Dysregulated genes and their functional pathways in luteinized granulosa cells from PCOS patients after cabergoline treatment

H Ferrero^{1,2,*}, P Díaz-Gimeno^{1,2,*}, P Sebastián-León^{1,2}, A Faus¹, R Gómez² and A Pellicer^{1,3}

¹Fundación IVI, Instituto Universitario IVI, Universidad de Valencia, Valencia, Spain, ²Instituto de Investigación Sanitaria INCLIVA, Valencia, Spain and ³Instituto de Investigación Sanitaria Hospital Universitario y Politécnico La Fe, Valencia, Spain

Correspondence should be addressed to R Gómez; Email: raulgomgal@gmail.com

*(H Ferrero and P Díaz-Gimeno contributed equally to this work)

Abstract

Polycystic ovarian syndrome (PCOS) is a common reproductive disorder frequently associated with a substantial risk factor for ovarian hyperstimulation syndrome (OHSS). Dopamine receptor 2 (D2) agonists, like cabergoline (Cb2), have been used to reduce the OHSS risk. However, lutein granulosa cells (LGCs) from PCOS patients treated with Cb2 still show a deregulated dopaminergic tone (decreased D2 expression and low dopamine production) and increased vascularization compared to non-PCOS LGCs. Therefore, to understand the PCOS ovarian physiology, it is important to explore the mechanisms that underlie syndrome based on the therapeutic effects of Cb2. Here, LGCs from non-PCOS and PCOS patients were cultured with hCG in the absence/presence of Cb2 ($n=12$). Subsequently, a transcriptomic-paired design that compared untreated vs treated LGCs within each patient was performed. After transcriptomic analysis, functions and genes were prioritized by systems biology approaches and validated by RT-qPCR. We identified that similar functions were altered in both PCOS and non-PCOS LGCs treated with Cb2; however, PCOS-treated LGCs exhibited more significant changes than non-PCOS. Among the prioritized functions, dopaminergic synapse, vascular endothelial growth factor (VEGF) signaling, apoptosis and ovarian steroidogenesis were highlighted. Finally, network modeling showed *CASP9*, *VEGFA*, *AKT1*, *CREB*, *AIF*, *MAOA*, *MAPK14* and *BMAL1* as key genes implicated in these pathways in Cb2 response, which might be potential biomarkers for further studies in PCOS.

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Introduction

Polycystic ovarian syndrome (PCOS) is a common reproductive disorder frequently associated with various metabolic abnormalities and substantially leading to the increased risk for ovarian hyperstimulation syndrome (OHSS) after administration of exogenous gonadotropins. According to the Rotterdam criteria established in 2004 (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004), women with PCOS are diagnosed by the presence of at least two of the three following criteria: clinical and biochemical signs of hyperandrogenism, anovulation (oligo- or amenorrhea) and/or polycystic ovaries. In general, PCOS is associated with infertility: a lower chance of conceiving and higher miscarriage rates (Pasquali & Gambineri 2006, Goverde *et al.* 2009). For this reason, women with PCOS often have to undergo assisted reproduction techniques, which involve conventional treatments to induce ovulation by exogenous gonadotropins and *in vitro* maturation (Norman & Clark 1998). However, these treatments may lead to a higher risk of OHSS (Van Wely *et al.* 2003) in these women: 15% of women with PCOS undergoing

ovulation treatment suffer from severe OHSS, compared to 3% of control women (Swanton *et al.* 2010). In this regard, dopamine receptor 2 (D2) agonists (D2-ags) have been successfully used in women with PCOS undergoing ovulation treatment to reduce the risk of OHSS (Papaleo *et al.* 2001, Guvendag Guven *et al.* 2013).

