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1 # Elevation of anti-müllerian hormone (AMH) in PCOS women in ART:
2 # Effect of Insulin

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18 **Abstract**

19 **Objective**: To measure blood and follicular AMH levels in PCOS woman in ART and
20 examine direct action of insulin on AMH expression in human granulosa cells

21 **Design:** Prospective clinical and experimental study.

22 **Setting:** University Hospital-based laboratory.

23 **Patients:** Women with (n=86) and without (n=172) PCOS in ART.

24 **Intervention(s):** Blood, follicular fluid and luteinized granulosa cells were collected from
25 PCOS or non-PCOS women in ART.

26 **Main Outcome Measure(s):** Hormone levels in blood and fluid and gene expression in
27 granulosa cells were measured.

28 **Result(s):** Serum levels of AMH were elevated and inversely correlated with embryo
29 cleavage rate in PCOS women in ART. Significant higher levels of AMH were also found in
30 small and large follicles collected from PCOS women as compared to those in non-PCOS
31 women. Luteinized granulosa cells from PCOS women showed higher expression of AMH
32 and its receptor AMHR2. Direct effect of insulin in increasing the expression of AMH in the
33 isolated luteinized granulosa cells was observed with the PCOS granulosa cells responding to
34 a high dose of insulin. Co-treatment of AMH attenuated insulin-induced aromatase
35 expression in the luteinized granulosa cells.

36 **Conclusion(s):** These results suggest insulin may contribute to AMH elevation in PCOS,
37 while AMH counteracts with insulin-promoted aromatase expression in granulosa cells.

38 **Key Words:** Polycystic Ovarian Syndrome (PCOS), Assisted reproductive technology
39 (ART), Anti-Müllerian hormone (AMH), Insulin.

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41

42 **Running title:**

43 AMH elevation and insulin in PCOS under ART

44

45 **Abstract capsule**

46 AMH is elevated in blood and follicular fluid in PCOS in ART. Insulin increases AMH
47 expression, while AMH counteracts with insulin-promoted aromatase expression in human
48 luteinized granulosa cells granulosa cells.

49

50 ## Introduction

51 Polycystic ovary syndrome (PCOS), characterized by ovarian follicular arrest and
52 hormonal disturbance, is a leading cause for female infertility affecting 5-10% women of
53 reproductive age (1, 2). A variety of hormones are dysregulated in PCOS including excessive
54 androgens (3), hypersecreted luteinizing hormone (LH) (4), reduced follicle-stimulating
55 hormone (FSH) (4) as well as disturbed metabolic hormones (e.g. insulin and glucagon) (5).
56 In particular, insulin resistance accompanied with compensatory hyperinsulinemia is
57 commonly found in PCOS women (up to 75% of lean and 95% of overweight, respectively)
58 (6, 7).

59 Anti-Müllerian hormone (AMH), a key regulator for sex differentiation during
60 embryonic development (8), is recently recognized to be elevated in PCOS contributing to the
61 ovarian dysfunction (9, 10). AMH is a homodimer glycoprotein belonging to the
62 transforming growth factor β (TGF-β) family (11, 12). In addition to its role in inhibiting
63 embryonic development of Müllerian duct and thus leading sex differentiation into males (13),
64 AMH is also known to be involved in folliculogenesis (14). In women, AMH is produced
65 exclusively by granulosa cells (15), starting at the primary follicle stage with its highest
66 expression detected in pre-antral and small antral follicles, which declines afterwards till
67 absent in large (> 8-10 mm diameter) follicles (16). Studies have shown that AMH inhibits
68 FSH-stimulated expression of aromatase, a key enzyme for ovarian steroidogenesis (17), as
69 well as the growth of pre-antral follicles(18). Therefore, the expression pattern of AMH
70 during folliculogenesis is believed to preserve most antral follicles from all being matured
71 and ovulated, which is evident by the accelerated folliculogenesis leading to early depletion
72 of ovarian follicles observed in AMH-null mice (19). Abnormally elevated AMH in PCOS,

however, is believed to be detrimental to FSH-stimulated growth of selectable follicles leading to anovulation (20).

While its contribution to PCOS pathogenesis is generally accepted, the cause of AMH elevation in PCOS remains unknown. It was thought that the increased number of small antral follicles in PCOS might produce an excessive AMH environment, although later studies have shown that AMH production is increased in individual follicles or granulosa cells from PCOS women (21-23) with the underlying mechanism largely unexplored. Interestingly, hyperinsulinemia is proposed a reason for AMH elevation in PCOS, as AMH levels were found to be particularly higher in PCOS women with insulin resistance (24, 25) and long-term metformin treatment was reported to correct AMH levels in PCOS (26). However, contradictory findings were also reported (27, 28) and the direct effect of insulin on AMH production by granulosa cells is not clearly demonstrated.

PCOS women often seek in vitro fertilization (IVF)-based assisted reproductive technology treatment (ART). In ART, the AMH level has been associated with ovarian responses (29), implantation, pregnancy and other qualitative ART outcomes (30, 31), although controversial results are also reported (32, 33)(34, 35).

We undertook the present study to better characterize the association between AMH level with embryo quality as well as ART outcomes in PCOS. We also examined possible action of insulin on AMH expression in human granulosa cells, while comparing the cells from PCOS and non-PCOS women.

Materials and Methods

Human subjects and clinical data collection

Total 258 women undergoing in vitro fertilization (IVF)-based assisted reproductive treatment (ART) at Shanghai First Maternity and Infant Hospital from June 2014 to June 2017 were recruited for the present study. The Rotterdam criteria (36): I, oligomenorrhea (cycle length > 35 days) or amenorrhea (cycle lengh > three months or no rise of progesterone before menstruation); II, hyperandrogenism as defined by hirsutism, and/or serum testosterone level > 0.7 ng/ml and/or androstenedione > 2.2 ng/ml; and III, the presence of more than 12 follicles of 2-9 mm in diameter in each ovary and/or unilateral ovarian volume >10ml under B-ultrasound, was used for diagnosis of PCOS. 86 women showed the presence of Rotterdam criteria- I and III and were diagnosed of PCOS. Androstenedione was not measured in them. 172 women who met the following inclusion criteria: i) age between 20 and 40, ii) both ovaries were present with no morphological abnormalities and adequately visualized in transvaginal ultrasound scans, iii) regular menstrual cycle, iv) no current or past diseases affecting ovaries or gonadotrophin or using sex-steroids for at least 3 months prior to inclusion, were grouped as non-PCOS for the present study. The cause of infertility in non-PCOS group was due to either tubal obstruct or male factor. The study was approved by the Ethics Committee of Tongji University and all the subjects gave informed consent. All the procedures were performed in accordance with the relevant guidelines and regulations. The subjects' clinical data including age and BMI were documented. Patients with oligo- or amenorrhea were pre-treated with progesterone to induce regular cycle before the blood test and ART treatment. A blood test was performed on the 3rd-5th day of the menstrual cycle before the treatment to determine basal levels of hormones.

