Title

Expression of genes controlling steroid metabolism and action in granulosa-lutein cells of women with polycystic ovaries.

Authors

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Running Title

Steroidogenic genes in PCOS

Declaration of interests: None

Abstract

Introduction

Aberrant function of granulosa cells has been implicated in the pathophysiology of PCOS.

Materials & methods

GL cells were collected during oocyte retrieval for IVF/ICSI. RT-qPCR was used to compare gene expression between 12 control women, 12 with ovulatory PCO and 12 with anovulatory PCOS. To examine which genes are directly regulated by androgens, GL cells from an additional 12 control women were treated *in-vitro* with 10nM dihydrotestosterone (DHT).

Results

Women with PCOS showed reduced expression of CYP11A1 3-fold (p=0.005), HSD17B1 1.8-fold (p=0.02) and increased expression of SULT1E1 7-fold (p=0.0003). Similar results were seen in ovulatory women with PCO. GL cells treated with 10nM DHT showed a 4-fold (p=0.03) increase in expression of SULT1E1 and a 5-fold reduction in SRD5A1 (p=0.03).

Conclusions

These findings support the notion that aberrant regulation of steroid metabolism or action play a part in ovarian dysfunction in PCOS

Key words: PCOS, granulosa cells, steroidogenic enzymes.

1. Introduction

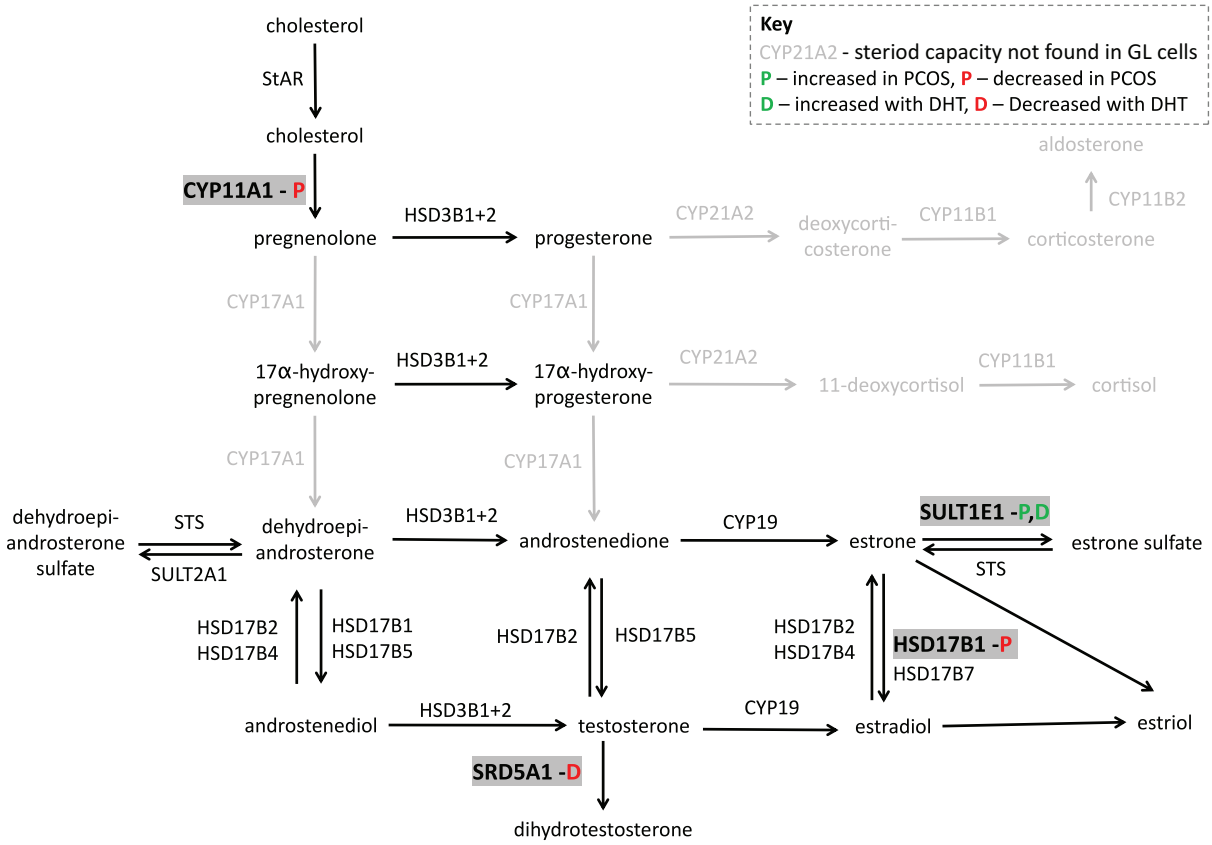
Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting 5-10% of women of reproductive age (Franks 1995). It is the principal cause of anovulatory infertility, menstrual disturbances and hirsutism and is associated with an adverse metabolic profile including obesity, insulin resistance and increased risk of type-2 diabetes mellitus (Barber et al. 2006; Franks 2006). The pathophysiology of PCOS is complex and multifactorial, involving ovarian dysfunction, insulin resistance and hyperandrogenism (Dumesic et al. 2015). Recent genome-wide association studies support the concept that PCOS has a strong genetic basis (Chen et al. 2011; Hayes et al. 2015; Day et al. 2015).

The biochemical hallmark of PCOS is the presence of increased ovarian androgen production. The importance of androgens in the aetiology of PCOS is supported by extensive animal studies showing that an increase in androgen exposure during foetal life in primate, sheep and rodent models induces reproductive, endocrine and metabolic features of PCOS in the offspring (Franks 2012; Walters, Allan, and Handelsman 2012; Tyndall et al. 2012; Abbott, Tarantal, and Dumesic 2009; Steckler et al. 2007). This suggests that many of the features of the syndrome are the result of androgen “programming”, including effects on ovarian steroidogenesis.