Granulosa cells (GCs) are important in ovarian folliculogenesis because they provide a suitable microenvironment for follicular development and oocyte maturation (Lan *et al.* 2015). Therefore, a recent study performed by our group assessed lutein GCs (LGCs) from women without and with PCOS treated with a D2-ag, cabergoline (Cb2) (the D2-ag most used in clinic treatment), to examine the mechanism of D2 inhibition. We demonstrated that PCOS LGCs present a deregulated dopaminergic tone (decreased D2 expression and low dopamine production) and increased vascularization compared to non-PCOS LGCs (Gómez *et al.* 2011). PCOS LGCs are known to exhibit alterations in vascular endothelial growth factor (VEGF) signaling (Agrawal *et al.* 2002), ovarian steroidogenesis (Eisner *et al.* 2002) and apoptosis (Karuputhula *et al.* 2013) pathways,

which could promote increased OHSS risk. Therefore, to understand the PCOS ovarian physiology, it is important to explore the mechanisms that underlie syndrome based on the therapeutic effects of Cb2.

Microarrays are useful tools to measure the expression of thousands of genes (the transcriptome) within a particular mRNA sample. These expression profiles can be used to understand the transcriptomics of cell behavior in diseases and identify diagnostic biomarkers and personalized responses to reproductive therapies. For these reasons, several studies have focused on describing the GC transcriptome in reproductive diseases. Microarray studies have observed differentially expressed genes in GCs at different stages of folliculogenesis (Douville & Sirard 2014, Golini *et al.* 2014), in relation to oocyte quality (Ouandaogo *et al.* 2011, Al-Edani *et al.* 2014) and in association with PCOS (Lan *et al.* 2015). Despite the relevance of Cb2 in the prevention of OHSS risk, to date, no complete study on GCs from women with PCOS treated with Cb2 has been reported to elucidate the molecular mechanism underlying Cb2's effect. Hence, the main goal of this study was to assess the transcriptomic profiles of LGCs from women with and without PCOS using Affymetrix microarray chips to provide novel information about the molecular changes that occur in these cells when they are treated with a D2-ag (Cb2). We also assessed the signal transduction pathways regulated by this treatment using systems biology as a novel approach in ovarian transcriptomics to better understand such a complex system.

Materials and methods

Sample collection

Follicular fluid-derived LGCs were isolated from women without PCOS ($n=3$) as a control group, and women with PCOS ($n=3$) as determined by achieving 2/3 of the Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004). LGCs were obtained at the time of oocyte retrieval from women aged 25 to 32 years, who had normal response profiles to controlled ovarian hyperstimulation (COH) protocols (oocytes retrieved, 7–20; E2 <189 pg/mL in follicular phase; body mass index (BMI) <30)

(Table 1). Women gave written informed consent, and the study protocol was approved by the ethics committee at CEIC-IVI Valencia (1503-VLC-014-AP).

LGC isolation and culture

LGCs were isolated using our previously described filter method (Ferrero *et al.* 2012). Isolated LGCs were seeded in 24-well cell culture plates at a density of 50,000 cells/well and incubated overnight (37°C, 5% CO₂) to enable removal of non-adherent cells. Subsequently, LGCs from each patient were washed and cultured in M-199 with 10% fetal bovine serum (Labclinics, Barcelona, Spain) and 5 IU/mL hCG (Profasi: Serono Laboratories, Madrid, Spain), in the absence or presence of the D2-ag (cabergoline; Cb2) at 100 µM (Dostinex: Pharmacia & Upjohn, North Peapack, NJ, USA) for 72 h (37°C, 5% CO₂). Cabergoline doses and administration days and doses of hCG were established in our previous study, demonstrating that LGCs are sensitive to hCG in the first culture days (Ferrero *et al.* 2014b).

Thus, this study included four different analysis groups: Cu: control untreated (non-PCOS, untreated ($n=3$)), Ct: control treated (non-PCOS, treated ($n=3$)), Pu: PCOS untreated ($n=3$) and Pt: PCOS treated ($n=3$).