118

119 ***Ovarian hyperstimulation***

120 All the subjects were given diphereline, (Ipsen Pharma Biotech, France, 1.25 mg) a
121 gonadotropin-releasing hormone agonist, at their mid-luteal phase of the menstrual cycle, to
122 suppress pituitary secretion of gonadotropin hormones and prevent premature ovulation.
123 After pituitary suppression was achieved, which was determined by E_2 ≤50pg/ml,
124 endometrial thickness ≤6mm and no follicles >10 mm under transvaginal ultrasound, subjects
125 were daily injected with Gonal-F, a recombinant human FSH (rhFSH, Merck-Serono,
126 Switzerland), starting from day 5 of a normal menstrual cycle (initial day). Serum sex
127 hormone levels were monitored and transvaginal ultrasound (5-MHz, Model SSD-620, Aloka
128 Co. Ltd, Tokyo Japan) was performed to evaluate the development of ovarian follicles and
129 adjust the dose of rhFSH. When the lead follicle achieved 18 mm in diameter, the lead two
130 were 17mm or the lead three were 16mm, patients were subcutaneously injected with
131 recombinant human chorionic gonadotropin (rhCG, 250μg, Ovidrel, MERCK SERONO,
132 Switzerland) to trigger oocyte maturation (hCG day). 36 hours afterwards, oocytes were
133 retrieved by transvaginal ultrasound-guided approach. Fertilization of the retrieved oocytes
134 was done either by IVF or intracytoplasmic sperm injection (ICSI), according to the sperm
135 quality. Successful fertilization and cleavage were determined by reported ART standards
136 (37). Quality of embryo were evaluated and graded into I to V: grade I – embryos with equal
137 blastomeres and less than 5% cytoplasm fragmentation, grade II – embryos with equal
138 blastomeres and 5-25% cytoplasm fragmentation, grade III – embryos with unequal
139 blastomeres and less than 5% cytoplasm fragmentation, grade IV –embryos with unequal
140 blastomeres and 5-25% cytoplasm fragmentation, grade V – embryos with a few blastomeres
141 and with a strong or complete cytoplasm fragmentation. Embryos of grade I and II were

142 considered as good, grade III as intermediate, grade IV and V as poor quality embryos. 3-5
143 days after cleavage was observed, up to 2 embryos were transferred in each patient per cycle.
144 Grade I and II embryos were transferred as priority. Grade III embryos were transferred in
145 case no grade I-II embryo was available. In rare cases, grade IV-V embryos were cultured till
146 blastocyst-stage before the transfer. Patients were intramuscularly injected with progesterone
147 (60 mg/day, Tongyong Pharmaceutical Co., Shanghai, China) from oocyte retrieval day till
148 14 days after embryo transfer. Clinical pregnancy was defined as the identification of a
149 gestational sac with fetal heart activity on ultrasound examination 4 to 5 weeks after embryo
150 transfer. Implantation rate was calculated as the number of gestational sacs (observed at week
151 4 post-transfer) divided by the number of embryos transferred in each patient. Other
152 downstream parameters - pregnancy, miscarriage, ectopic and live birth rates were calculated
153 with the total number of patients in each group as the denominator.

154

155 ***Follicular fluid and granulosa cell collection***

156 The ovarian follicles were collected after hCG injection from the patients as we
157 previously reported (38). Follicles were classified into two groups, small (<10mm diameter)
158 and large (>18mm), according to their size measured by ultrasonography. Under the guidence
159 of ultrasonography, a sterile needle, connected to a vaculum device, was transvaginally
160 inserted. With gental suction, each follicle was individually collected through the needle to a
161 collection vial. Large follicles were collected before the collection system was washed
162 sufficiently and small follicles were collected afterward. For each individually collected
163 follicle, the oocyte was carefully dissected out under a dissecting microscope, the remaining
164 granulosa cells with fluid were transferred into sterile tubes (Axygen Scientific, Union City,
165 CA, USA) for centrifugation at 400g for 10 min. Afterwards, the supernatant was used as

166 follicular fluid. Cell pellets were washed with PBS, subsequently a red blood cell lysis buffer
167 and further centrifuged at 400 g for 5 min. For ELISA measurements of the follicular fluid, a
168 single large follicle with sufficient amount of fluid was used for one measurement, whereas
169 follicular fluid gathered from 3-4 small follicles from a patient was used for one
170 measurement. The collected granulosa cells and follicular fluid were stored at -80 ºC until
171 mRNA analysis or re-suspended for culture.

172

173 ***Hormonal immunoassays***

174 Blood was collected from subjects on the 3rd-5th day of the menstrual cycle prior to the
175 treatment (Basal), the day when FSH treatment started (Initial day) and the day when hCG
176 was given (hCG day) to determine testosterone, FSH, LH or E_2 levels by an automated
177 chemiluminescence immunoassay analyzer (Siemens, Tarrytown,NY, USA). On hCG
178 injection day and before the injection, blood was collected to examine serum AMH levels by
179 an ELISA kit, Anti-Mullerian Hormone ELISA (Biomatik), according to the supplier's
180 instructions. For follicular fluid samples which are of small volume and have lower AMH
181 levels, we used a more sensitive and specific chemiluminescence kit (Roche, Basel,
182 Switzerland) according to manufacturer's instruction. Insulin in follicular fluid was measured
183 using a specific ELISA-kit (Mai Bio, Shanghai, China) according to manufacturer's
184 instruction. The plates were read by an ELISA reader (Thermo Fisher Scientific, Inc.).

185

186 ***Human luteinized granulosa cell culture***

187 The collected granulosa cells were cultured in DMEM/F12 (Gibco, Rockville, MD,
188 USA) with 10% fetal bovine serum (Gibco), 100 U/ml penicillin (Life Technologies), 100
189 ug/ml streptomycin sulfate (Life Technologies), and 1×GlutaMAX (Life Technologies) at 37

190 ºC in CO_2 incubator. Cells were deprived of serum for 24 hours before stimulated with 5, 10,
191 100ng/ml of insulin (Sigma; bovine pancreatic insulin) or GSK1904529A (5μM, Selleck), an
192 insulin receptor (INSR) inhibitor.
193
194 ***RNA extraction, reverse transcription and quantitative real-time PCR***
195 Total RNAs from cells were extracted using the Trizol reagent (Life Technologies, Inc.)
196 following the manufacturer's protocol. The concentration of RNA was measured using the
197 NanoDrop 2000cUV-Vis spectrophotometer (Thermo Fisher Scientific, Inc.).1 μg RNA was
198 transcribed to cDNA using the PrimeScript RT reagent Kit (Takara, Shiga, Japan). Briefly, a
199 master mix containing 4 μL 5×PrimeScript Buffer, 1 μL Rime Script RT Enzyme Mix I, 1 μL
200 Oligo dT Primer (50μM), 1 μL Random 6 mers (100μM) and RNase Free water was prepared
201 for each 20 μL reaction, which followed by 37 ºC for 30 min, at 85 ºC for 5 s and cooling at 4
202 ºC. Gene expression levels were evaluated by the manufacturer's protocol on the
203 StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Inc.) using the SYBR
204 reagent (Tiangen, China). The primers were from Sangon Biotech (Shanghai, China) and the
205 sequences are listed in Table 1. The general thermal profile of the reaction was for all the
206 genes tested 10s at 95°C to activate the enzyme followed by 40 cycles of 95°C for 5s and
207 60°C for 34s for each cycle. The dissociation stage was initiated at 95°C for 15s, followed by
208 one cycle at 60°C for 60s and 95°C for 15s. Each reaction was assayed in triplicate and three
209 separate experiments were performed on different cultures. A mean value (generated from the
210 triplicate data of the target genes and reference gene of each sample) was used for the
211 determination of mRNA levels by the comparative Ct (2-ΔΔCt) method with β-actin as the
212 housekeeping gene, ΔΔCt = [Ct of target (sample A) – Ct of reference (sample A)] – [Ct of
213 target (sample B) – Ct of reference (sample B)]. This equation can be used to compare the

214 gene expression in two different samples (sample A and sample B); each sample is related to
215 a housekeeping gene (β-actin).