Granulosa cells provide an essential environment for healthy follicle development and oocyte maturation. They are the main source of ovarian estrogens and progesterone, produced under the influence of FSH and LH (Barnes et al. 2000). Abnormal function of granulosa cells has also been implicated in the pathophysiology of PCOS. Granulosa cells from women with PCOS show an increased production of estradiol in response to FSH and premature LH responsiveness and production of progesterone (Willis et al. 1998), (Mason et al. 1994;

74 Erickson et al. 1992). The action mechanism of this enhanced steroidogenesis has not been
75 elucidated.
76
77 More recently, there have been several studies that have shown that GL cells from women
78 with PCOS show differential gene expression profiles. In one study, cDNA microarray
79 technology was used to compare GL cell gene expression profiles in women with and without
80 PCOS. Comparative analysis revealed genes involved in the mitogen-activated protein
81 kinase/extracellular regulated kinase (MAPK/ERK) signalling pathways and suggests that
82 they might influence the function of granulosa cells in women with PCOS (Lan et al. 2015).
83 A second study showed an enhanced inflammatory transcriptome in GL cells of women with
84 PCOS with increased levels of genes encoding cytokines, chemokines, and immune cell
85 markers (Adams et al. 2016).
86
87 The aim of this study was firstly to investigate the differential expression of the network of
88 genes involved in steroid biosynthesis, metabolism and action (the 'steroidogenic network',
89 Figure 1) in human GL cells from three groups of women: those with regular cycles and
90 normal ovaries, women with regular cycles and polycystic ovaries (ovulatory PCO) and
91 women with irregular cycles and polycystic ovaries (anovulatory PCOS). Secondly, we
92 treated granulosa lutein cells from women with normal ovaries *in-vitro* with DHT to
93 investigate whether differences in gene expression between groups could be replicated by the
94 direct action of androgens. Lastly we looked at the impact of body mass index on steroid
95 gene expression. Only steroidogenic enzymes to be present and active in granulosa cells were
96 examined. For example, CYP17A1, the enzyme that is critical to androgen production in
97 theca cells, was not studied as androgens are not produced in granulosa cells.
98

Figure 1. Steroidogenesis and associated gene network



The steroid network highlighting the steroidogenic genes and their roles. A green "P" signifies increased gene expression and a red "P" decreased gene expression in women with PCOS. A green "D" signifies increased gene expression and a red "D" decreased gene expression in GL cells treated with 10nM of DHT. Grey shading indicates steroids and genes that are absent (or found in insignificant levels) in human GL cells.

2. Materials and Methods

2.1 Study approval

The study was performed in accordance with the Declaration of Helsinki. Written informed consent was also obtained from all subjects prior to collection of GL cells. The Hammersmith

and Queen Charlotte's Research Ethics Committee, London, UK, approved the study (Reference 10/H0707/2).

2.2 Study participants

The indications for IVF were tubal problems, male factor infertility or infertility of unknown cause. Also included were anovulatory women with PCOS who were offered IVF, either because there were other infertility factors or had proved unresponsive to induction of ovulation with clomiphene or in whom low dose FSH treatment was either ineffective or inappropriate. In addition, these women met the NHS criteria for assisted reproduction, i.e. had a body mass index <30 kg/m^2, were aged ≤40 years and were non-smokers. Polycystic ovaries (PCO) were defined sonographically on the basis of follicle number and/or increased ovarian volume (>10 mL) (Conway et al. 2014). PCOS was defined according to the Rotterdam diagnostic criteria (i.e. two or more of the following: oligo- or anovulation, hyperandrogenism and PCO (Conway et al. 2014) (Rotterdam 2004).

2.3Ovarian stimulation protocol

The women underwent IVF cycles under two distinct protocols; Long Day 21 (LD-21) or GnRH antagonist. In a LD-21 cycle gonadotropin-releasing hormone agonist (Buserelin 0.5 mg, Sanofi, Guildford UK) was used to suppress the pituitary production of gonadotropins for two weeks starting from Day 21 of the menstrual cycle. Ovarian suppression was confirmed with an ultrasound scan and stimulation was started using recombinant follicular stimulating hormone. FSH dose varies based on ovarian reserve and can range between 112.5 and 450 units. hCG 5000 units was administered once at least three follicles reached ≥18 mm in diameter; oocyte retrieval followed 36 hours later. The antagonist cycle was more commonly used in PCO cases to reduce the risk of OHSS (table 1). Ovarian stimulation was started on the second day of the menstrual cycle, a GnRH antagonist (Cetrorelix or Ganirelix 0.25 mg daily, Merck Serono, Feltham, UK) was used from Day 5 to prevent a premature LH surge. hCG (Ovitrelle and Pregnyl, Merck Serono) or a GnRHa (Buserelin 2 mg) was

administered when at least three follicles reached ≥18 mm in diameter, and transvaginal oocyte retrieval was carried out 36 hours thereafter.

2.4 GL cell collection and isolation

Follicular fluid was aspirated and pooled from follicles of individual women during the retrieval of oocytes for IVF/ICSI. GL cells were subjected to centrifugation at 1000 rpm for 5 min to separate the follicular fluid from cells, as previously described (Rice et al. 2005). The cell pellets were re-suspended in M199 (Thermo Fisher Scientific, Waltham, MA USA) and layered onto a Percoll gradient (GE healthcare, Chicago, USA) and then centrifuged at 1600 rpm for 30 min to separate red blood cells. GL cells at the interface were collected and washed with Dulbecco's phosphate buffered saline (DPBS, Thermo Fisher Scientific). The cells were then either frozen and stored at −80°C or RNA was extracted immediately.

2.5 RNA extraction, cDNA synthesis and Real-Time quantitative PCR (RT-qPCR)

To compare steroidogenic gene expression levels, RNA was extracted from GL cells from women with regular cycles and normal ovaries, women with regular cycles and polycystic ovaries (ovulatory PCO) and women with irregular cycles and polycystic ovaries (anovulatory PCOS) using the RNeasy® plus Mini Kit (Qiagen Inc., CA, USA) according to manufacturer's instructions. The RNA samples were stored at −80 °C until all samples were collected. The quantity, quality and integrity of samples were analysed using Agilent Technologies 2200 TapeStation (Agilent, CA, USA) and Nanodrop 1000 (NanoDrop Technologies, Wilmington, DE, USA). RNA was reverse transcribed into cDNA using Invitrogen superscript IV First Strand synthesis system according to manufacturer's instructions (Thermo Fisher, Massachusetts, USA).

RT-qPCR was carried out on 384-well plates using POWER SYBR Green (Applied Biosystems, CA, USA) according to the manufacturer's instructions on an Applied Biosystems 7900 HT instrument. Primer sequences are listed in supplementary table 1. Relative expression levels were determined using the delta-delta Ct method (Schmittgen and Livak 2008). The housekeeping genes GAPDH and β2-microglobulin were selected as internal reference genes, after we performed a geNorm primer analysis (Hellemans et al. 2007) showed that they were the most stable housekeepers. All samples were analysed in duplicate and dissociation curves were used to ensure that a single product was formed.

