). A
291 similar response was seen for SMAD-7, in that AMH had no effect on SMAD-7 protein in normal
292 cells, but increased protein levels significantly in cells from women with PCOS compared to normal
293 (figure 5E).

294 Discussion

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

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

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

319 of AMH expression. It must be pointed out that whilst the effects were modest, we were measuring
320 AMH protein secreted into media and that the cellular protein levels would in fact have been higher.
321 The fact that the presence of flutamide made no discernible difference to T's actions, indicates that
322 the reduction in AMH expression seen with T could be occurring indirectly through its conversion to
323 E2. There is a growing body of evidence to support the assertion that E2 down-regulates the
324 expression of AMH & AMHRII (Grynberg *et al.*, 2012; Pierre *et al.*, 2017), and that this effect was
325 associated with alterations in the ratio of ER α :ER β (Grynberg *et al.*, 2012; Pierre *et al.*, 2017).
326 Grynberg *et al* (2012) clearly showed that when cells were transfected with ER α and treated with
327 increasing doses of E2, there was an increase in AMH promoter activity, with a significant attenuation
328 occurring on transfection with ER β . This supports our findings that blocking ER β with the antagonist
329 PHTPP, and hence increasing the ratio of ER α :ER β , produced a significant increase in AMH
330 expression. Interestingly, the non-aromatisable 5 α -DHT produced a dose-related increase in AMH
331 protein production at levels associated with HA. This further corroborates the findings of Pierre *et al*
332 (2017), who demonstrated that 5 α -DHT increased AMH mRNA expression in GC from women with
333 PCOS overexpressing the AR, but not in normal ovaries.

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

344 One limitation of our study is that we used luteinised GC pooled across follicle sizes for each
345 woman undergoing IVF which have a relatively low expression of AMH/AMHR11, as they have
346 already progressed to form *corpora lutea* due to exogenous gonadotrophin stimulation during IVF.
347 We know however that these cells maintain their phenotype with respect to ovarian morphology *in*
348 *vitro*, which does mean that they are suitable for investigating the legacy of the impact of PCOS on
349 cellular functions, hence allowing for extrapolation of the changes to the non-IVF cycle. To counter
350 balance this limitation we also used KGN cells that have been characterised as equivalent to
351 immature GC (Nishi *et al.*, 2001) and produce more AMH than GLCs. This was especially important
352 regarding experiments investigating attenuation of AMH expression, since any reduction of the
353 already low expression levels would push the limits of even sensitive laboratory techniques such as
354 qPCR. In addition the robustness of this cell line made them suitable for prolonged culture.

355 We and others have previously shown that in both un-luteinised and luteinised GCs from
356 normal ovaries, AMH had no effect on unstimulated levels of aromatase, but reduced FSH-
357 stimulated aromatase expression (Pellatt *et al.*, 2011; Sacchi *et al.*, 2016). Likewise, in this study,
358 increasing doses of AMH had no effect on aromatase expression in normal GLCs, but surprisingly
359 reduced aromatase mRNA expression by approximately 25-50% in PCOS GLCs, although these
360 differences did not reach significance due to high inter-patient variability. Interestingly however, AMH
361 (10, 20ng/ml) significantly increased aromatase expression in PCOM compared to PCOS cells,
362 which was probably due to the increased expression of FSHR in PCOM cells compared to PCOS,
363 suggesting that *in vivo* this would have the effect of indirectly increasing aromatase. This highlights
364 the fact that women with anovulatory PCOS are a distinct sub-group, and that the polycystic ovarian
365 morphology *per se* is not associated with diminished FSH responsiveness (Homburg, 2008).

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

370 numbers and induced apoptosis; though this effect was only at supra-physiological doses of 10 &
371 25µg/ml AMH which far exceeded the average AMH level of 0.68ng/ml that they measured in culture
372 media (Anttonen *et al*, 2011). This lack of an apoptotic effect of AMH *in vivo* explains the ability to
373 “rescue” stalled follicles with super-ovulation regimes, as shown by Hayes *et al* (Hayes *et al.*, 2016).
374 We speculate that the difference in effects in comparison to the Müllerian ducts may be due to either
375 an indirect action by AMH on the epithelial cells in the latter occurring after initial interaction with
376 mesenchymal cells, which is does not occur in the follicle (Roberts *et al.*, 1999); or a dosing effect
377 as shown by the ability of AMH to promote Sertoli cell proliferation in mice at low doses (10-50ng/ml)
378 and apoptosis at high concentrations (50-800ng/ml) (Rehman ZU *et al*, 2017)

379 Given the similarity in spectrum of the PCOM cells to normal, we decided to investigate the
380 AMH intra-cellular signalling pathway in normal ovaries and PCOS. Using specific anti-SMAD
381 antibodies, we have demonstrated for the first time that there was no difference in the basal levels
382 of SMAD proteins between normal and PCOS cells. However, AMH treatment at an equivalent
383 concentration to that measured in GC-conditioned media from women with anovulatory PCO
384 (20ng/mL), increased pSMAD-1/5/8 and SMAD-4 protein levels in normal GLCs, but strikingly,
385 significantly *decreased* levels in PCOS GLCs. This indicates that the AMH-mediated activation and
386 nuclear translocation of pSMAD-1/5/8 and SMAD-4 to alter downstream target gene expression is
387 reduced in PCOS cells compared with normal. It must be remembered that other members of the
388 TGF-β family of signalling proteins also utilise the SMAD signalling pathway, eg BMP-15 secreted
389 from the oocyte activates SMAD-1/5/8 (Knight and Glister, 2006; Liu *et al.*, 2018), adding to the
390 cross-talk and complexity of AMH signalling.