216

217 *Western blot*

218 Cells were washed with PBS and lysed in RIPA buffer (Beyotime, Shanghai, China)
219 containing protease inhibitor cocktail and PMSF (Sigma-Aldrich) on ice. The extract was
220 centrifuged at 15000g for 30 min at 4ºC to remove cellular debris and the protein
221 concentration was quantified using the BCA assay (Thermo Fisher Scientific, Inc.). The same
222 amount of protein (20μg) of each sample was separated by SDS-PAGE (Beyotime, Shanghai,
223 China) with 5% stacking gel and 10% separating gel at 120V for 2 hours and then transferred
224 onto the polyvinyl difluoride membranes (PVDF) at 250 mA for 1 hour. Membrane
225 nonspecific binding was blocked with 5% nonfat milk in TBST (0.01M Tris-HCl, 0.15M
226 NaCl, 0.1%Tween 20, PH7.4, Beyotime), containing 0.05% Tween-20 for 1 hour at room
227 temperature and then incubated with rabbit antihuman aromatase (Cell Signal Technology,
228 USA, 1:1000) and rabbit antihuman GAPDH (Abmart, Shanghai, China, 1:1000) diluted in
229 TBST with 5% milk overnight at 4°C. Next, the membranes were washed three times with
230 TBST and then incubated for 1 h with the horseradish peroxidase –conjugated secondary
231 antibody (Abmart, 1:2000). Immunoreactive bands were detected using an enhanced
232 chemiluminescent substrate (Millipore, Watford, UK). The intensities of the bands were
233 quantified using Image- J software.

234

235 ***Statistical analysis***

236 Data were mean ± SEM of at least three independent experiments. Comparisons of two

237 independent groups were using the Student's t-test. One-way ANOVA followed by Tukey's
238 post hoc tests was used to compare more than two groups. The correlation analysis was done
239 by the Spearman's coefficient. $P<0.05$ was considered statistically significant. All statistic
240 procedures were run on SPSS 20.0 (IBM, Armonk, NY, USA). Since there were differences
241 in age and BMI as well as in the duration of infertility between PCOS and non-PCOS in
242 Cohorts I and III, we corrected a priori for these differences by analysis of covariance and
243 multiple liner regression.

244

245 **Results**

246 ***Clinical characteristics of PCOS and non PCOS women in ART***

247 Blood, follicular fluid and granulosa cells were collected from 3 cohorts of woman
248 subjects respectively. Clinical characteristics are summarized in Supplementary Table 2. In
249 general, the PCOS women, when examined, were about 2 years younger with a higher BMI
250 in average, as compared to the non-PCOS women. The PCOS women in average were given
251 a lower initial dose of rhFSH with longer treatment duration than the non-PCOS, although the
252 total dose of rhFSH were comparable with no significant difference between the two groups.

253 Hormonal profiles of the two groups are shown in Supplementary Table 2. Prior to the
254 treatment (basal), the PCOS group exhibited lower FSH and higher LH levels as compared to
255 the non-PCOS group. The serum FSH, LH and E_2 in both PCOS and non-PCOS subjects
256 were largely decreased to a low level before rhFSH treatment started (Initial day), indicating
257 successful pituitary suppression. After rhFSH treatment and right before hCG injection (hCG
258 day), the LH level remained low while E_2 level was highly elevated in both groups with no
259 significant difference. Progesterone levels on hCG day were also found to be comparable

260 between PCOS and non-PCOS women.

261 Clinical outcomes are shown in Supplementary Table 2. More PCOS patients were
262 considered to have risk of ovarian hyperstimulation syndrome (OHSS), and therefore chose
263 to cancel embryo transfer within the same cycle and freeze the formed embryos for later
264 treatment. Among the assessed parameters including fertilization, cleavage, embryo quality,
265 implantation and pregnancy, PCOS and non-PCOS women exhibited similar ART outcomes.

266

267 ***AMH is elevated and negatively correlated with oocyte/embryo quality in PCOS women***

268 We collected blood samples on hCG-injection day from 31 PCOS and 59 non-PCOS
269 women (Cohort I, Supplementary Table 2) to determine AMH blood levels. Results showed
270 that the averaged blood level of AMH in PCOS women (39.77 ± 1.84) was significantly
271 higher than that in non-PCOS women (28.26 ± 1.00) (Fig.1A). To explore possible effect of
272 AMH on embryo quality, correlation analysis was performed between the AMH blood level
273 and the number of oocytes retrieved (Fig.1B), fertilization rate (Fig.1C), cleavage rate
274 (Fig.1D), number of good quality embryo (Fig.1E) and implantation rate (Fig.1F) in PCOS
275 and non-PCOS subjects, respectively. Results showed significantly negative correlation of the
276 AMH blood levels with the cleavage rate (Fig.1D) and number of good quality embryos
277 (Fig.1E) in PCOS women. In non-PCOS women, only a weak inverse correlation of AMH
278 with number of good quality embryos was found.

279

280 ***AMH is elevated in follicular fluid and granulosa cells in PCOS women***

281 Since the follicular environment is particularly important to oocyte development and
282 maturation, we further examined the AMH level in the follicular fluid in another cohort of

283 PCOS (n=19) and non-PCOS women (n=36) (Cohort II, Supplementary Table 2). Both small
284 (<10mm) and large (>18mm) follicles were achieved under ultrasonography during oocyte
285 pick-up and the follicular fluid was collected. In both PCOS and non-PCOS groups, the small
286 follicles contain higher concentrations of AMH as compared to the large follicles, consistent
287 with a role of AMH during folliculogenesis (Fig.2A). Importantly, PCOS follicles exhibited
288 higher AMH levels (large: 6.15 ± 0.98 ng/ml, small: 16.51 ± 1.48 ng/ml), as compared to
289 non-PCOS group (large: 2.29 ± 0.32 ng/ml, small: 6.29 ± 1.00 ng/ml) in large and small
290 follicles respectively (Fig.2A). Since AMH is known to be exclusively produced by granulosa
291 cells in women (8), we tested whether the elevated AMH in follicular fluid would be due to
292 upregulation of AMH expression in PCOS granulosa cells. Indeed, in collected luteinized
293 granulosa cells from large follicles, significantly higher mRNA level of AMH was observed
294 in PCOS women as compared to non-PCOS group (Fig.2B). Moreover, the AMH receptor,
295 AMHR2, was also found to be increased at the mRNA level in PCOS granulosa cells, as
296 compared to non-PCOS (Fig.2C).