2.6 Androgen treatment *in-vitro*

GL cells isolated from pooled follicular fluid collected from patients with regular cycles, and without PCO, were cultured in 12-well plates in culture medium (supplemented DMEM/F12 Ham mixture with 10% FBS, Thermo Fisher Scientific). On day three, cells were then treated with either 10nM DHT (Sigma) or vehicle control (ethanol) in media without serum for 24 hours. This 3-day protocol has been shown to allow recovery of responsiveness to gonadotropins and steroidogenesis *in-vitro* and to minimise the impact of exogenous gonadotropins and maturation triggers, that are routinely given as part of an IVF treatment cycle (Owens et al. 2018). After this 24-hour treatment period, cells were lysed, collected for RNA extraction and RT-qPCR, as described above.

2.7 Statistical analysis

For analysis of RT-QPCR data either a Student's t-test or Mann Whitney test was used to compare results between different patient sets depending on whether or not data were normally distributed. A one-way ANOVA was used to compare patient demographics. The

relationship between gene expression and BMI was analysed using either a Pearson correlation or Spearman's rank–order correlation. A P-value of <0.05 was considered to indicate statistical significance. Statistical analysis was performed using GraphPad, Prism, version 7 (La Jolla, CA, USA).

3. Results

3.1 Patient demographics (table 1)

	Control women n= 24	Anovulatory PCOS n=12	Ovulatory PCO n=12	P-value (ANOVA)
Age	35 ± 4.3	30.4 ± 4	32.4 ± 4.6	<0.01
Mean BMI (kg/m^2)	22.9 ± 2.9	23.6 ± 2.8	26 ± 2.6	<0.05
Mean FSH dose	2998 ± 1401	1671 ± 748	1740 ± 136	<0.0001
Maturation trigger	hCG	7 GnRH agonist 4 hCG 1 Kisspeptin	8 hCG 3 GnRH agonist 1 kisspeptin	
IVF protocol	5 Antagonist 7 Long Day-21	10 Antagonist 2 Long Day 21	12 Antagonist	
Number of eggs collected	12 ± 5	17 ± 9	11 ± 8	<0.05

Samples were included from 24 control women, 12 with anovulatory PCOS and 12 with ovulatory PCO. PCO/PCOS women were younger and had a higher body mass index. Control women received a higher mean dose of FSH. All control women received hCG to trigger final oocyte maturation, whereas PCO/PCOS women received a combination of hCG, GnRH agonist and kisspeptin. The number of eggs collected was higher from women with anovulatory PCOS.

3.2 GAPDH/B2M are the most stable reference genes in GL cells (Supp. Figure 1)

To ensure the generation of accurate and robust RT-qPCR data from GL cells, we used the geNorm software package to identify the most stable reference genes when comparing women with and without PCOS (Vandesompele et al. 2002). The geNorm algorithm

212 determines the medium reference target stability measure (M-value), as the average pair-wise
213 variation of each reference gene in relation to all the other reference genes enabling the
214 elimination of the least stable gene. This is followed by recalculation of the M values
215 resulting in ranking of the most stable genes, i.e. the lower the M value, the higher the gene
216 stability. The software indicates that a good stable reference gene should have an average
217 geNorm M value ≤1.0 (Hellemans et al. 2007). GAPDH and B2M had the lowest M-values
218 and were the most stably expressed gene pair, while 18S and SDHA had the highest M-
219 values and were the least stable genes across all samples (supp. Figure 1A). We also
220 computed the optimal number of reference genes based on the pairwise variation value (V-
221 value). geNorm defines a pairwise variation of 0.15 as the cut-off value, below which the
222 inclusion of an additional reference gene for normalisation is not needed. As shown in supp.
223 Figure. 1B, the V2/3 value is far below the cut-off of 0.15, indicating that the top two ranked
224 reference genes GAPDH and B2M (with the lowest M-values) are suitable for gene
225 expression normalisation in GL cells from women with PCOS.
226
227 **3.3 Expression of steroidogenic network genes in GL cells from women with ovulatory**
228 **PCO and anovulatory PCOS (Figure 2)**
229 RT-qPCR was used to compare expression of genes involved in the steroidogenic network
230 between cells from women with regular cycles and normal ovaries and women with ovulatory
231 PCO and anovulatory PCOS (Figure 2). Of the 20 steroidogenic network genes (see Figure 1)
232 that were investigated in human GL cells, 17 showed no significant differences in expression
233 between cells from normal women and women with anovulatory PCOS. The three genes that
234 showed differential expression are CYP11A1, HSD17B1 and SULT1E1.
235

Steroidogenic acute regulatory protein (STAR) expression was not different between GL cells from the two groups. CYP11A1 (encoding the cytochrome P450 enzyme effecting cholesterol side-chain cleavage), was, however, reduced 3-fold (p=0.005) in women with anovulatory PCOS and HSD17B1 (encoding 17-Beta-Hydroxysteroid Dehydrogenase Type 1) was reduced 1.8-fold (p=0.02) in women with anovulatory PCOS. By contrast, expression of SULT1E1 (encoding sulfotransferase Family 1E Member 1) was increased 7-fold (p=0.0003) in women with anovulatory PCOS. Similar to women with anovulatory PCOS, women with ovulatory PCO showed a 2.7-fold ($p<0.05$) reduction in CYP11A1 gene expression (Figure 3). Likewise, SULT1E1 expression was increased 5-fold (p=0.005) in women with ovulatory PCO. HSD17B1 expression showed a similar trend to that observed in women with anovulatory PCOS, but, in this case, the reduction was not significant.

Figure 2.