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

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

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

422 1/5/8 and the co-SMAD-4 (figure 6b). We speculate that *in vivo*, these high levels of AMH may cause
423 uncoupling or desensitization of the AMH signalling pathway leading to the dysregulated follicle
424 growth seen in PCOS.

425 Declaration of Interest

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

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429 Authors Roles

430 ND, LP & SR performed data acquisition, analysis and contributed to the drafting of the article. EJ
431 and MO performed additional data acquisition and analysis. All the other authors, in particular HM
432 and SR who supervised the experimental work, were involved in critical intellectual input and study
433 design, interpretation of data, and drafting, critical revision and approval of the paper for final
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578

579 Figure & Table Legends

580 **Figure 1. Effect of androgens on AMH production. (A)** Treatment of KGN cells with a
581 range of testosterone (T) doses (10^{-9} to 10^{-5} M; black bars) reduced AMH production below
582 basal levels as measured by ELISA ($p=0.03$; ANOVA), with significance at the lower doses
583 (10^{-9} to 10^{-7} M) (* $p<0.05$; ** $p<0.005$; multiple uncorrected comparisons Fishers LSD), but
584 there was no attenuation of AMH above 10^{-6} M in comparison to untreated control (white
585 bar). In the presence of the selective AR antagonist flutamide (F; grey bars) (10^{-6} M) there
586 was no further reduction in AMH production in comparison to basal (* $p<0.05$), apart from
587 at 10^{-6} M (** $p<0.05$). **(B)** Treatment of cells with DHEA (black bars) \pm F (grey bars) had no
588 effect on AMH production. **(C)** Treatment with 5α -DHT (10^{-9} to 10^{-5} M; shaded grey bars)
589 produced a dose-related increase in AMH production compared to control (white bar)
590 (* $p=0.022$; ANOVA). Low dose 5α -DHT (10^{-8} M) significantly reduced AMH production
591 (* $p<0.05$), but as the concentration of 5α -DHT increased to 10^{-5} M, there was a small but
592 significant increase in AMH levels (* $p<0.05$; multiple uncorrected comparisons Fishers
593 LSD) (mean \pm SEM, $n = 3$).

594

595 **Fig 2: Effect of altering ER α :ER β on AMH expression.** KGN cells were treated with T
596 (500nM) and a range of PHTPP doses (10^{-8} to 10^{-6} M). The highest dose of PHTPP
597 produced a significant 30-fold increase in ER α :ER β (black bars) expression compared to
598 basal, with a corresponding 5-fold increase in AMH mRNA expression (white bars)
599 ($p=0.014$, Two-way ANOVA) (mean \pm SEM, $n=4-6$).

600

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

604 (dotted line) women AMH at high doses *stimulated* aromatase mRNA levels compared to
605 normal cells (* $P < 0.05$, one-way ANOVA). **(B & E)** AMH treatment had no significant
606 effect on the expression levels of AMH or LHR mRNA in any group. **(C)** Though basal
607 levels of AMHRII were significantly lower in PCOS than normal or PCOM cells, AMH
608 treatment did not affect expression of its receptor in any cell type. **(D)** AMH had no effect
609 on FSHR in GLCs from women with normal (solid line) or PCOS (dashed line), but
610 interestingly, AMH (>5ng/ml) significantly stimulated FSHR mRNA expression in cells from
611 women with PCOM (dotted line) (* $P < 0.0001$ one-way ANOVA; mean \pm SEM, normal =
612 7 experiments (from 18 women), PCOM = 5 experiments (from 7 women), PCOS = 4 (from
613 6 women)).

614

615 **Figure 4. Effect of AMH on KGN cell proliferation and apoptosis. (A & B)** AMH at 20
616 & 50ng/ml (\blacktriangle) decreased KGN cell proliferation over 8 days of culture compared to non-
617 treated control cells (\bullet). MTT cell proliferation assays were performed on day 3, 6 and 8,
618 absorbance measured, and cell density calculated (mean \pm SEM, $n = 6$, where 1n is the
619 mean of triplicate wells; *, $P < 0.005$, Two-way ANOVA). **(C – E)** The ApoTox-Glo triplex
620 assay was performed on day 3, 6 and 8. The fluorescence and luminescence was
621 measured on day 3, 6 and 8 to assess the quantity of viable cells and the level of
622 cytotoxicity and apoptosis induced by 50ng/ml of AMH (mean \pm SEM, $n = 4$, where 1n is
623 the mean of triplicate wells; **, $P < 0.0006$, Two-way ANOVA. Control (\bullet); AMH 50ng/ml
624 (\blacktriangle)).

625

626 **Figure 5. The effect of AMH on SMAD signalling proteins in normal and PCOS cells.**
627 **(A)** Representative western blot images using anti-pSMAD 1/5/8, -4, -6 & -7 antibodies on
628 total protein lysates extracted from GLCs from women with normal ovaries or PCOS
629 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 (\circ), while in contrast in PCOS cells (\bullet)
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 \pm 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 ER β . The non-aromatisable 5 α -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 TGF β 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 (5 α -DHT) or indirectly via T
655 conversion to E2 and action through the increased expression of ER α . Prolonged