297

298 ***Insulin induces upregulation of AMH in PCOS granulosa cells***

299 We next explored possible reason for AMH upregulation in granulosa cells in PCOS.
300 Hyperinsulinemia is proposed to be responsible for AMH elevation in PCOS (25). We also
301 detected the presence of insulin in both PCOS and non-PCOS follicular fluid, with a
302 relatively higher level detected in the PCOS group (Fig.3A). We next examined the direct
303 effect of insulin on AMH expression in culture human granulosa cells. Luteinized granulosa
304 cells from 24 non-PCOS and 6 PCOS women (Cohort III, Supplementary Table 2) were
305 isolated and cultured as we previously reported (38). The expression of insulin receptor
306 showed no significant difference between PCOS and non PCOS granulosa cells (Fig. 3B).

307 After 24-hour insulin treatment, the mRNA levels of AMH in granulosa cells were
308 significantly increased by insulin in a dose-dependent manner in both non-PCOS and PCOS
309 (Fig.3C) groups. At 100ng/ml, insulin no longer increased AMH expression in non-PCOS
310 cells, suggesting desensitization to a high dose of insulin in these cells (Fig. 3C). Whereas,
311 PCOS cells continued to respond to insulin at 100 ng/ml resulting in further increases in
312 AMH mRNA levels as compared to those at 10 ng/ml, suggesting their loss of desensitization
313 to high dose insulin (Fig.3C). In addition, pretreatment with an antagonist of insulin receptor,
314 GSK1904529A (5μM), for 8 hours, blocked the insulin-induced AMH upregulation in
315 non-PCOS granulosa cells (Fig.3E). No significant changes in AMHR2 mRNA were
316 observed after the insulin treatment in either non-PCOS or PCOS cells (Fig.3D).

317

318 ***AMH inhibits insulin-stimulated aromatase expression in granulosa cells***

319 Granulosa cells function is essential to oocyte development and maturation. Given the
320 presence of both insulin and AMH in follicular fluid and the direct effect of insulin in
321 increasing AMH expression as suggested above, we wondered whether the expression of
322 genes essential to in granulosa cell function would be subject to regulation by either the
323 follicular insulin or AMH. Aromatase (CYP19), CYP17, FSH receptor (FSHR), androgen
324 receptor (AR), vascular endothelial growth factor receptor (FLK), insulin-like growth factor 1
325 receptor (IGF1R) and LH receptor (LHR) were examined in luteinized granulosa cells from
326 non-PCOS women after treatment with insulin (10 ng/ml), AMH (20 ng/ml) or their
327 combination for 24 hours. Results showed that insulin (10 ng/ml) alone significantly
328 increased mRNA levels of CYP19 (Fig.4A), FSHR (Fig.4B), AR (Fig.4C) and CYP17
329 (Fig.4D), as compared to controls cells. AMH (20 ng/ml) alone did not alter any of these
330 genes (Fig.4). Co-treatment of AMH, however, substantially attenuated the effect of insulin

331 (10ng/ml) in increasing CYP19 mRNA expression (Fig.4A). Slight effect of AMH
332 co-treatment in inhibiting insulin-induced FSHR expression was also observed (Fig.4B). No
333 obvious changes in FLK, IGF1R and LHR by either insulin or AMH were observed in these
334 cells (Fig.4E-G). To confirm the inhibitory effect of AMH on insulin-induced CYP19, we
335 also examined the protein level of CYP19, which consistently showed that as compared to
336 cells treated with insulin (10ng/ml) alone, cells treated with combination of AMH (20 ng/ml)
337 and insulin (10ng/ml) exhibited significantly lower CYP19 protein levels (Fig.4H).
338 Antagonizing insulin receptor by GSK1904529A (5µM Fig. 4I) also inhibited insulin-induced
339 upregulation of CYP19 in these cells.

340

341 ## Discussion

342 Collectively, the present study has indicated a higher blood AMH level in PCOS than
343 non-PCOS women during ART treatment. The blood AMH levels are in negative correlations
344 with the cleavage rate and number of good quality embryos in PCOS women. AMH is also
345 elevated in collected follicular fluid and upregulated in isolated luteinized granulosa cells
346 from PCOS women. Direct effect of insulin in increasing the expression of AMH in the
347 isolated granulosa cells is observed with the PCOS cells responding to a high dose of insulin.
348 The presence of AMH exerts an inhibitory effect on insulin-induced aromatase expression in
349 isolated luteinized granulosa cells.

350 Consistent with others (10, 39), the present study demonstrates that AMH is abnormally
351 upregulated in PCOS. First, protein levels of AMH are increased in the blood and follicular
352 fluid of either small (presumably pre-antral/antral) or large (matured) follicles. Second, we
353 were only able to collect analysable granulosa cells from large but not small follicles, which
354 are usually believed luteinized cells and supposed to have reduced AMH expression.

355 Nevertheless, in these cells, significantly increased mRNA expression of both AMH and its
356 receptor AMHR-II is observed in PCOS compared to non-PCOS. It should be also noted that
357 the blood AMH level was examined on hCG day, when pituitary suppression and exogenous
358 FSH was already applied and other hormones became comparable between PCOS and
359 non-PCOS. AMH is elevated in PCOS even after hormonal correction in ART, which in
360 together suggests intrinsic changes in PCOS granulosa cells resulting in abnormally
361 upregulated AMH and its downstream signaling.

362 It has been controversial whether AMH can affect outcomes of ART (40-44). The
363 present study has found negative correlations of AMH blood level with the cleavage rate and
364 number of good quality embryos in PCOS women. Therefore, a negative impact on embryo
365 quality, particularly at early stages of embryo development, by the elevated AMH particularly
366 in PCOS is suggested. However, since only cleaved or good quality embryos are selected to
367 proceed with embryo transfer and implantation in ART, the later stages and ultimate ART
368 outcomes seem not influenced by the elevated AMH in PCOS. Interestingly, a most recent
369 study has shown that prenatal AMH exposure may account for the pathogenesis of PCOS in
370 adulthood (45), suggesting the influence of AMH on embryo development in PCOS too. Of
371 note, it was previously reported in a general population that no relationship between AMH
372 and embryo quality was found (46). Others showed that low maternal AMH level could be a
373 predicting marker for fetal aneuploidy (47), suggesting that a low AMH level is not good to
374 embryos either. In the present study, although a high AHM level correlates with poor embryo
375 quality in PCOS women, the maternal AMH levels in non-PCOS women have no or weak
376 correlation with embryo quality parameters. It seems consistent that AMH level can hardly
377 predict embryo quality within a general population. Nevertheless, detailed mechanisms
378 underlying the effect of AMH on embryo quality in PCOS await further investigation.