LGC collection, RNA extraction and microarray hybridization

After treatment, total mRNA from the cells was extracted using the Quick-RNA Microprep kit (Zymo Research, Freiburg, Germany), according to the manufacturer's instructions. Quality and concentration of the mRNA samples were determined using the RNA Nano chip (Agilent Technologies) and analyzed by the Agilent 2100 Bioanalyzer (Agilent Technologies). mRNA extracts were stored at –80°C until further use for array analysis. Microarray experiments were conducted according to the manufacturer's instructions (Central Research Unit-INCLIVA; University of Valencia, Spain). RNA was hybridized to Human 1.0 ST GeneChip Arrays (Affymetrix). The array includes >750,000 unique 25-mer oligonucleotide transcripts, thus constituting >28,000 well-annotated genes. Analyses were performed by using one patient sample per GeneChip. Files were captured using an Affymetrix GeneChip Scanner 3000 7G. To verify if microarray results were reliable and sample quality was good, RNA samples from four of the 12 conditions were randomly selected to be replicated as a technical control.

Table 1 Characteristics of individual non-PCOS and PCOS patients.

	Age	BMI	Oocytes	Follicular Phase			PRL 1.39–24.2 (ng/mL)	Menstrual cycle	Ovaries
				E2: 39–189 (pg/mL)	LH: 1–18 (IU/mL)				
Non-PCOS 1	25	22.76	7	29	6.25		5.5	Eumenorrhea	Normal
Non-PCOS 2	30	22.58	17	45.65	11.6		10.26	Eumenorrhea	Normal
Non-PCOS 3	27	20.78	15	80.21	1.6		3.5	Eumenorrhea	Normal
PCOS 1	23	19.48	20	39	80.89*		51.02*	Amenorrhea*	Polycystic ovaries*
PCOS 2	32	24.12	5	49.8	1.7		16.85	Oligomenorrhea*	Polycystic ovaries*
PCOS 3	29	23.57	9	36	6.96*		11.19	Amenorrhea*	Polycystic ovaries*

*Meets with Rotterdam criteria.

BMI, body mass index; E2, estradiol; LH, luteinizing hormone; PRL, prolactin.

Microarray preprocessing and normalization

Analyses were performed using the R programming language, version 3.2.0 (R Core Team 2013). Affymetrix raw data (CEL files) was preprocessed by background correction, logarithmic transformation and normalization. Background correction was assessed using the robust multi-array average (RMA) method (Irizarry *et al.* 2003) and microarray intensity normalization was performed using the quantile method (Bolstad *et al.* 2003). Box plot diagrams were analyzed for detecting outliers. Microarray data are available at Gene Expression Omnibus (GSE98595).

Exploratory and differential expression analysis

Principal component analysis (PCA) was used as an exploratory method to detect sample behavior and the effects of other uncontrolled variables, using the *Prcomp* function from R, version 3.2. Differential gene expression assessment of all comparisons was carried out using Limma moderated *t*-statistics (Ritchie *et al.* 2015). Standard microarray analysis techniques for comparisons were carried out using one test for each gene (or probe-set) in the microarray. To minimize false positives in the study, raw *P* values were corrected for multiple testing to derive adjusted *P* values (false discovery rate (FDR)) (Benjamini & Hochberg 1995).

Functional profiling

Gene Set Enrichment Analysis (GSEA) was carried out for each of the comparisons explored in the study. To discover functional blocks enriched in the conditions, we used logistic regression models (Sartor *et al.* 2009, Montaner & Dopazo 2010). The conventional multiple testing *P* value correction procedure proposed by Benjamini and Hochberg (1995) was used to derive adjusted *P* values. Functional blocks from the Gene Ontology Biological Process (Ashburner *et al.* 2000) (GO BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa *et al.* 2012) were used in this study. Ovarian-specific databases were consulted to contextualize our results (Hsueh & Rauch 2012, Mary *et al.* 2016). To analyze the main regulator genes between the prioritized functions, a network analysis was implemented using Cytoscape (Cline *et al.* 2007). Network modeling allowed us to identify main genes related to their position in the network, based on network medicine principles, in which genes that connect various functions are highlighted as key regulators (Barabási *et al.* 2011).