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

662

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

669

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

673

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

Supplementary Figure Legends

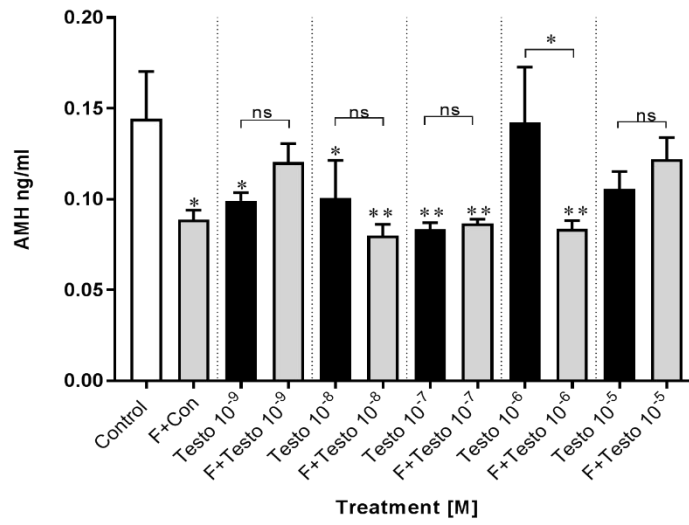
Supplementary Figure 1. The effect of flutamide on basal expression of AMH from all androgen experiments combined. For all the androgen experiments, the average basal (untreated controls, white bar) AMH values ($0.14 \pm 0.03 \text{ ng/ml}$) were compared with the average value of the flutamide-treated cells (grey bar) ($0.13 \pm 0.05 \text{ ng/ml}$) (mean \pm 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)).

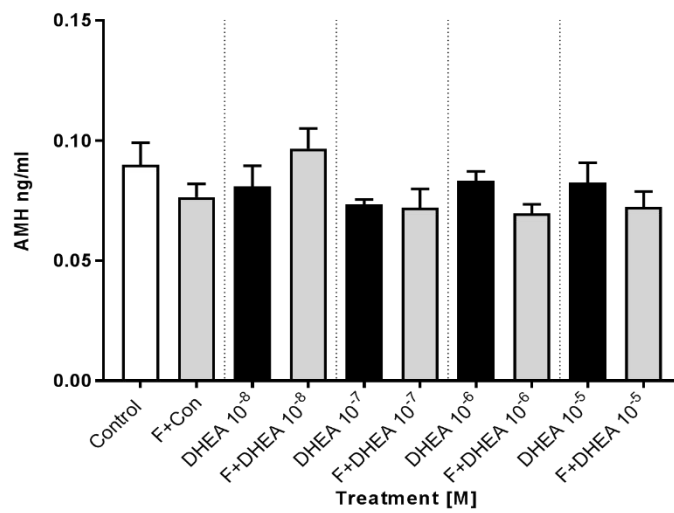
Supplementary Figure 3. A comparison of the basal levels of SMAD proteins between normal and PCOS. The basal levels of all SMAD proteins from the normal ($n=6$, white bars) and PCOS ($n=3$, black bars) samples were compared and showed no statistically significant differences between the two groups (unpaired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