379 An association between the environmental insulin and granulosa cell production of
380 AMH is suggested. A direct effect of exogenous insulin, mediated by insulin receptor, in
381 increasing AMH expression is observed in both PCOS and non-PCOS luteinized granulosa
382 cells. Interestingly, luteinized granulosa cells isolated from PCOS women seem to be quite
383 sensitive to insulin, rather insulin-resistant, in terms of AMH expression (Fig.3C). PCOS
384 cells respond to insulin even at a high dose that already desensitizes non-PCOS cells (Fig.3C).
385 Given that the expression level of the insulin receptor in PCOS cells showed no difference
386 with that of non-PCOS cells (Fig.3B), the difference observed might due to functional change
387 of the insulin receptor or alterations in its regulatory pathways, which is possibly a cause for
388 the observed elevation of AMH in PCOS. However, the detailed mechanisms underlying the
389 discrepancy in responses to insulin between PCOS and non-PCOS granulosa cells await
390 further investigation. Of note, it was suggested that the overexpression of AMH and
391 AMHR-II in PCOS women could be due to increased LH levels, especially in PCOS women
392 with oligo/anovulation (48). Since insulin and LH are believed to have synergy effects (49),
393 hyperinsulinemia may enhance the effect LH in increasing AMH expression in PCOS
394 women.

395 It should be noted that insulin is known for long to increase aromatase activity in
396 granulosa cells from either normal and PCOS women (50, 51). Similar effect of insulin in
397 increasing aromatase expression is also observed presently. Whereas, aromatase is known to
398 be dysfunctional or downregulated (38) contributing to a hyperandrogenic environment in
399 PCOS. In addition to previously reported effect of AMH in reducing FSH-induced aromatase
400 expression (52), the present study has shown the capacity of AMH in counteracting with
401 insulin's action on aromatase expression. Together with the finding of insulin's role in

402 promoting AMH production, it may provide an explanation to the reduced aromatase activity
403 despite a high insulin environment in PCOS.
404

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410

411 Author contribution

412 Y.J.Z. and Y.C.R.: conception and design; X.Y.L, Y.J.Y., C.L.T, K.W., J.C., and X.M.T.,
413 experiments and/or data analysis; Y.C.R. and Y.J.Z.: article writing with contributions from
414 other authors.
415

416 Competing financial interests

417 The authors declare no conflict of interests.
418

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570

571 # Figure legends

572 **Fig.1. Blood AMH is elevated in PCOS women**

573 **(A)** ELISA measurement of AMH levels in the blood collected on hCG injection day (before
574 the injection) from PCOS and non-PCOS women. n is shown in each column. ***P < 0.001,
575 t-test. **B-F)** Correlation analysis of the blood AMH level and oocytes retrieved (B),
576 fertilization(C), cleavage (D), good quality embryo(E) and implantation (F) in PCOS and
577 non-PCOS women. *P < 0.05, **P < 0.01, Spearman's coefficient correlation test.

578

579 **Fig.2. AMH is elevated in follicular fluid and granulosa cells in PCOS women**

580 **(A)** ELISA measurement of AMH levels in follicular fluid from non-PCOS and PCOS
581 women. Data are mean ± SEM, n is indicated in each column. *** P<0.001, t-test**. B-C)**
582 Quantitative PCR (qPCR) analysis of AMH(B) and AMHR-II(C) mRNA levels in luteinized
583 granulosa cells freshly isolated from non-PCOS and PCOS women. β-actin was used as the
584 internal control for relative mRNA level measurement by qPCR. Data are mean ± SEM. n is
585 indicated in each column, *P<0.05, **P<0.01, t-test.

586

587 **Fig.3. Effect of insulin on AMH expression in human luteinized granulosa cells.**

588 **(A)** ELISA measurement of insulin levels in follicular fluid from non-PCOS and PCOS
589 women. Data are mean ± SEM, n is indicated in each column. t-test**. (B)** qPCR analysis of
590 mRNA levels of insulin receptor (INSR) in cultured luteinized granulosa cells isolated from
591 non-PCOS and PCOS women**. (C-D)** qPCR analysis of mRNA levels of AMH (C) and
592 AMHR2 (D) in cultured luteinized granulosa cells isolated from non-PCOS and PCOS
593 women, after incubation with insulin (0-100 ng/ml) for 24 hours. Cells isolated from 14

594 non-PCOS women and 6 PCOS women were pooled together for the experiments. n =3
595 (number of independent experiments). *$P<0.05$, ***$P<0.001$. One-way ANOVA with
596 post-tests. **(E)** qPCR analysis of mRNA levels of AMH in non-PCOS granulosa cells
597 pretreated with or without GSK1904529A (5μM, an insulin receptor antagonist) for 8 hours
598 before incubated with or without insulin (10 ng/ml) for 24 hours. Cells isolated from 10
599 women were pooled together for the experiments. Data are mean ± SEM, n = 3 (number of
600 independent experiments). *$P<0.05$ one-way ANOVA.

601

602 **Fig.4. Effect of insulin and AMH on genes expression in human luteinized granulosa
603 cells**. **(A-I)** qPCR analysis of mRNA levels of CYP19 (A), FSHR (B), AR (C), CYP17 (D),
604 FLK (E), IGF1R (F) and LHR (G) in luteinized granulosa cells from non-PCOS women,
605 treated with or without insulin (10 ng/ml) and AMH (20 ng/ml) for 24 hours. β-actin was
606 used as the internal control for relative mRNA level measurement by qPCR. Cells isolated
607 from 9 women were pooled together for the experiments. Data are mean ± SEM, n = 3
608 (number of independent experiments). *$P<0.05$ and ***$P < 0.001$, one-way ANOVA**. (H)**
609 Western blotting for CYP19 in luteinized granulosa cells isolated from non-PCOS women,
610 treated with or without insulin (10ng/ml) or AMH (20ng/ml). β-actin was used as a loading
611 control for western blot. **(I)** Western blotting for CYP19 in luteinized granulosa cells isolated
612 from non-PCOS women, treated with or without GSK1904529A (5μM, an insulin receptor
613 antagonist) for 8 hours before incubated with or without insulin (10 ng/ml) for 24 hours. Cells
614 isolated from 10 women were pooled together for the experiments. Data are mean ± SEM, n
615 = 3 (number of independent experiments). *$P<0.05$, one-way ANOVA.