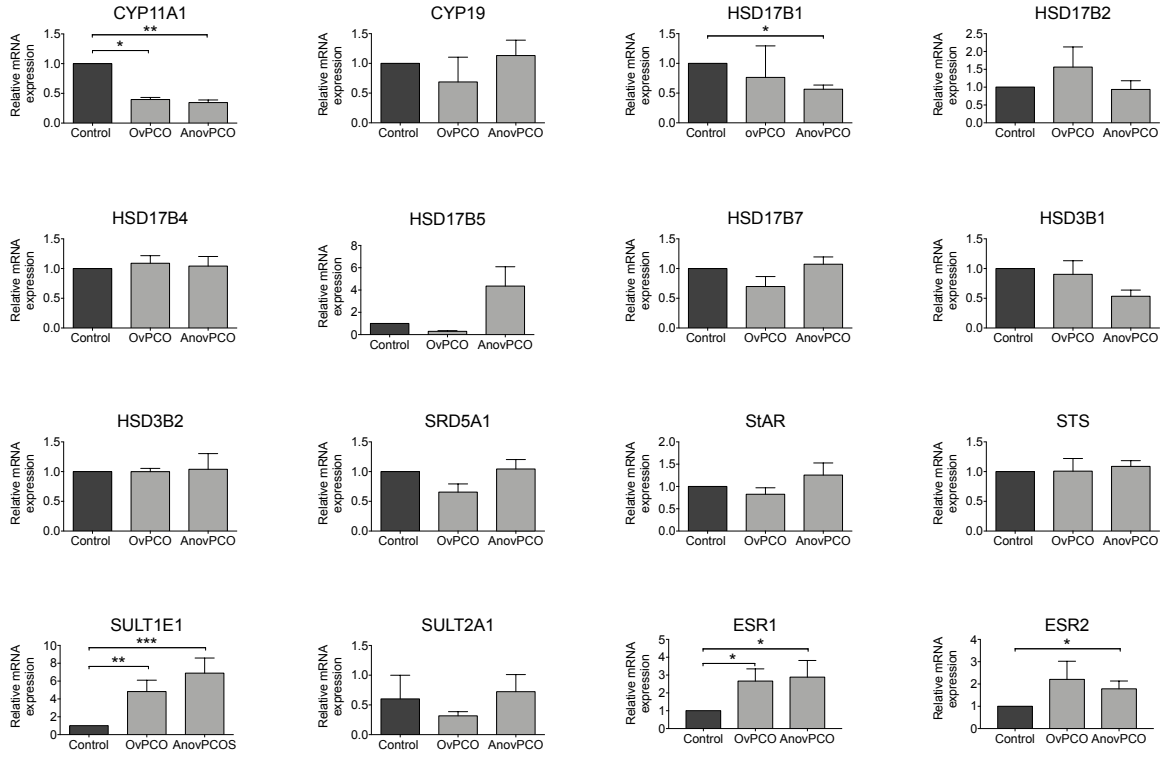


Figure 2. Differential expression of steroidogenic network genes in women with ovulatory and anovulatory PCOS

qRT-PCR results showing expression of steroidogenic genes in women with normal ovaries (control), women with polycystic ovaries and regular cycles (OvPCO) and women with polycystic ovaries and irregular cycles (AnovPCOS). CYP11A1 is reduced 3-fold in women with both ovulatory and anovulatory PCOS. HSD17B1 is reduced 1.8-fold in women with anovulatory PCOS. SULT1E1 is strongly increased 8-fold in women with anovulatory PCOS and is increased 5-fold in in women with ovulatory PCO. Both estrogen receptors are increased in women with PCOS. t-test, *P<0.05, **P<0.01, ***P<0.005. Data shown are mean +/- SEM, n=12 patients per group.

3.3 Expression of ESR1 and ESR2 is increased in GL cells from women with both ovulatory PCO and anovulatory PCOS (Figure 2)

Both SULT1E1 and HSD17B1 encode enzymes that are important in catalysing reactions that determine the bioavailability of estradiol. To investigate estrogen signalling further we examined expression of estrogen receptor genes ESR1 and ESR2 in GL cells from women with both anovulatory and ovulatory PCO. ESR1 expression was increased 2.6-fold (p=0.02) in women with ovulatory PCO and 2.9-fold (p=0.03) in women with anovulatory PCOS (Figure 4.A). ESR2 was increased 1.8-fold (p=0.02) in women with anovulatory PCOS (Figure 2) Expression of ESR2 in ovulatory PCO showed the same trend but this change was not significant.

3.4 Treatment of cultured GL cells with androgen *in-vitro* (Figure 3).

GL cells from normal women with regular cycles and normal ovaries treated with 10nM dihydrotestosterone (DHT) for 24 hours showed a 4-fold (p=0.03) increase in SULT1E1 and a 4-8 fold (p=0.017) reduction in SRD5A1. All other steroid genes and ESR1 and ESR2 were unaffected by direct androgen treatment under these conditions.

Figure 3.

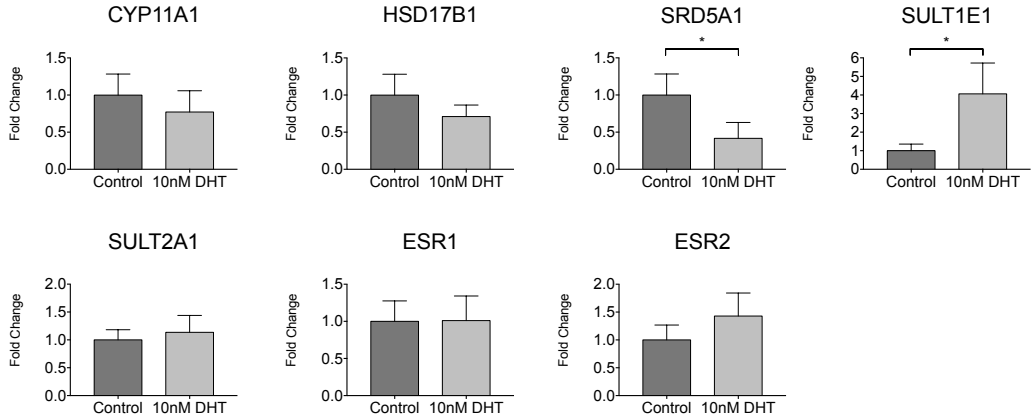


Figure 3. *in-vitro* treatment of GL cells with DHT

GL cells were treated with 10nM DHT for 24 hours (n=20). Direct androgen treatment increased SULT1E1 expression 4-fold and reduced expression of SRD5A1 by 2-fold. t-test, *P<0.05. Data shown are mean +/- SEM.

3.5 Effect of BMI, FSH does and IVF maturation trigger on gene expression

We examined the effect of BMI on steroid gene expression in women with ovulatory PCO and anovulatory PCOS. None of the differentially expressed genes correlated with BMI and the only gene to show a significant correlation with BMI was SULT2A1 ($r=0.54$, $p<0.005$) (Figure 4). The use of different dose regimens for FSH in women with and without polycystic ovaries is a possible confounding variable, as is the use of various maturation triggers (hCG, GnRHa and kisspeptin). We examined the relationship between dose of FSH used for superovulation and expression of the differentially expressed genes. There was no correlation between FSH dose and CYP11A1, SULT1E1, HSD17B1, ESR1, ESR2 expression (Supplementary Figure 2). Similarly, adjusting for the maturation trigger had no significant effect on gene expression (Supplementary Figure 3).

Figure 4.