Figure 1

1A



1B



1C

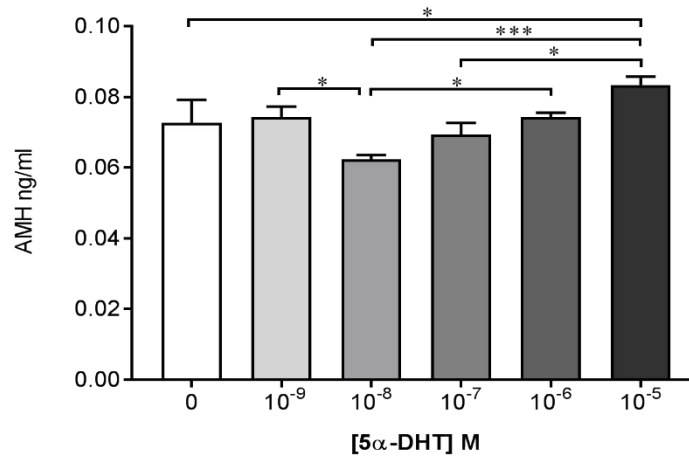


Figure 2

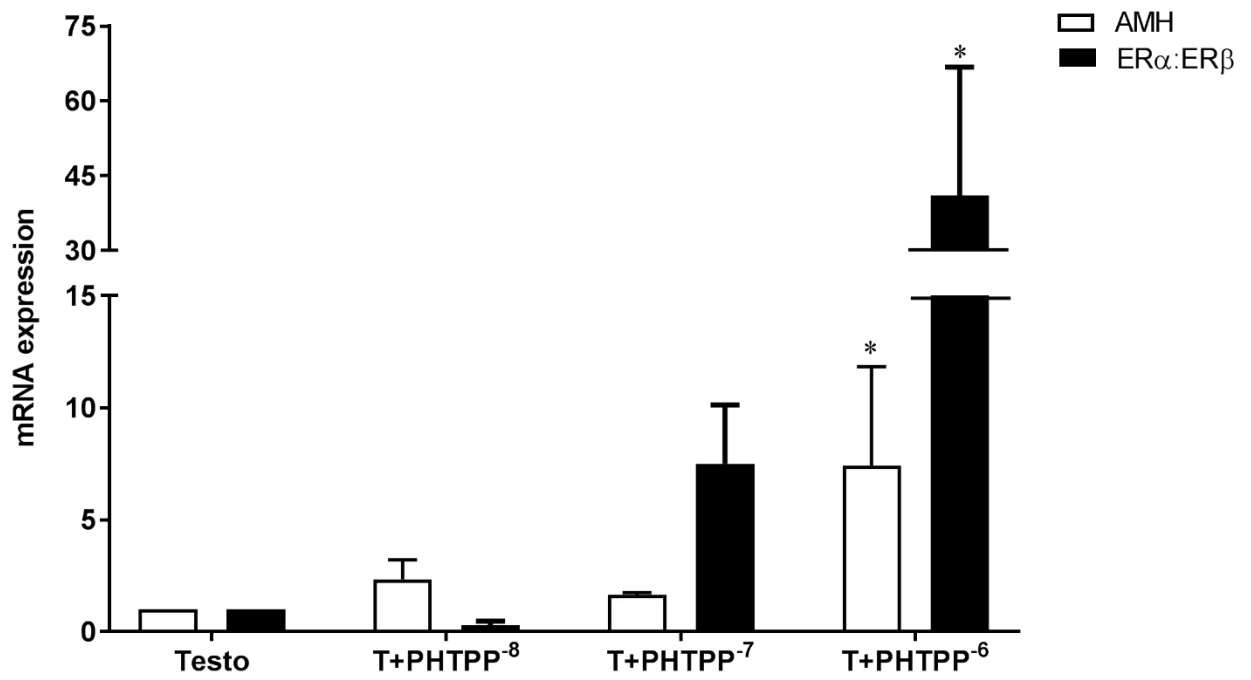
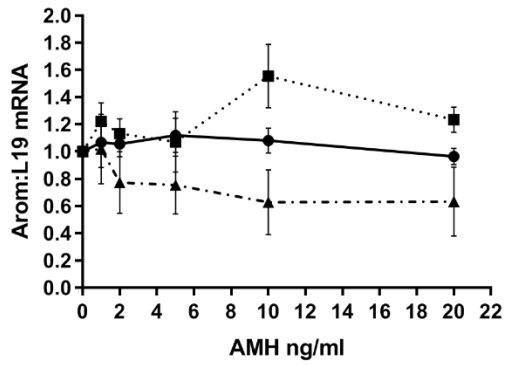
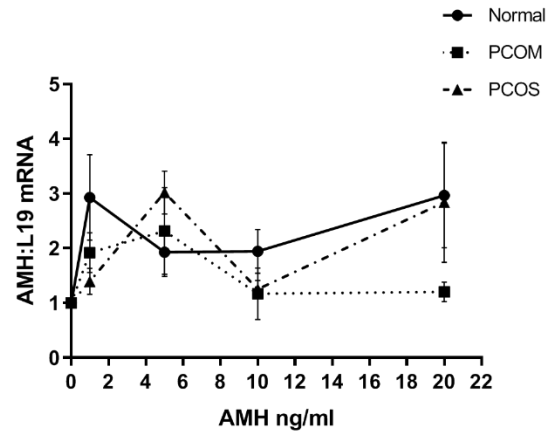