Quantitative fluorescent real-time PCR

To corroborate the gene expression obtained in the array analysis, 300 ng of mRNA from the same samples used in the microarray (Cu and Ct; Pu and Pt) were reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio) and quantified by Qubit. cDNA expression levels of the genes caspase 9 (*CASP9*), *VEGFA*, RAC- α serine/threonine protein kinase (*AKT1*), apoptosis-inducing factor (*AIF*), monoamine oxidase A (*MAOA*), mitogen-activated protein kinase 14 (*MAPK14*) and brain and muscle Arnt-like protein-1 (*BMAL1*)

were estimated by quantitative real-time PCR (RT-qPCR) using the Power-Up SYBR Green master mix (Applied Biosystems). Expression levels were normalized to the house keeping gene β -actin (*ACTB*) quantified by the $\Delta\Delta C_t$ method and expressed as the fold change in each group.

Statistical analysis

Statistical analysis comparing both RNA expression methodologies was performed using R statistical computing (R Core Team 2013) and data were expressed as fold changes. To compare treated and untreated groups in each condition (non-PCOS and PCOS), a paired *t*-test comparison was used to analyze the expression of *CASP9*, *VEGFA*, *AKT1*, *MAOA* and *BMAL1*, which were normally distributed. The non-parametric paired-samples Wilcoxon test was used to analyze the expression of *AIF* and *MAPK14* that were not normally distributed.

Results

Transcriptomic analysis

Full human transcriptome analysis revealed a clustering effect in the LGCs treated with or without Cb2. Equally, the transcriptomes in LGCs from non-PCOS and PCOS were grouped depending on the presence/absence of the syndrome, showing a gene expression response (Fig. 1A) and indicating that there were baseline differences between groups.

Subsequently, microarray data revealed 137 genes differentially expressed between Ct vs Cu and 290 genes between Pt vs Pu (Fig. 1B) (FDR < 0.05), demonstrating that Cb2 influenced the expression of more genes in PCOS than non-PCOS (control) samples. Notably, only 10.07% (43 genes) of the differentially expressed genes were shared in both treated vs untreated comparisons (Fig. 1C), showing the different effect of Cb2 in PCOs. In addition, analysis in Pu vs Cu showed that most of the differentially expressed genes at baseline in untreated PCOS vs untreated controls were independent of dysregulated genes in the Cb2 treatment, corroborating that the baseline differences due to PCOS did not affect our results (Supplementary Fig. 1A, see section on supplementary data given at the end of this article).

Functional dysregulation in Cb-2-treated PCOS cells

The main functions affected after Cb2 treatment are shown in Fig. 2. There were 388 GO terms enriched from the Ct vs Cu comparison and 661 from the Pt vs Pu comparison. For the functional interpretation of all these enriched GO terms (FDR < 0.05), 17 functions were assigned manually to group and summarize all the terms: Vesicles transport; Response, transduction and signaling; Reproductive related process; Ossification; Neurosystem; Lipid metabolism; Immune system; homeostasis and transport; Energetic and respiratory

Figure 1 Transcriptomic analysis. (A) Principal component analysis (PCA) of full transcriptomes in LGCs from women without (non-PCOS) and with PCOS treated and untreated with D2-ag (Cb2). Ct: non-PCOS (control) treated ($n=3$); Cu: non-PCOS (control) untreated ($n=3$); Pt: PCOS treated ($n=3$); Pu: PCOS untreated ($n=3$). PCA Component 1 (PC1)/Component 2 (PC2) revealed a clustering effect related to treatment with (dark shapes) or without D2-ag (Cb2) (bright shapes) (top left), and PCA Component 2 (PC2)/Component 3 (PC3) clustered the samples related to the disease (triangles=PCOS; circles=non-PCOS) (top right). PC1, PC2 and PC3 represent the % of explained variances. Technical replicates are shown with a red outline and overlap their replicate, therefore indicating the same gene expression. (B) Volcano plots of differential expression analysis (DEA). On the left is the Ct vs Cu comparison, on the right, the Pt vs Pu comparison. Red color indicates upregulated genes and blue downregulated genes in the treated samples ($\text{FDR} < 0.05$). Gray indicates non-differentially expressed genes ($\text{FDR} > 0.05$). (C) Venn diagram intersection between the Cb2 effects in PCOS and control samples.