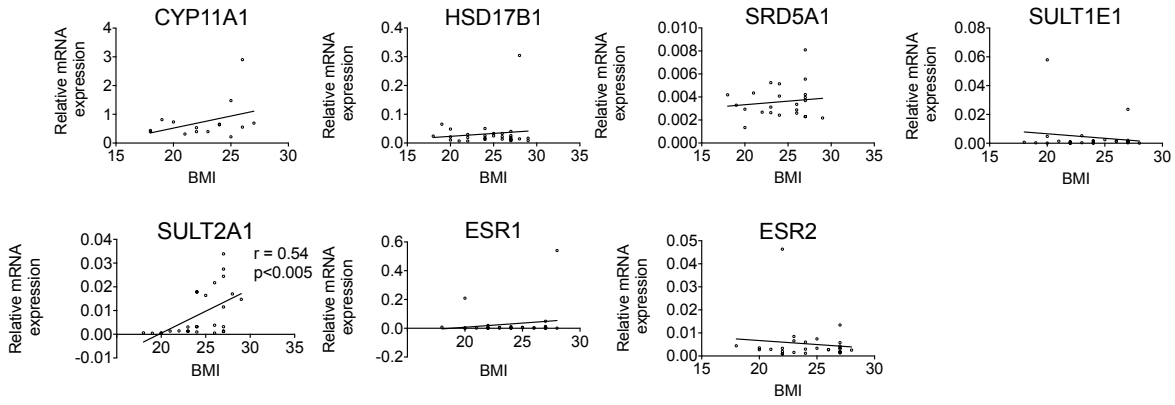


Figure 4. Effect of BMI steroidogenic gene expression.

BMI had a limited impact on steroid gene expression in women with ovulatory and anovulatory PCOS. SULT2A1 is positively correlated with increased BMI ($r=0.54$, $p<0.005$, $n=27$).

4. Discussion

In this study, we have used RT-qPCR data to determine the expression of genes encoding the steroidogenic network in GL cells from women with normal ovaries and regular cycles, women with ovulatory PCO and women with anovulatory PCOS. We also looked at the effect of *in-vitro* DHT treatment on expression of steroidogenic genes in cells from women without PCO/PCOS, to assess whether the findings PCOS GL cells could be attributed to a direct effect of androgen. Our findings show that while most genes are unchanged by the woman having PCO/PCOS or by the presence of androgen *in-vitro* in GL cells, there was differential regulation of CYP11A1, SULT1E1 and HSD17B1 in women with PCOS, and SULTE1 and SRD5A1 after DHT treatment *in-vitro*. These genes encode enzymes which are critical to ovarian steroidogenesis. Although the women with PCO had a slightly higher body

mass index (BMI), SULT2A1 was the only gene to show significant correlation of gene expression with BMI.

CYP11A1 was reduced 3-fold in GL cells from women with both ovulatory PCO and anovulatory PCOS. CYP11A1 is a Family 11 Subfamily A Member 1 that catalyses the conversion of cholesterol to pregnenolone, the first step in the synthesis of the steroid hormones These results are consistent with those of Adams and colleagues (Adams et al. 2016) who also showed that CYP11A1 is reduced in GL cells from women with anovulatory PCOS but this is the first time it has been shown to be consistently reduced in ovulatory women with PCO. This suggests that the reduction in CYP11A1 is associated with the presence of polycystic ovaries, irrespective of cycle regularity. CYP11A1 is a rate-limiting step in the steroidogenic pathway and therefore an intrinsic reduction in women with PCO could have a significant impact on the ability of granulosa cells to produce progesterone. An alternative explanation is that the reduction in gene expression represents a compensatory mechanism (at the transcriptional level) to reduce increased CYP11A1 protein or enzyme activity and help modulate the bioavailability of progesterone. Nevertheless, in animal experiments, excess androgens have also been shown to have an inhibitory action on CYP11A1 expression. In mouse follicles treated *in-vitro* with testosterone or DHT there is a reduction in Cyp11a1 protein (Laird et al. 2017). Furthermore, CYP11A1 gene expression is reduced in ovaries of day 90 fetal sheep whose mothers treated in-utero with testosterone (Hogg, McNeilly, and Duncan 2011). The importance of CYP11A1 has also been shown in studies that associate polymorphisms in CYP11A1 with increased risk of PCOS (Reddy et al. 2014; Zhang et al. 2012; Pusalkar et al. 2009).

We describe a 7-fold increase in SULT1E1 expression in GL cells from women with both ovulatory or anovulatory PCOS. SULT1E1 is an estrogen sulfotransferase that catalyses the transfer of a sulfate group to estrone and estradiol thereby reducing its biological activity (Adjei et al. 2003). Sult1e1 has been shown to play a role in female fertility through ERK1/2 signalling in mice granulosa cells (Fan et al. 2009). Sult1e1 null female mice showed impaired ovulation due to an excess of estrogen and a low ovulatory response to hCG treatment (Gershon et al. 2007). A plausible effect of increased SULT1E1 in human granulosa lutein cells would be an increase in sulfoconjugation, a subsequent increase in the inactivation of estradiol and, as a result, a reduction of bioavailable estradiol (Figure 5). Potentially this represents a compensatory mechanism to attenuate the effect of higher production of estradiol in granulosa cells in women with PCOS (Erickson et al. 1992; Mason et al. 1994). Furthermore, the significant reduction of HSD17B1 in GL cells from women with anovulatory PCOS lends further support to a mechanism of estradiol inactivation. HSD17B1 is a key oxido-reductase enzyme in granulosa cells and catalyses the conversion of estrone into the more potent estradiol (Ghersevich, Nokelainen, et al. 1994; Ghersevich, Poutanen, et al. 1994).

As SULT1E1 and HSD17B1 both play a role in estrogen metabolism and biosynthesis we investigated additional components of the estrogen signalling pathway. Estradiol mediates its actions by binding to classical nuclear receptors, estrogen receptor α (ESR1) and estrogen receptor β (ESR2). We showed that expression of both ESR1 and ESR2 were increased in GL cells from women with anovulatory PCOS. Estradiol can also signal in a non-genomic fashion via a third estrogen receptor known as the G protein-coupled estrogen receptor 1 (GPER) (Pavlik et al. 2011). Interestingly, GPER has been shown to be increased in cumulus granulosa cells of women with PCOS (Zang et al. 2016). The functional role of these gene

expression increases is unclear but it gives further support to aberrant regulation of estrogen metabolism and signaling in granulosa cells of women with PCOS.