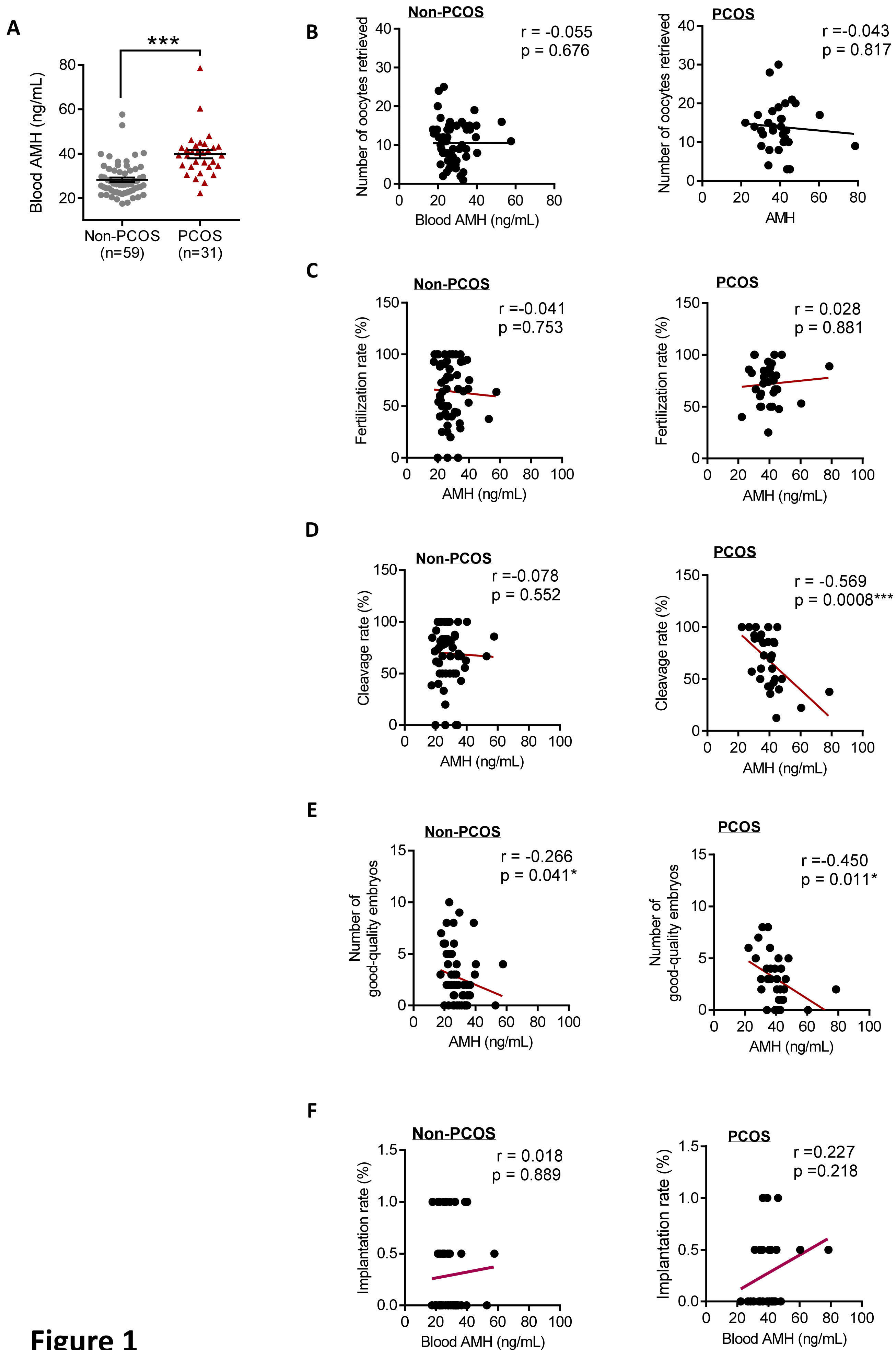

Figure 1

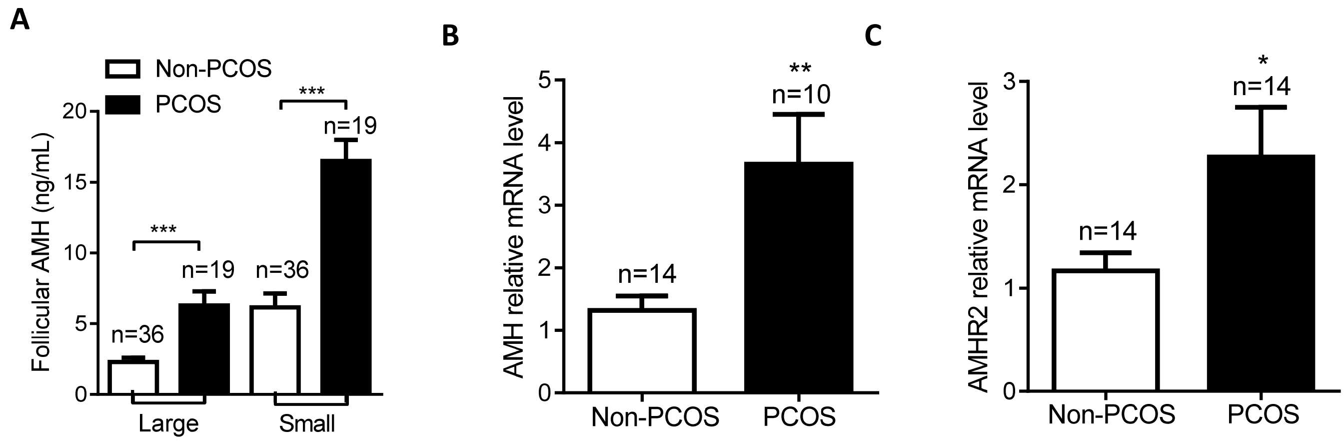

Figure 2

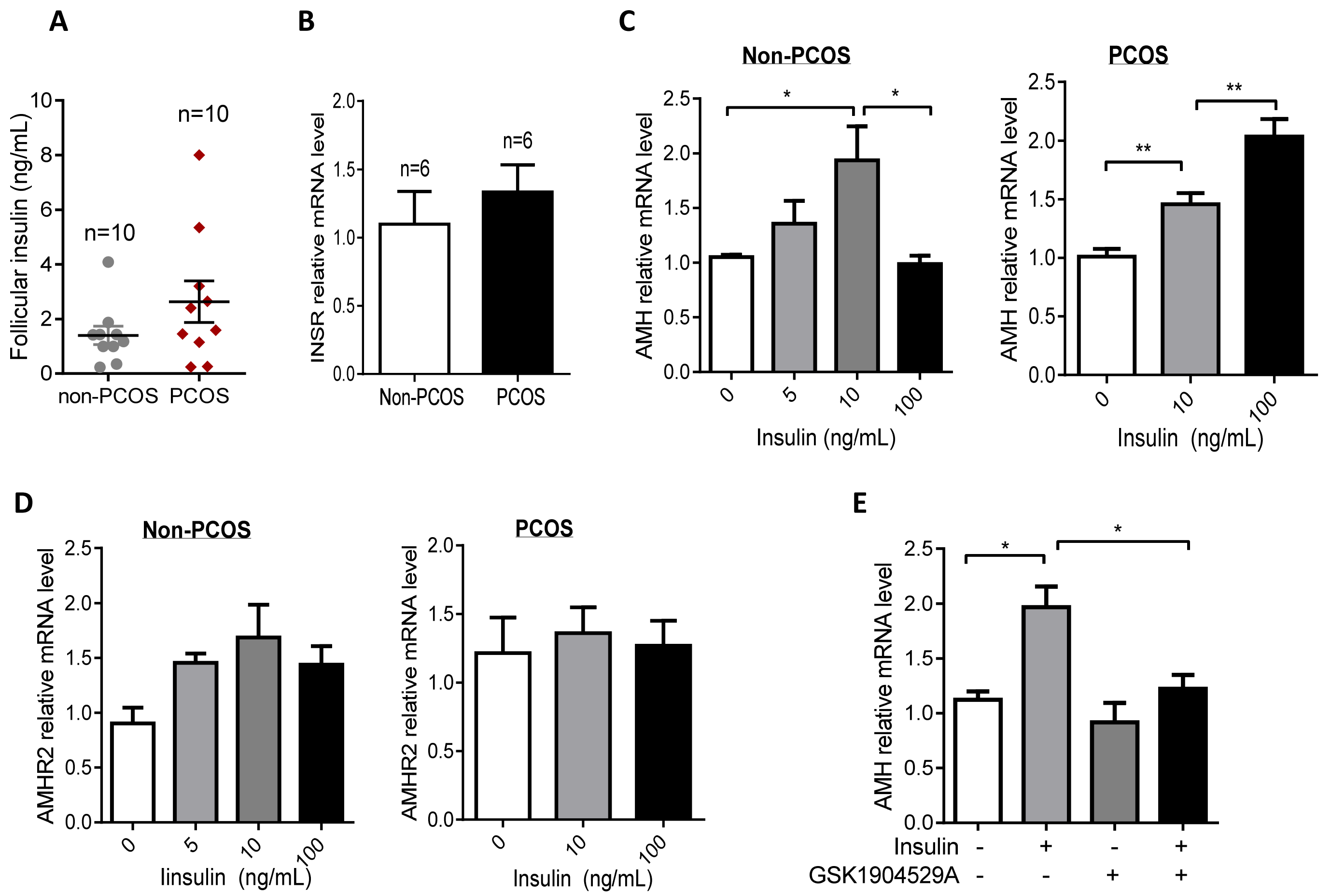

Figure 3

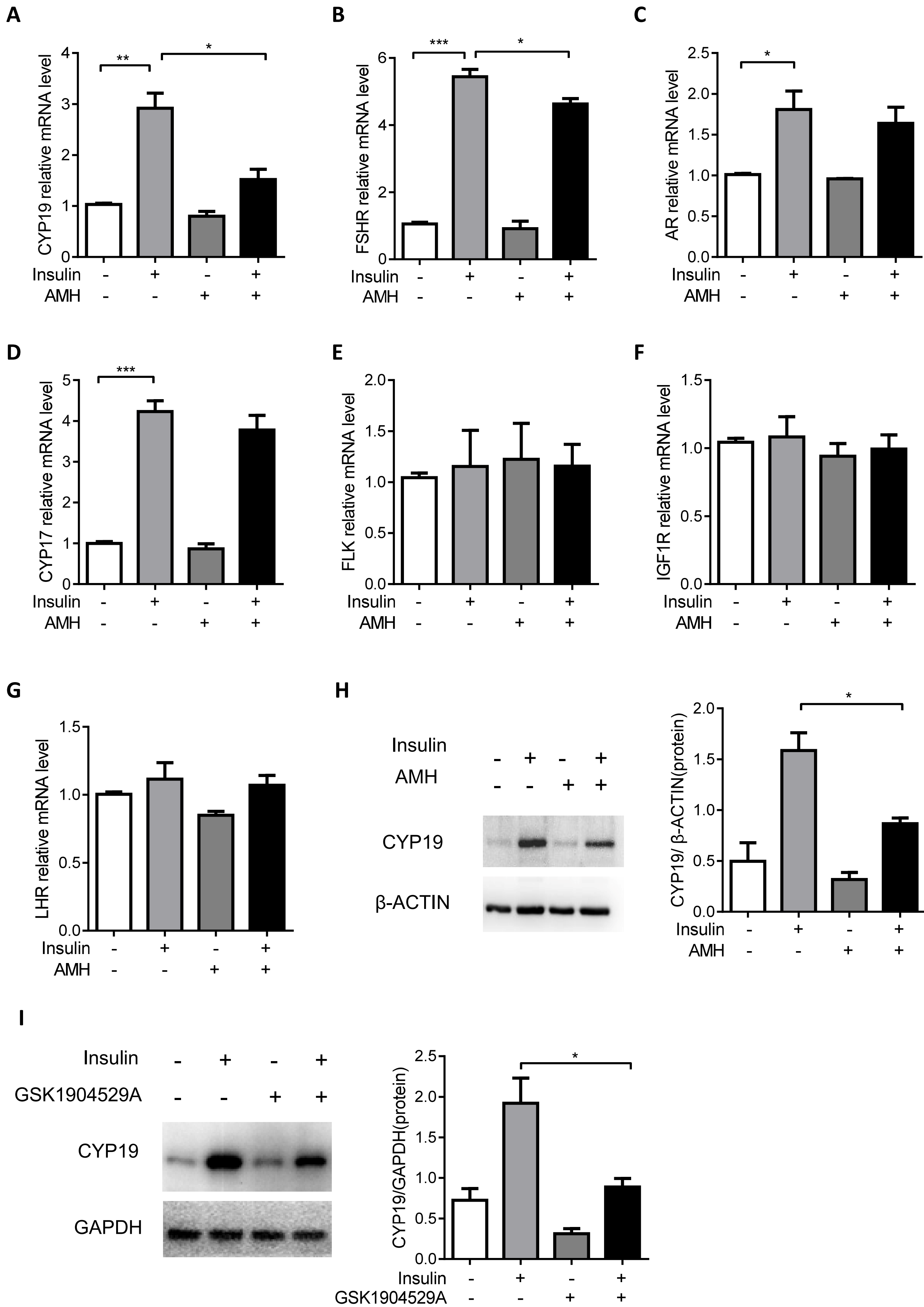

Figure 4

Table.1 Sequences of primers

Gene	Forward sequence	Reverse sequence	Product size (bp)
AMH	TGGCCTCATCTTCCGAGAAG	GGAGCTGCTGCCATTGCT	100
AMHR-II	TTGGGGCTTTGGGCATTACTT	AGCTCTCCCAGTGTCTTTGTG	113
CYP19	TGGAAATGCTGAACCCGATAC	AATTCCCATGCAGTAGCCAGG	161
CYP17	GCTGCTTACCCTAGCTTATTTGT	ACCGAATAGATGGGGCCATATTT	174
FSHR	TCTGTCACTGCTCTAACAGGG	TGCACCTTTTTGGATGACTCG	131
AR	CCTGGCTTCCGCAACTTACAC	GGACTTGTGCATGCGGTACTCA	168
LHCGR	ATTTGTCAATCTCCTGGAGGC	CACTCAGTTCACTCTCAGCA	191
FLK	GGCCCAATAATCAGAGTGGCA	TGTCATTTCCGATCACTTTTGGA	105
INSR	AAAACGAGGCCCGAAGATTTC	GAGCCCATAGACCCGGAAG	90
β-actin	GCATGGGTCAGAAGGATTCCT	TCGTCCCAGTTGGTGACGAT	106