process; DNA/RNA regulation; Differentiation, proliferation and development; Circulatory system and development; Cell cycle; Carbohydrate metabolism; Biosynthetic process; Apoptosis; and Amino acid metabolism and protein folding, transport and localization (Fig. 2A). The GO terms associated to each category assigned by us is detailed in [Supplementary Table 2](#), among which GO BP terms related to apoptosis were only enriched in PCOS cells (Fig. 2A).

Additionally, KEGG pathway analysis showed 29 pathways only dysregulated in PCOS LGCs treated and 17 pathways only dysregulated in non-PCOS treated.

Figure 2 Functional enrichment of the Cb2 effects. (A) Gene Set Enrichment Analysis (GSEA) from Gene Ontology (GO). GO terms of the enriched biological processes (BP) ($\text{FDR} < 0.05$) related to 17 general functions (Y axis) for the effects of Cb2 treatment on non-PCOS (Ct vs Cu) and PCOS (Pt vs Pu) samples are indicated. The number of enriched GO BP terms is indicated on the X-axis. (B) GSEA according to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for the effects of Cb2 on non-PCOS (Ct vs Cu) and PCOS (Pt vs Pu) LGCs. Significance is indicated on the X-axis ($-\log \text{FDR}$). Only significant pathways are described on the Y-axis ($\text{FDR} < 0.05$).

Interestingly, PCOs-treated LGCs shared 14 common Cb2-disrupted pathways with non-PCOS-treated LGCs. Protein digestion and absorption, the intestinal immune network for IgA production, the complement and coagulation cascade, cell adhesion molecules (CAM), the proteasome, RNA transport and ribosome biogenesis in eukaryotes were commonly dysregulated in Cb2-treated non-PCOS and PCOS LGCs but more highly enriched in the PCOS samples. Lysosomal and cell cycle pathways were also shared, but were more highly enriched in Cb2-treated non-PCOS (controls) LGCs (Fig. 2B). Metabolism of sugar, lipids and amino

acids was also dysregulated after Cb2 treatment in both non-PCOS and PCOS cells.

Functional results from databases analyses (GO terms and KEGG pathways) showed more highly dysregulated pathways following Cb2 treatment in PCOS than in non-PCOS (control) LGCs (Fig. 2). In addition, the paired design removes changes related to the baseline and inter-patient variability, improving statistical power to detect differences and highlighting the treatment effect. KEGG pathways analysis in Pu vs Cu showed pathways that were dysregulated in Pu vs Cu, but were not dysregulated in Pt vs Pu or Ct vs Cu, indicating that the baseline differences in PCOS did not affect our results (Supplementary Fig. 1C).

Functional network modeling between the main pathways altered in LGCs treated with Cb2

After the functional description of Cb2 treatment effect in PCOS vs controls, we highlighted the apoptosis function (hsa04210) because its enrichment was evident only in treated PCOS LGCs and because most of the enriched processes, such as cytokine–cytokine interactions, protein processing in the endoplasmic reticulum (ER), calcium signaling pathway, MAPK pathways, P53 signaling, lysosomes and the cell cycle, are directly involved in apoptotic responses (Supplementary Fig. 2). Moreover, these enriched processes (Fig. 2) are also directly involved in important pathways implicated in PCOS and Cb2 treatment, such as VEGF signaling (hsa04370), ovarian steroidogenesis (hsa04913) and circadian rhythm (hsa04710), which were previously described in the literature. For this reason, we focused our study on intersecting the mentioned pathways with the main pathways related to Cb2 response in the KEGG drug database (neuroactive ligand–receptor interactions (hsa04080) and the dopaminergic synapse (hsa04728)).