The potential importance of increased androgen action in development of PCOS is supported by a range of studies in experimental animals (Franks 2012; Walters, Allan, and Handelsman 2012; Tyndall et al. 2012; Abbott, Tarantal, and Dumesic 2009; Steckler et al. 2007; Laird et al. 2017; Nelson et al. 1999). We therefore asked whether the changes that we observed in gene expression in PCO GL cells could be replicated *in-vitro* by the direct action of DHT in cells from women without PCO. Direct treatment with a non-aromatizable androgen caused an upregulation of SULT1E1. Several animal studies support the idea that androgens directly upregulate SULT1E1 expression and action. Snyder et al., showed that in *in-vivo* androgen treated mice there was increased SULT1E1 expression in the caput epididymides (Snyder et al. 2009). In another study, Qian and Song treated mouse Leydig cells with DHT and showed that SULT1E1 gene expression was increased and that there was increased was blocked with co-treatment of flutamide (Qian and Song 1999). In addition, they showed a DHT dose-dependent increase in SULT1E1 action and conversion from estradiol to estradiol sulphate. Furthermore, using a testicular feminisation mouse model that contains a mutated and non-functioning AR resulted in a loss of SULT1E1 expression in the testes (Qian and Song 1999). In contrast to SULT1E1 other genes such as CYP11A1, HSD17B1, ESR1 and ESR2 that are differentially expressed in women with PCOS are unaffected by direct androgen treatment suggesting an inherent difference in these cells rather than a direct androgen effect."

DHT treatment also resulted in an almost 5-fold reduction in expression of SRD5A1 but there was no difference in expression between GL cells from PCOS and controls. SRD5A1 is one of three 5α reductase enzymes involved in reducing testosterone to dihydrotestosterone and progesterone to dihydroprogesterone (Andersson et al. 1991). In prostate cancer cells line DHT treatment *in-vitro* has been shown to upregulate or have no effect on SRD5A1 expression (Li et al. 2011).

We examined the impact of BMI on steroid gene expression in women with and without PCOS and found that none of the differentially expressed genes correlated with BMI. This is important for two reasons; firstly, raised BMI is associated with reduced fertility in women, the cause of which is incompletely understood. This infertility may be in part related to the inhibitory effect of leptin on steroidogenesis (Ghizzoni et al. 2001; Zachow and Magoffin 1997). Secondly the women in the PCO/PCOS group were marginally heavier, so this was a potential confounding variable for our results. Given that publicly funded IVF is only available for those with a BMI<30 however, we were only able to look at the impact on BMI in non-obese women. BMI did correlate strongly with SULT2A1, which encodes the enzyme responsible for converting DHEA to DHEAS (Bonser et al. 2000). It has been previously shown that human granulosa cells are able to utilise DHEAS as a precursor for estradiol and androstenedione synthesis. It has also been shown that DHEAS concentrations are positively associated with obesity in pre-menopausal women (Mazza et al. 1999). Ovarian SULT2A1 action in ovaries may be a novel mechanism contributing to this phenomenon.

One of the strengths of this study was the ability to compare gene expression between three distinct groups of women; women with normal ovaries and regular cycles, women with polycystic ovaries and regular cycles and women with polycystic ovaries and irregular cycles. This allowed us to better understand whether oligo- or anovulation affects the PCOS phenotype in terms of steroidogenic gene expression. A further strength is examination of potential direct effects of androgens on gene expression. A limitation of this and all studies involving granulosa lutein cells is that women with PCOS are given different doses of recombinant FSH during ovarian stimulation, which could plausibly affect gene expression results. In addition, women with PCOS are often giving a GnRH agonist to stimulate oocyte maturation (or Kisspeptin was given as part of a clinical trial) rather than hCG and this

likewise could affect gene expression in GL cells. However, we found no significant correlation between dose of FSH and expression of any of the genes explored in this study, including those that were differentially expressed. Likewise, the maturation trigger had little effect on differential gene expression. We used granulosa cells from pooled follicles from each patient rather than individual follicles. It was beyond the scope of this study to measure protein expression or enzyme activity of steroidogenic enzymes and receptors. Although steroid gene expression does not necessarily give an indication of steroid enzyme activity, these data still provide valuable and novel insight into the role of differential regulation of steroid metabolism and action in the pathogenesis in PCOS.

This descriptive study is the first to compare a comprehensive network of genes implicated in steroid synthesis, metabolism and action in women with ovulatory and anovulatory PCOS and to look at the effect of body mass index and the direct effect of androgen treatment *in-vitro*. We have shown that genes involved in steroid metabolism and action; CYP11A1, SULT1E1, HSD17B1, ESR1 and ESR2 are differentially regulated in GL cells from women with ovulatory PCO or anovulatory PCOS. The net effect of these changes point towards reduction of the bioavailability of estradiol and progesterone, which may be compensatory. Finally, we have demonstrated the increase in SULT1E1 expression may be explained, at least in part, by the direct action of androgen on GL cells.