Supplemental Table 1

	Cohort I (Blood)			Cohort II (Follicular fluid)			Cohort III (Granulosa cells)			Corhort I + II + III		
Variables	**Non-PCOS (n=59)**	**PCOS (n=31)**	***P***	**Non-PCOS (n=36)**	**PCOS (n=19)**	***P***	**Non-PCOS (n=77)**	**PCOS (n=36)**	***P***	**Non-PCOS (n=172)**	**PCOS (n=86)**	***P***
Basic characteristics												
Age (years)	31.9 ± 0.6	29.4 ± 0.7	**	31.8 ± 0.6	30.1 ± 0.9		31.3 ± 0.5	29.5 ± 0.6	*	31.6 ± 0.3	29.6 ± 0.4	***
BMI (kg/m^2)	20.9 ± 0.4	22.4 ± 0.5		22.07 ± 0.5	23.4 ± 0.7		20.5 ± 0.5	23.5 ± 0.8	**	21.2 ± 0.2	22.6 ± 0.4	***
Infertility causes												
Primary infertility	28 (47.5%)	17 (54.8%)		26 (72.2%)	9 (47.4%)		41 (53.2%)	22 (61.1%)		95 (55.2%)	48 (55.8%)	
Secondary infertility	31 (52.5%)	14 (45.2%)		10 (27.8%)	10 (52.6%)		36 (46.8%)	14 (38.9%)		77 (44.8%)	38 (44.2%)	
Initial dose of rhFSH (IU)	173.3 ± 5.3	162.4 ± 7.4		170.8 ± 8.1	153.3 ± 6.5		178.2 ± 5.2	153.5 ± 8.3	*	170.4 ± 4.0	154.2 ± 5.9	*
Total dose of rhFSH (IU)	2065.1 ± 150.1	2062.8 ± 205.2		2208.3 ± 116.4	2154.6 ± 182.3		2139.3 ± 98.1	2038.1 ± 157.1		2076.7 ± 76.85	1998.6 ± 113.5	
Duration of rhFSH (day)	10.6 ± 0.6	11.9 ± 0.9		11.7 ± 0.4	12.6 ± 0.8		11.1 ± 0.3	12.6 ± 0.5	**	10.9 ± 0.3	11.6 ± 0.5	
Hormonal test												
Basal												
Testosterone (ng/ml)	0.4 ± 0.1	0.6 ± 0.1	**	0.5 ± 0.1	0.6 ± 0.01	*	0.4 ± 0.0	0.7 ± 0.0	***	0.4 ± 0.0	0.6 ± 0.0	***
FSH (IU/l)	6.9 ± 0.3	6.45 ± 0.4		7.8 ± 0.3	6.5 ± 0.4	*	7.4 ± 0.2	6.5 ± 0.3	*	7.3 ± 0.1	6.5 ± 0.2	**
LH (IU/l)	4.2 ± 0.5	7.3 ± 07	***	6.1 ± 0.8	6.7 ± 1.0		4.7 ± 0.3	7.6 ± 0.5	***	4.9 ± 0.3	7.1 ± 0.4	***
E_2 (ng/ml)	42.0 ± 3.1	44.4 ± 4.0		48.0 ± 3.1	46.4 ± 4.2		46.8 ± 2.8	47.9 ± 4.2		45.4 ± 1.8	47.1 ± 2.6	
Initial day												
FSH (IU/l)	2.6 ± 0.6	4.0 ± 0.9		3.3 ± 0.5	3.3 ± 0.3		3.5 ± 0.3	2.6 ± 0.6		3.1 ± 0.3	3.5 ± 0.4	
LH (IU/l)	1.4 ± 0.1	0.9 ± 0.2	*	2.2 ± 0.4	1.7 ± 0.3		2.2 ± 0.2	1.1 ± 0.3	**	1.9 ± 0.1	1.2 ± 0.2	***
E_2 (ng/ml)	20.3 ± 1.7	20.3 ± 2.5		41.70 ± 20.4	27.1 ± 5.5		22.0 ± 1.6	26.3 ± 3.0		21.6 ± 1.2	23.5 ± 1.9	
hCG day												
LH (IU/l)	2.0 ± 0.2	1.4 ± 0.3		1.8 ± 0.4	1.4 ± 0.4		1.5 ± 0.3	2.4 ± 0.5		1.8 ± 0.1	1.4 ± 0.2	
E_2 (ng/ml)	2615.7 ± 223.0	3081.8 ± 311.8		2387.6 ± 264.5	2419.0 ± 403.6		2933.6 ± 190.9	2398.4 ± 300.9		2622.1 ± 133.0	2794.9 ± 195.8	
Progesterone (ng/ml)	0.8 ± 0.1	0.8 ± 0.1		0.8 ± 0.1	1.0 ± 0.3		0.9 ± 0.1	0.7 ± 0.1	*	0.8 ± 0.0	0.8 ± 0.1	
Clinic outcomes												
Fertilization methods												
IVF	35 (59.3%)	23 (74.2%)		25 (69.4%)	17 (89.5%)		50 (64.9%)	22 (61.1%)		110 (64.0%)	62 (72.1%)	
ICSI	24 (40.7%)	8 (25.8%)		11 (30.6%)	2 (10.5%)		27 (35.1%)	14 (38.9%)		62 (36.0%)	24 (27.9%)	
Oocyte number	10.7 ± 0.7	13.6 ± 1.0	*	8.3 ± 0.8	9.6 ± 1.4		12.7 ± 0.8	10.4 ± 1.1		10.8 ± 0.5	12.4 ± 0.7	
Fertilization	63.3 ± 3.4	73.6 ± 4.8		75.9 ± 3.7	66.4 ± 5.8		71.0 ± 2.4	70.6 ± 3.6		69.3 ± 1.8	70.1 ± 2.7	
Cleavage	69.2 ± 3.5	65.0 ± 4.9		91.5 ± 2.8	91.8 ± 2.9		89.8 ± 2.4	85.7 ± 3.6		83.7 ± 1.9	79.2 ± 2.8	
Number of good-quality embryos	2.7 ± 0.3	3.0 ± 0.5		2.2 ± 0.4	2.6 ± 0.6		3.5 ± 0.4	3.3 ± 0.5		2.8 ± 0.2	3.4 ± 0.3	
Cancellation	12 (20.3%)	10 (32.3%)		9 (25.0%)	8 (42.1%)		21 (27.3%)	16 (44.4%)	*	42 (24.4%)	34 (39.5%)	*
OHSS risk	7 (11.9%)	8 (25.8%)		3 (8.3%)	6 (31.6%)	*	15 (19.5%)	12 (33.3%)		25 (14.5%)	26 (30.2%)	*
No embryos formed	5 (8.5%)	2 (6.5%)		16.7 (6/36)	2 (10.5%)		6 (7.8%)	4 (11.1%)		17 (9.9%)	8 (9.3%)	
Implantation rate (%)	37.8 ± 6.1	36.9 ± 9.5		27.7 ± 7.3	13.6 ± 7.0		30.7 ± 4.7	19.1 ± 8.2		31.8 ± 3.4	31.0 ± 5.5	
Pregnancy	24 (40.7%)	13 (41.9%)		11 (30.6%)	3 (15.8%)		30 (39.0%)	5 (13.9%)	*	65 (37.8%)	21 (24.4%)	
Miscarriage	2 (3.4%)	1 (3.2%)		0 (0%)	0 (0%)		2 (2.6%)	0 (0%)		4 (2.3%)	1 (1.2%)	
Ectopic pregnancy	2 (3.4%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)		2 (1.2%)	0 (0%)	
Live-birth	20 (33.9%)	12 (38.7%)		11 (30.6%)	3 (15.8%)		28 (36.4%)	5 (13.9%)		59 (34.3%)	20 (23.3%)	

Values are Mean ± SEM or Number (Percentage).
Chi-square test was used for categorical variables.
Unpaired t-test was used to analyze Age and BMI before the rest data were analyzed by covariance analysis with Age and BMI adjustment.
P values are indicated (*<0.05, **<0.01, ***<0.001) or otherwise >0.05

Supplemental Table 2