Finally, network analysis showed the principal genes that intersected between these pathways (Fig. 3). In this regard, we described genes involved in the selected pathways that might be key genes in Cb2 effect in PCOS, and they could help us to understand the underlying mechanism in PCOS. Among these key genes, *AKT*, *MAPK* and *CREB* were of particular interest because they were the genes most shared between pathways.

Validation of microarray data

To validate whether there were significant differences between Cb2-treated and -untreated samples, seven key genes were selected by GSEA analysis, based not only on the significance of these genes, but also on their intersection with the selected pathway. These were validated by RT-qPCR from the same RNA samples used in the microarray. These genes could be grouped as biomarkers of the dopaminergic synapse (*MAOA*), VEGF signaling (*VEGF*, *AKT1*, *MAPK14*) and apoptosis (*CASP9*, *AIF*). The circadian gene *BMAL1* was also selected and validated due to its importance in the activation of estrogen synthesis and aromatase expression.

The RT-qPCR results for all validated genes showed the same trend as found in the microarray experiment, confirming the microarray data. In addition, most of the changes were significant (P value < 0.05) (Fig. 4). RT-qPCR data showed that Cb2 treatment affected the apoptosis pathway by decreasing *CASP9*, which was significant in the non-PCOS samples (P value < 0.05), while increasing *AIF*, which was significant in the PCOS samples (Fig. 4A and D). In addition, Cb2 significantly decreased *BMAL1* but increased *MAOA* in the PCOS samples, showing the same tendency as found in the microarray experiment (Fig. 4B and E). Finally, Cb2 treatment significantly decreased *VEGFA* in the non-PCOS and PCOS groups (non-PCOS P value < 0.05 ; PCOS P value < 0.01), while

Figure 3 Network relationship between the main pathways and key intersected genes. Gene relationships between six pathways related to PCOS and the Cb2 drug response are indicated. On the top left, network expression values for non-PCOS treated with a D2-ag (Cb2) and on the top right, network expression values for PCOS treated with Cb2. The differences in color intensity of the genes indicate higher or lower fold changes (FC). Upregulation is shown in red and down-regulation in blue. Table indicates fold changes in the microarray experiment (FCm), the false discovery rates (FDR) after the differential expression analysis (DEA), and the FC and P values for real-time PCR (FCp). FDR < 0.1 or P value < 0.1 is highlighted in gray. FDR < 0.05 or P value < 0.05 is in bold.

Figure 4 Validation of microarray data of genes up or downregulated in non-PCOS and PCOS LGCs treated with Cb2. Expression levels of genes related to the pathways: Apoptosis: (*CASP9*, *AIF*) in (A) non-PCOS and (D) PCOS; dopaminergic synapse (*MAOA*, *BMAL1*) in (B) non-PCOS and (E) PCOS and VEGF signaling pathway (*VEGFA*, *AKT1*, *MAPK14*) in (C) non-PCOS and (F) PCOS. Expression levels were determined by quantitative real-time PCR and normalized to the house keeping gene *ACTB*, quantified by the $\Delta\Delta Ct$ method and expressed as the fold change in each group (mean $\pm$ s.d.). Ct: non-PCOS (control) treated ($n=3$); Cu: non-PCOS (control) untreated ($n=3$); Pt: PCOS treated ($n=3$); Pu: PCOS untreated ($n=3$). * $P<0.05$; ** $P<0.01$ comparing Cb-2 treated LGCs vs untreated group.