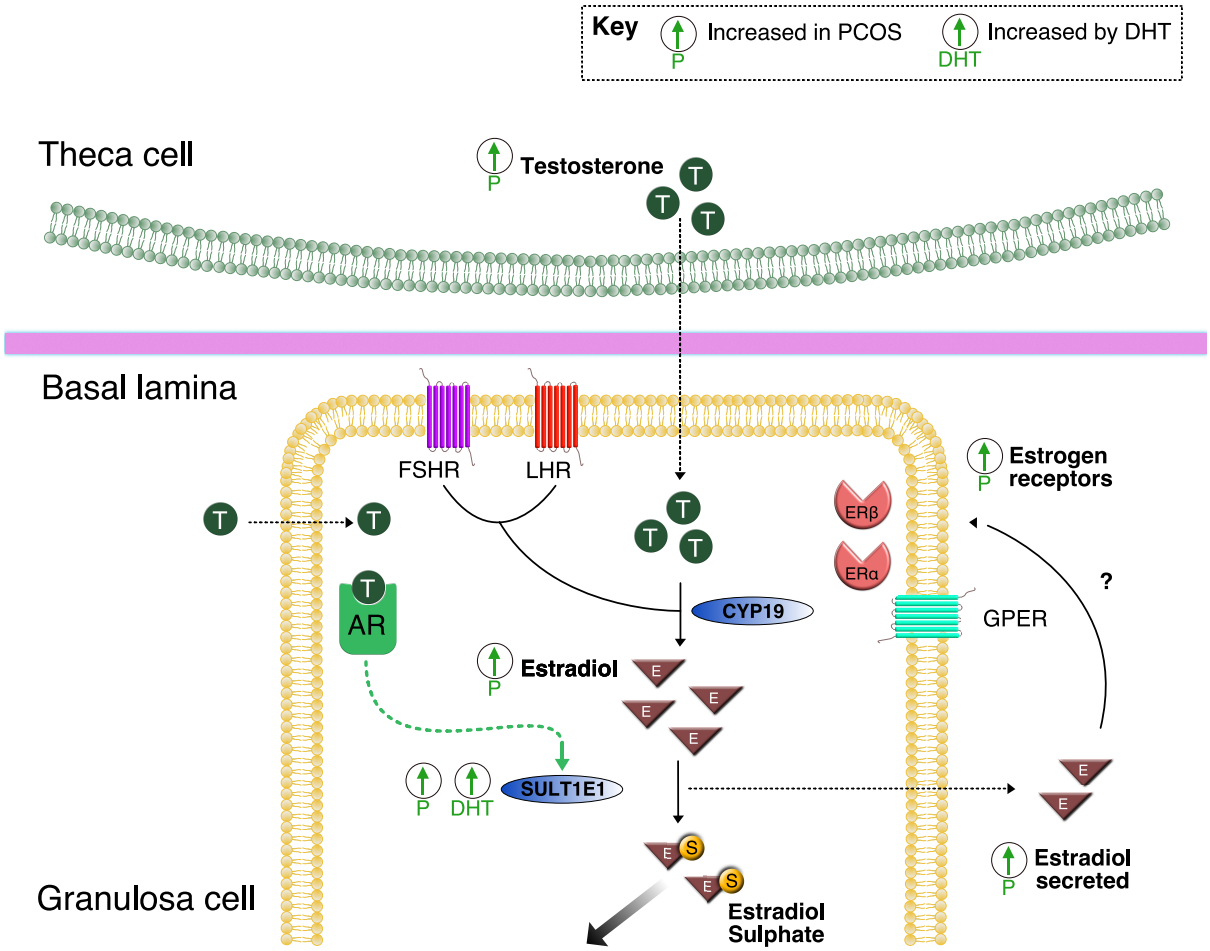


Figure 5. Proposed model of estradiol bioavailability in GL cells in PCOS

Women with PCOS have (1) increased basal SULT1E1 expression and (2) increased SULT1E1 expression from direct androgen action due to increased testosterone in women with PCOS (3) increased SULT1E1 leads to increased conversion of estrogen to the inactive estrogen sulphate and thus reduces the bioavailability of estrogen in the granulosa cells (4) this helps counteract increased basal estrogen production and increased levels of testosterone from adjacent theca cells that can be converted to additional estrogen.

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Author contributions

A Lerner is joint first author. He was involved in concept, data acquisition, analysis, article drafting and final approval.

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M Coates, C Simpson, J Velupillai, G Poole were involved in lab experiments, data interpretation, analysis, article drafting and revision.

M Liyanage, G Christopoulos and S Lavery were involved in study design, patient consent, sample collection, manuscript revision and approval.

K Hardy was involved in study design, data analysis/interpretation, drafting and revising the manuscript.

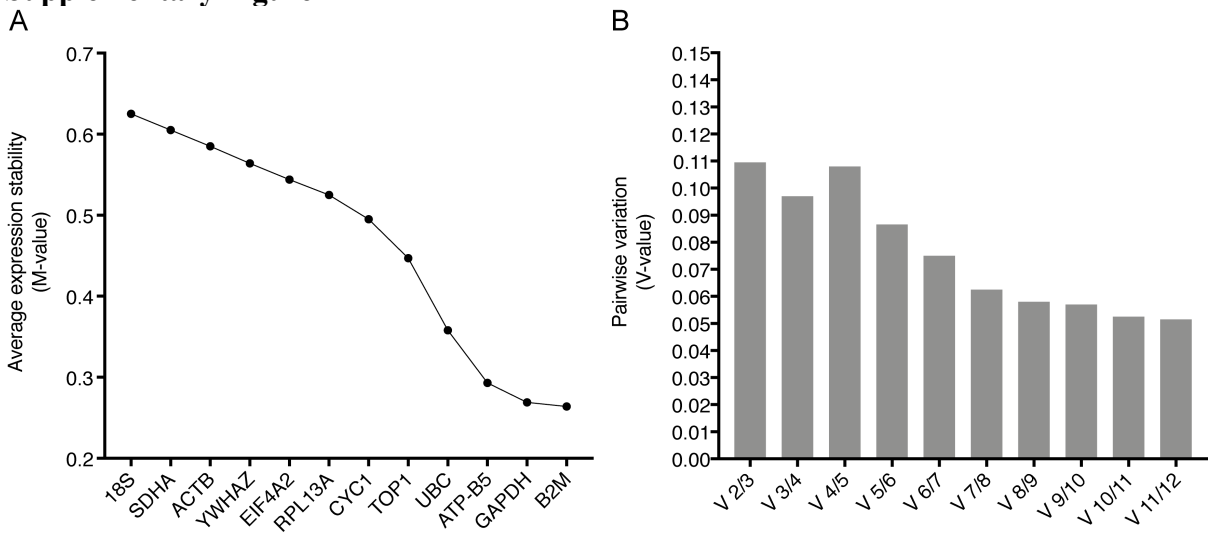
S Franks is the senior author and was involved in study conception, data analysis, manuscript writing and revision.