Figure 3

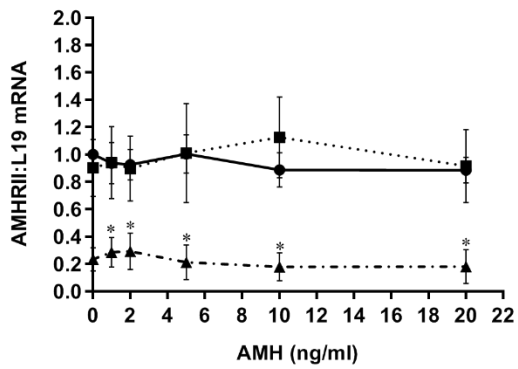
3A



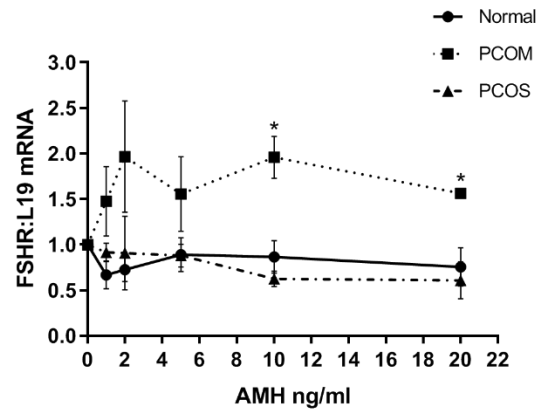
3B



3C



3D



3E

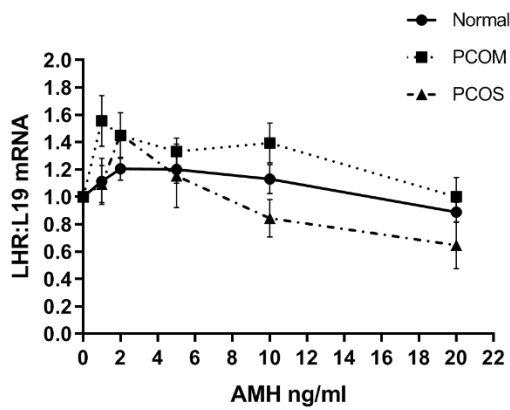


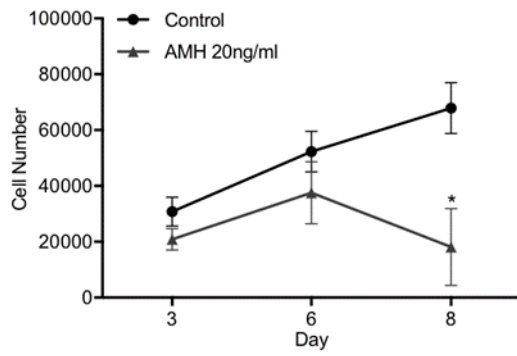
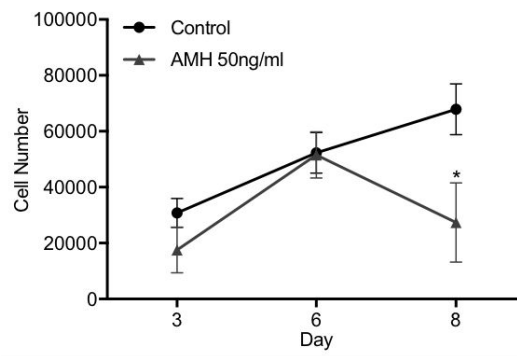
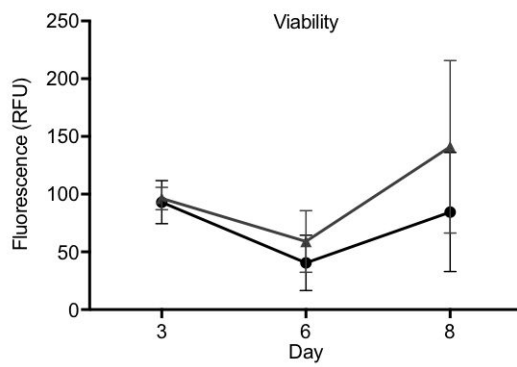
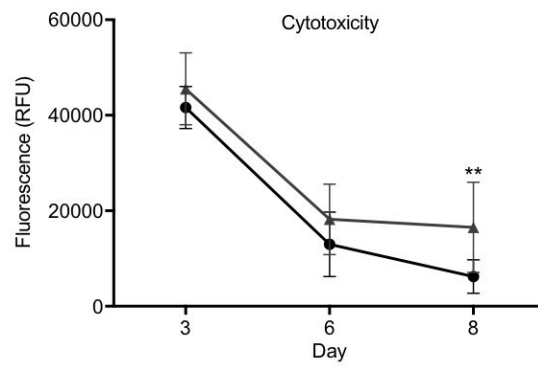
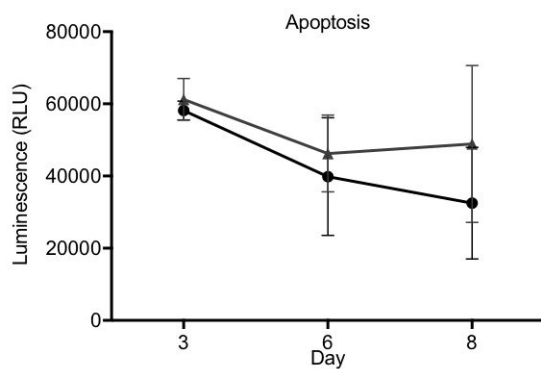
Figure 4**4A****4B****4C****4D****4E**

Figure 5

5A

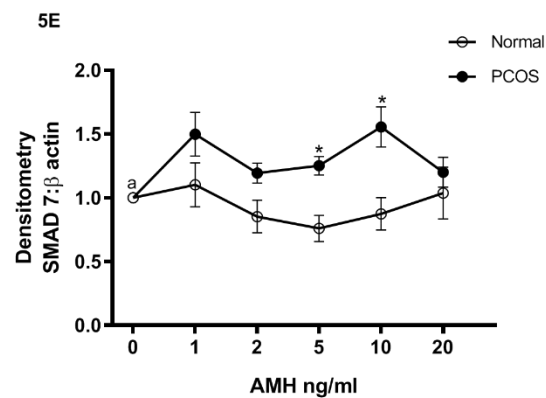
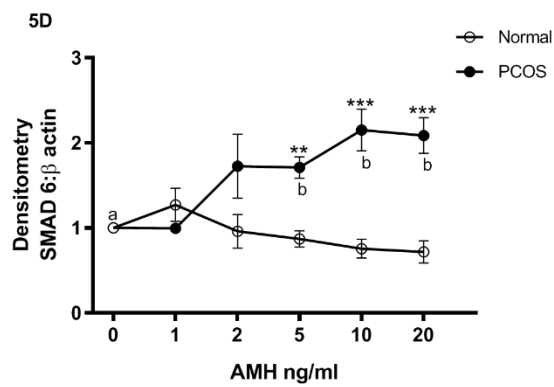
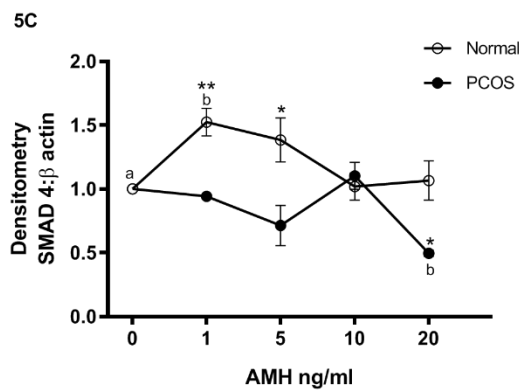
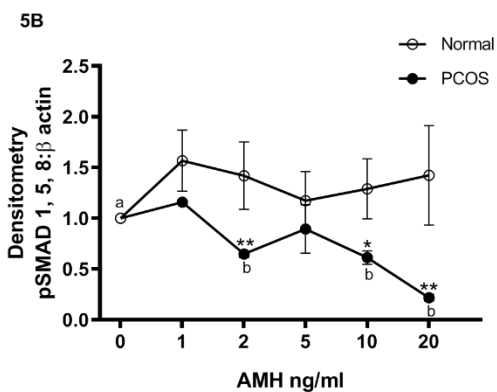
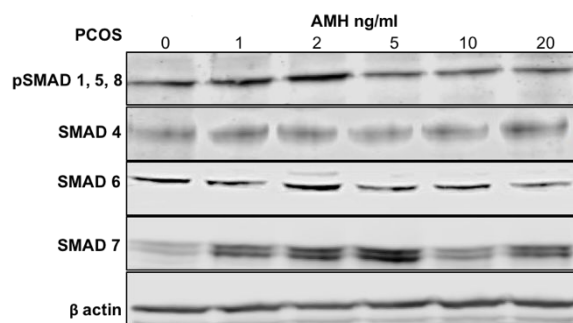
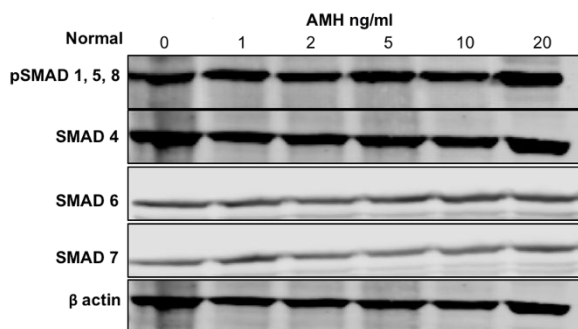