AKT1 and *MAPK14* increased in the non-PCOS and decreased in the PCOS samples. The change in *AKT1* was significant in PCOS cells (P value <0.05), and the change in *MAPK14* was significant in non-PCOS cells (P -value <0.05) (Fig. 4C and F).

Discussion

The goal of this study was to understand the PCOS ovarian physiology from an 'omic' point of view, through exploring the mechanisms that underlie syndrome based on the therapeutic effects of Cb2. For this purpose, we used a transcriptomic and systems biology approach to better understand how Cb2 treatment influences ovarian function in PCOS in a holistic manner. One of important aspects of systems biology approaches is to identify the biological pathways or networks that connect the differing elements of a system. We have thus identified genes and their functional interactions in non-PCOS and PCOS LGCs treated with a D2-ag (Cb2) to provide novel information about the mechanisms associated with Cb2 treatment and its relationship in both physiological and pathological conditions.

Because genes are not independent variables, Gene Set Enrichment Analysis (GSEA) better mirrors the biology underlying the systems we seek to study than artificial thresholds as fold changes and/or adj- P values (Subramanian *et al.* 2005, Dalman *et al.* 2012, Khatri *et al.* 2012). GSEA using GO and KEGG databases showed common and specific dysregulated processes in non-PCOS (control) and PCOS LGCs treated with Cb2. It is important to note that, although some functions were more highly affected in non-PCOS samples, the Cb2-affected biological functions and pathways were generally more significantly altered in PCOS LGCs, suggesting that the Cb2 treatment response was more robust for the PCOS samples. In this regard, our functional enrichment study provides novel information

about processes affected by Cb2 treatment. In addition, we wanted to better understand the enhanced effect of Cb2 on PCOS cells and highlight new key genes using other systems biology approaches, such as network intersection between pathways that exploits pathway knowledge in public repositories such as GO or KEGG, rather than on methods that infer pathways from molecular measurements (Khatri *et al.* 2012). For this purpose, we intersected pathways implicated in PCOS and/or Cb2 treatment associated with the enriched processes we identify, and which have been previously described in the literature, such as VEGF signaling, ovarian steroidogenesis, circadian rhythm and apoptosis. These pathways intersected with the main pathways related to Cb2 response in the KEGG drug database, such as neuroactive ligand–receptor interactions and the dopaminergic synapse.

Follicular development is associated with intense angiogenesis and increased vascular permeability under the control of angiogenic factors, such as VEGFA, which is essential for corpus luteum angiogenesis (Ferrara 1995), increases proliferation and inhibits apoptosis of GCs (Irusta *et al.* 2010). Previous studies from our group demonstrated a D2-ag dose-dependent inhibition in VEGF production and secretion in LGCs (Ferrero *et al.* 2014a,b) at a post-transcriptional rather than transcriptional level (Ferrero *et al.* 2015), suggesting that efficacy of D2-ag in preventing OHSS might rely on their capacity to inhibit VEGF protein secretion by LGCs. However, this study demonstrated that *VEGFA* mRNA levels were significantly decreased in non-PCOS and PCOS LGCs treated with Cb2. One possible explanation for not finding mRNA-level differences in our previous study might be attributable to the derivation of LGCs from non-PCOS and PCOS patients treated with or without Cb2 from separate women; in contrast, this new study is a paired design that compared for the first time untreated vs treated LGCs from the same patient. In

this way, it avoids inter-patient variability and improves statistical power to detect differences, making this new study more sensitive and accurate.

Of note, despite the fact that women with PCOS overexpress and oversecrete VEGF during the luteal phase after the induction of ovulation (Neulen *et al.* 1995, Agrawal *et al.* 2002), we found that Cb2 efficiently inhibits VEGF in both PCOS and non-PCOS LGCs, and thereby could prevent OHSS.