Supplementary table 1 Primer sequences

Gene name	Gene ID	Prime sequence	Product length (bp)
ACTB	60	F- GACGACATGGAGAAAATCTG R- ATGATCTGGGTCATCTTCTC	131
B2M	567	F- AAGGACTGGTCTTTCTATCTC R- GATCCCACTTAACTATCTTG	118
CYP11A1	1583	F- AGCATCAAGGAGACACTAAG R-GCAGGAATCATGTAATCTCG	95
CYP11B1	1584	F- ATCTTCCACTACACCATAGAAG R- GTGGATTTGAACATGACCTC	128
CYP11B2	1585	F- AGACACTAACTCAAGAGGAC R- AGTTAATCGCTCTGAAAGTG	90
CYP17A1	1586	F- CAAATGGCAACTCTAGACATC R- ATGATCTTCTCCAGCTTCTG	146
CYP19	1588	F- FGGTGAGAGAGACATAAAGATTG R- TTCAGGATAATGTTTGTCCC	170
ESR1	2099	F- GGAGTGTACACATTTCTGTC R- CAAAGTGTCTGTGATCTTGTC	90
ESR2	2100	F- GTATGCAGAACCTCAAAACAG R- CCTTTTCAATGTCTCCCTG	85
HSD17B1	3292	F- GACGTAAGGGACTCAAAATC R- ACAGTCCCTACTACATTCAC	164
HSD17B2	3294	F- GGCTTCCTAACAAATATCGC R- GTAGTCTTCCTGTAACCTCAG	97
HSD17B4	3295	F- CTCAGAAGGCATTCTTTAGTG R- TAATAGTGTAGTGTGCCCTTC	117
HSD17B5	8644	F- AGGTTTTTGAGTTCCAGTTG R- GGCTAGCAAAACTATCACTG	95
HSD17B7	51487	F- AATGCATTCACTTTGACACC R- TCTTCATCTAGGTCCATCTTC	158
HSD3B1	3283	F- CTCTTCTGTCCAGCTTTTAAC R- CATCCAAAGTAGCAGGAATC	116
HSD3B2	3284	F- AAGTGTTTCCTGCTACTTTC R- CTGGAGCTTAGAAAATTCCTC	181
SRD5A1	6715	F- CTGGCCCAACTGCAT R- AAGCTCCGTTGCGCA	95
SRD5A2	6716	F- CTTTTCACCACCATAGGTTC R- AGCTTGACAGTTTTCATCAG	140
STAR	6770	F- GACAAATGTATGAGTAAAGTGG R- CAGGCTCGTGAGTAATGAATG	196
STS	412	F- AAGGAGGAAAAGCAAACAAC R- TAGTGGGCTCATCAATCTTC	102
SULT1E1	6783	F- AAGGGAATTACAGGAGACT R- TAGATTCCTTCATTTGCTGC	91
SULT2A1	6822	F- GAGAAGATCTGTCAATTCCTG R- CCTTTTCTCAGAAGTTGTGC	161

478

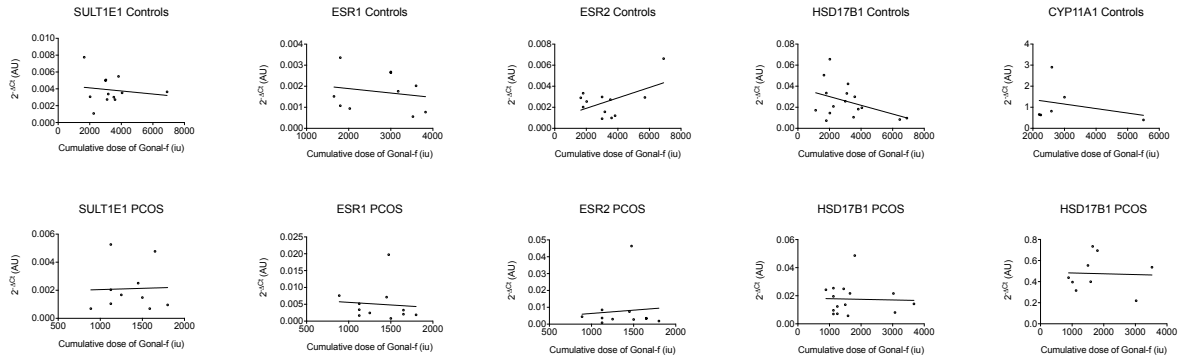
Supplementary Figure 1



Supplementary Figure 1: Reference gene selection and steroidogenic gene relative abundance

Gene expression stability analysis by GeNorm. (A) The mean expression stability value (M) of the 12 candidate reference genes. (B) Determination of the optimal number of reference genes for normalization using pairwise variation analysis by GeNorm.

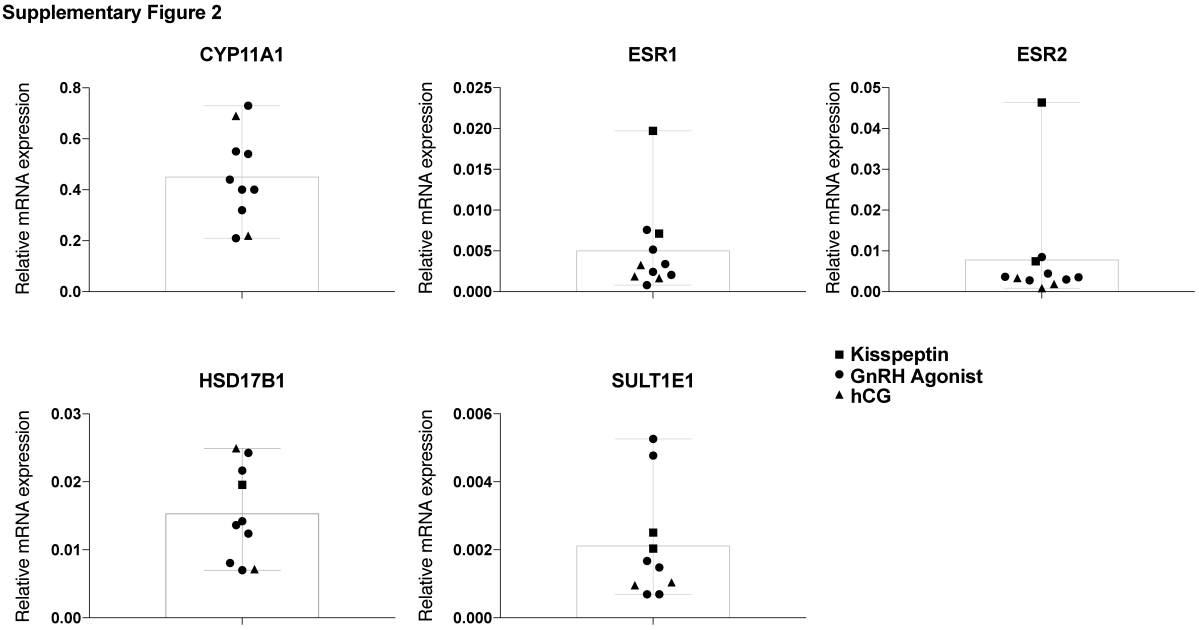
Supplementary Figure 2



Supplementary Figure 2: Effect of FSH dose on gene expression

FSH dose had no significant on any of the differentiated genes. The relationship between gene expression and Godal-F was analysed using either a Pearson correlation or Spearman's rank–order correlation

Supplementary Figure 3



Supplementary Figure 3: Supplementary Figure 3: Gene expression identified by maturation trigger

Individual gene expression data points identified by maturation trigger. Square = Kisspeptin, Circle = GnRH agonist, Triangle = HCG

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