Figure 6A (Normal)

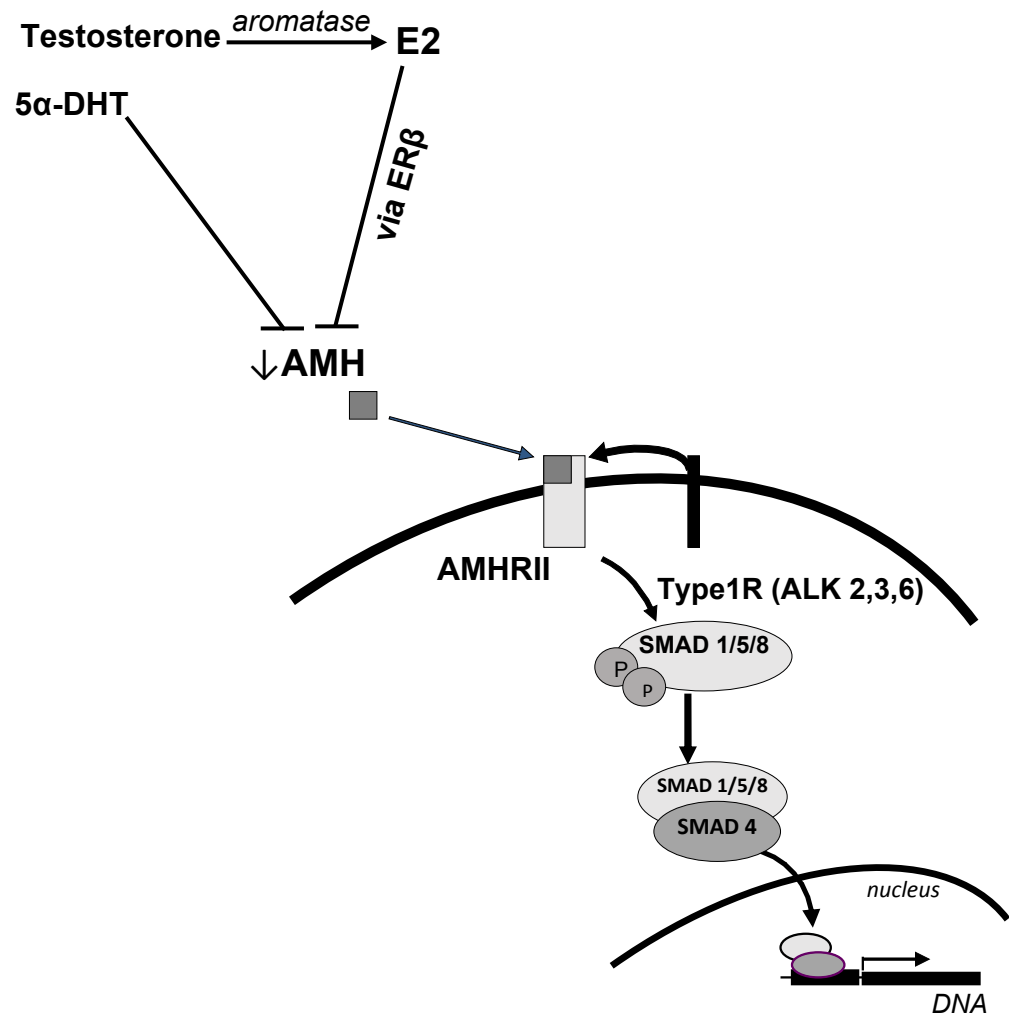


Figure 6B (PCOS)