Functions related to apoptosis, including cytokine–cytokine interactions, protein processing in the endoplasmic reticulum (ER) and calcium signaling, were especially dysregulated in our GSEA in PCOS, while MAPK pathways and P53 signaling were in non-PCOS. Lysosomes and the cell cycle were dysregulated in both (Supplementary Fig. 2). Our results confirmed findings in previous studies, which showed that dopamine induces apoptosis in PCOS-derived GCs (Saller *et al.* 2014). However, our mRNA expression data showed a significant decrease in *CASP9*, which plays an important role in apoptosis. Recently, a previous study reported that *AIF* might serve as a key factor during follicular atresia in GCs associated with ER stress (Yang *et al.* 2017). In this regard, we observed a significant increase in *AIF* in both LGCs from women without PCOS and women with PCOS treated with Cb2, suggesting for the first time that *AIF* could be regulating the apoptosis of PCOS GCs treated with Cb2 through ER stress. This caspase-independent apoptotic sub-pathway could be an alternative pathway that is not described in KEGG and is involved in the ovarian PCOS physiology. Thus, we bring new insight for future studies to analyze how Cb2 regulates apoptosis.

Microarray data showed that *AKT* and *MAPK14* were increased in non-PCOS and decreased in PCOS LGCs treated with Cb2. Several studies have described the important role of *AKT* and *MAPK14* in LGCs during folliculogenesis, due to their involvement in cellular processes including cell growth, survival, proliferation, metabolism and apoptosis (Villa-Díaz & Miyano 2004, Cecconi *et al.* 2010). Our systems biology results corroborate and reinforce the main role of these genes, which intersect directly with the dopaminergic synapse, apoptosis, VEGF signaling and circadian rhythm pathways. New studies will be necessary to explain why Cb2 treatment has differing effects on non-PCOS and PCOS cells.

Women with PCOS present increased MAO activity in hyperluteinized ovaries and GCs, which might be attributed to the increased number of transformed corpus luteum as a consequence of diminished luteolysis (IvaniĀeviĀ-MilovanoviĀ *et al.* 2003, Saller *et al.* 2014). This increased MAO-A/B activity might be associated with the increase in dopamine metabolism in GCs derived from women with PCOS previously described by our group (Gómez *et al.* 2011). Here, we observed an increase in *MAOA* expression levels in non-PCOS and in LGCs from PCOS patients treated with Cb2, which was

significant in the PCOS samples, suggesting increased metabolism of dopamine in Cb-2 treated derived from PCOS patients.

Finally, our transcriptomic data from LGCs treated with Cb2 showed that *BMAL1*, a circadian gene involved in activation of estrogen synthesis and aromatase expression, was decreased in both PCOS and non-PCOS samples treated with Cb2. Hyperandrogenism is implicated in the genesis of PCOS and affects the expression of circadian genes, such as *BMAL1*. PCOS LGCs exhibit hyperandrogenism (Eisner *et al.* 2002), low *BMAL1* expression (Zhang *et al.* 2016) and low follicular estrogen levels, which cause antral follicle arrest and anovulation. In this regard, our results suggest that Cb2 treatment in PCOS further decreases *BMAL1*, increasing the hyperandrogenism risk in these patients. Future studies will be necessary to analyze whether Cb2 has side effects during PCOS treatment related to genes involved in ovarian steroidogenesis, such as cyclooxygenase 2 (*COX2*) and cytosolic phospholipase A2 *cPLA2*, and decreasing *BMAL1* that is involved in estrogen synthesis.

This is a preliminary descriptive *in vitro* study with a limited number of samples. However, we have designed a paired study and validated genes by two mRNA quantification techniques that reinforce our findings.

In summary, LGCs from PCOS cells treated with Cb2 present more significant changes at the transcriptomic level than non-PCOS cells. The network analysis between the main pathways highlighted *CASP9*, *VEGFA*, *AKT1*, *CREB*, *AIF*, *MAOA*, *MAPK14* and *BMAL1* as key genes implicated in the Cb2 response and in the molecular mechanisms of PCOS.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0027>.

Declaration of interest

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

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