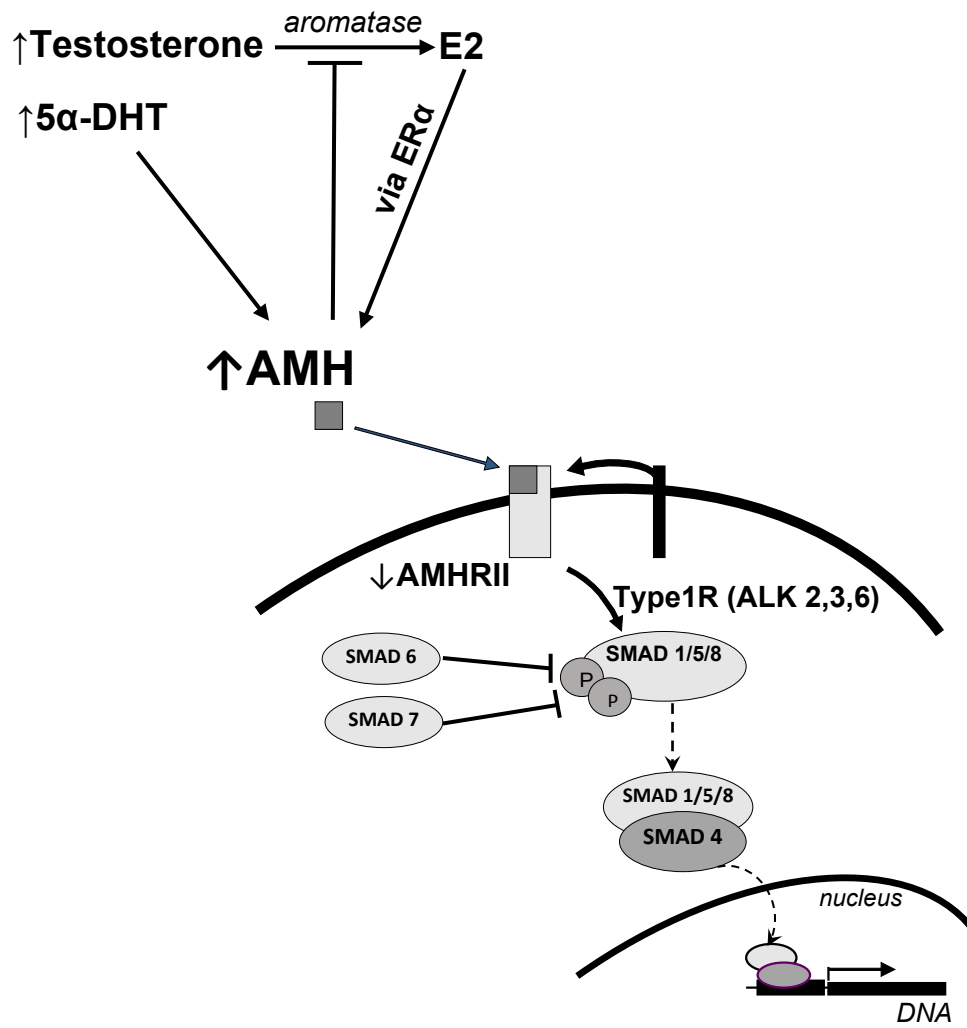


Table 1

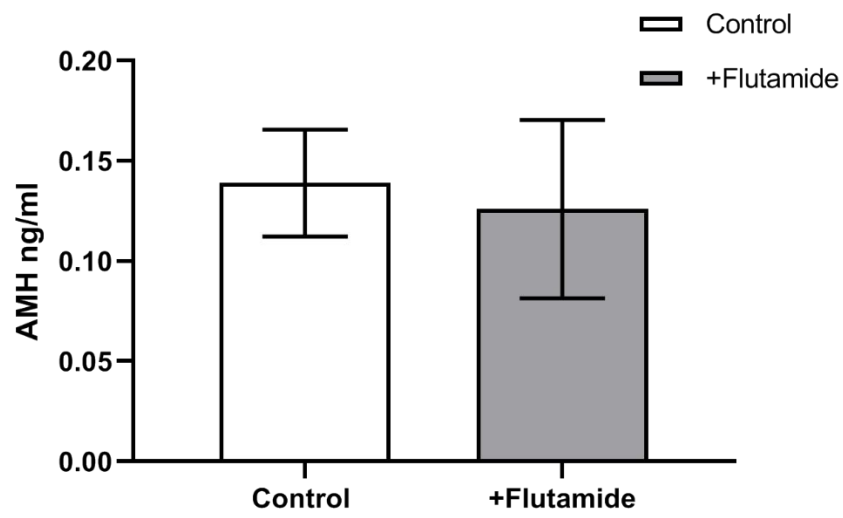
Ovarian Status	Age (yrs)	BMI (kg/m²)	AMH (pmol/L)	FSH (IU/L)	LH (IU/L)	AFC
Normal (n=26)	35.2±0.76	25.9±0.70	12.4±1.68	6.4±0.5	4.0±0.34	11.75±0.81
PCOM (n=7)	33±2.02	25.2±1.19	36.1±11.57 **p=0.001	5.0±0.73	3.2±0.37	22.86±2.38 ****p=00004
PCOS (n=9)	30.3±1.49 **p=0.003	24.8±1.10	33.7±6.98 ***p=0.0001	4.5±0.49	6.3±1.49 *p=0.03	37.3±8.32 *****p=000002

Table 2

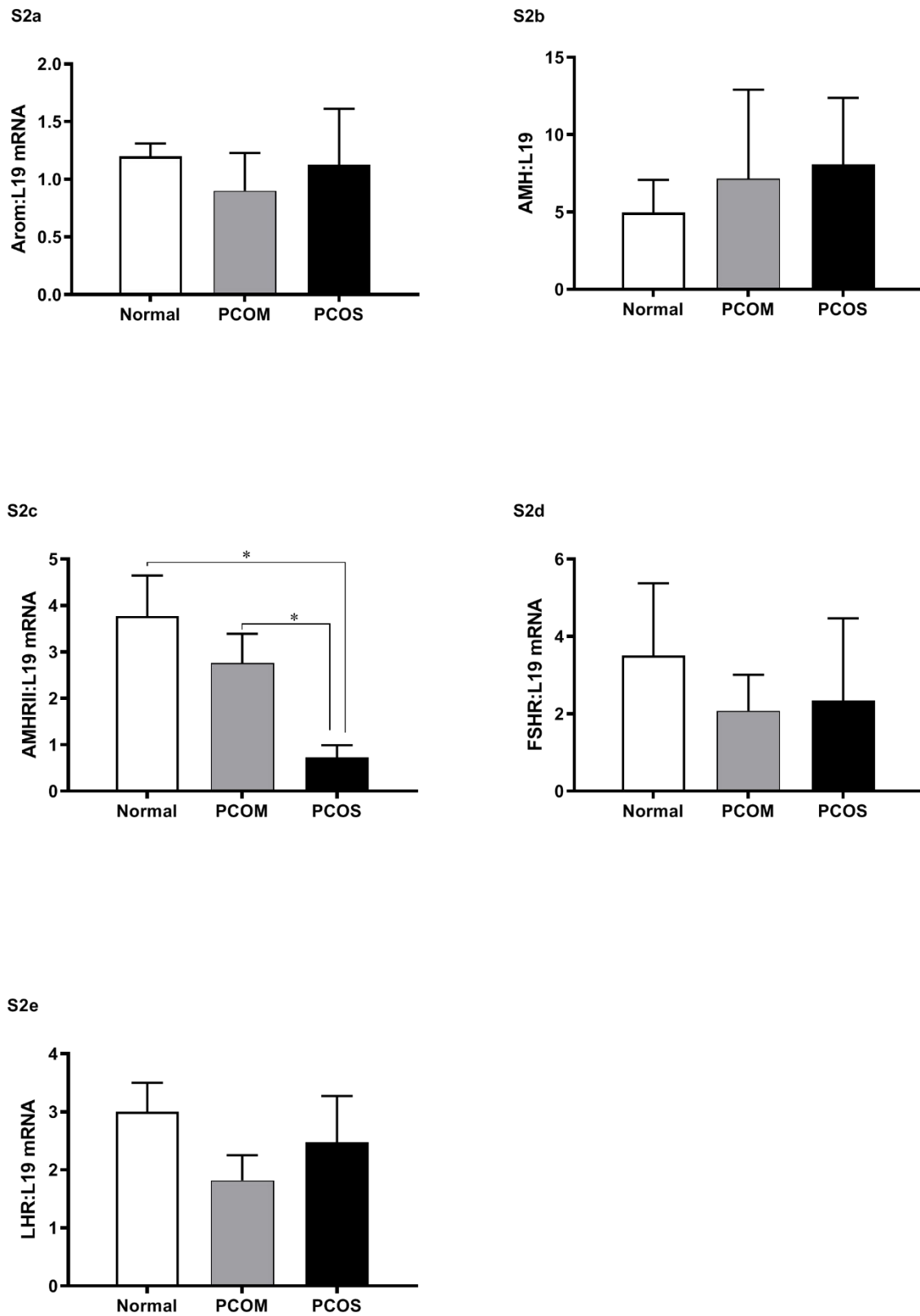
Gene (Accession Number)	Primer Sequence	Primer Concentration (nM)	Annealing Temperature (°C)
<i>AMH</i> (NM_000479)	F – GCATGTTGACACATCAGGC R – GAGTGGCCTTCTCAAAGAGC	100	60
<i>AMHRII</i> (NM_020547)	F – CCCTGCTACAGCGAAAGAAC R – ATGGCAACCAGTTTTTCCTTG	150	60
<i>Aromatase</i> (NM_000103)	F – GACTCTAAATTGCCCCCTCTG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

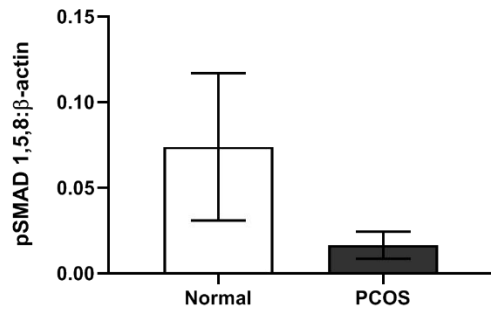
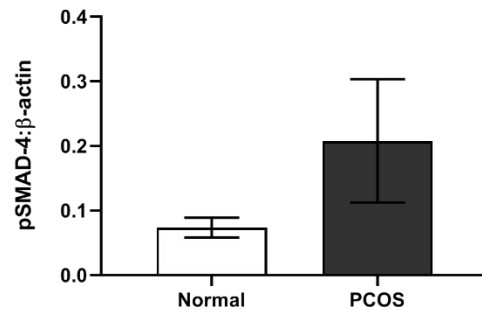
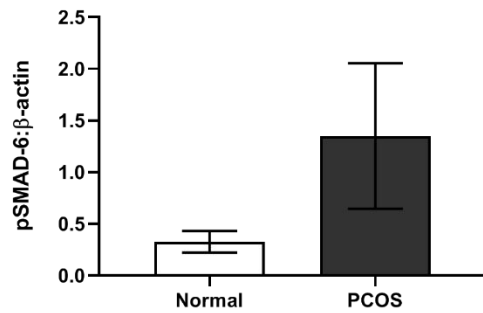
Table 3

Antibody	Molecular Weight (kDa)	Antibody dilution	Supplier of Primary antibody	Poly- or Monoclonal	Secondary Antibody (Licor IRDye 800/680)
AMHR II	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



Supplementary Figure 3**S3a****S3b****S3c****S3d**