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ORIGINAL ARTICLE ***Reproductive biology***

Single-cell RNA sequencing of oocytes from ovarian endometriosis patients reveals a differential transcriptomic profile associated with lower quality

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STUDY QUESTION: Do oocytes from women with ovarian endometriosis (OE) have a different transcriptomic profile than those from healthy women?

SUMMARY ANSWER: Oocytes from endometriosis patients, independently of whether they came from the affected ovary, exhibited a differential transcriptomic profile compared to oocytes from healthy egg donors.

WHAT IS KNOWN ALREADY: Studies of endometriosis have sought to determine whether OE affects oocyte quality. While many reports indicate that oocytes recovered from endometriotic ovaries may be affected by the disease, other studies have found no significant differences among oocyte/embryo quality and fertilization, implantation and pregnancy rates in women with endometriosis.

STUDY DESIGN, SIZE, DURATION: This prospective study compared metaphase II (MII) oocytes ($n = 16$) from endometriosis patients ($n = 7$) to oocytes ($n = 16$) from healthy egg donors ($n = 5$) by single-cell RNA sequencing (scRNA-seq). Participants were recruited between December 2016 and February 2018 at IVI-RMA Valencia and Vigo clinics.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human MII oocytes were collected from healthy egg donors and OE patients aged 18–34 years, with a body mass index of <30 and >6 pre-antral follicles. RNA was extracted, cDNA was generated and libraries were constructed and sequenced. scRNA-seq data libraries were processed and statistically analysed. Selected genes were validated by quantitative real-time PCR.

MAIN RESULTS AND THE ROLE OF CHANCE: Our scRNA-seq results revealed an effect of endometriosis on global transcriptome behaviour in oocytes from endometriotic ovaries. The highest number of differentially expressed genes (DEGs) was found when oocytes from women with OE were compared to oocytes from healthy donors [520 DEGs (394 upregulated and 126 downregulated)], independently of whether oocytes came from an affected or unaffected ovary. Among the top 20 significant DEGs in this comparison, most were upregulated, including *APOE*, *DUSP1*, *G0S2*, *H2AFZ*, *ID4*, *MGST1* and *WEE1*. PXK was the only downregulated gene. Subsequently, functional analysis showed 31 enriched functions deregulated in endometriosis patients (Benjamini $P < 0.1$), being 16 significant enriched functions considering Benjamini $P < 0.05$, which involved in biological processes and molecular functions, such as steroid metabolism, response to oxidative stress and cell growth regulation. In addition, our functional analysis showed enrichment for mitochondria, which are an important cellular component in oocyte development. Other functions important in embryo development, such as angiogenesis and methylation, were also significantly enriched.

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LARGE SCALE DATA: All raw sequencing data are submitted in Gene Expression Omnibus (GEO) under accession number (PRJNA514416).

LIMITATIONS, REASONS FOR CAUTION: This study was restricted only to OE and thereby other anatomical entities, such as peritoneal and deep infiltrating endometriosis, were not considered. This is a descriptive study with a limited number of samples reflecting the difficulty to recruit human oocytes, especially from women with endometriosis.

WIDER IMPLICATIONS OF THE FINDINGS: This study suggests that OE exhibits a global transcriptomic effect on oocytes of patients in OE, independently if they come from an affected or unaffected ovary and alters key biological processes and molecular functions related to steroid metabolism, response to oxidative stress and cell growth regulation, which reduce oocyte quality.

STUDY FUNDING/COMPETING INTEREST(S): This research was supported by IVI Foundation, the Spanish Ministry of Economy and Competitiveness through the Miguel Servet programme (CPII018/00002 to F.D.), the Sara Borrell Program (CD15/00057 to H.F.) and the VALi+d Programe (Generalitat Valenciana); ACIF/2016/444 to A.C.). The authors have no conflicts of interest to declare.

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Key words: oocyte quality / ovarian endometriosis / single-cell RNA-seq / steroid metabolism / oxidative stress / cell cycle

Introduction

Endometriosis is one of the most common reproductive disorders affecting women in their reproductive years. This oestrogen-dependent disorder is characterized by the presence of endometrial tissue outside the uterus, most commonly in the ovaries and peritoneal cavity (Sensky and Liu, 1980; Zondervan et al., 2018). Endometriosis is heterogeneous and includes different anatomical entities such as ovarian, peritoneal and deep-infiltrating endometriosis, all of them cause in a different range pelvic pain, dysmenorrhea, dyspareunia, painful defecation and/or infertility (Practice Committee of the American Society for Reproductive Medicine, 2004). While the estimated prevalence of endometriosis is 6%–10% in the general female population, an estimated 35%–50% of infertile women are affected by this disease (Giudice and Kao, 2004).

Endometriosis patients have reduced implantation and pregnancy rates compared to healthy women (Leone Roberti Maggiore et al., 2016). One of the recurrent paradigms of the endometriosis has been elucidating if ovarian endometriosis (OE) could affect the quality of the oocytes and future embryos obtained from these ovaries in *in vitro* fertilization treatments. A retrospective study classifying results of oocyte donation according to oocyte origin demonstrated that patients who receive embryos derived from endometriotic ovaries had a significantly reduced implantation rate, indicating reduced quality of oocytes produced by affected ovaries (Simón et al., 1994).

Subsequently, a prospective study demonstrated that embryos derived from endometriotic ovaries had a significantly reduced number of blastomeres and increased incidence of arrested embryos *in vitro*, showing a decreased ability to implant (Pellicer et al., 2001). Moreover, when oocytes from healthy women were transferred to endometriosis patients, they showed pregnancy and implantation rates similar to patients without endometriosis (Sung et al., 1997; Díaz et al., 2000), indicating that endometriosis might not alter endometrial receptivity. Another study showed that oocyte quality and fertilization rate are decreased in women with endometriosis (Barnhart et al., 2002). Further, morphological features have been described in oocytes from endometriosis-affected ovaries, showing increased cortical granule loss and zona pellucida hardening, possibly interfering with fertilization, dissolution of the zona pellucida and ability of the future embryo to hatch and implant (Goud et al., 2014). Oocytes from women with endometriosis also have both a higher percentage of abnormal mitochondria and an overall lower number of mitochondria, affecting oocyte quality and fertilization (Xu et al., 2015).

However, there is conflicting evidence about the effect of endometriosis on endometrium and oocyte/embryo quality (Garrido et al., 2002; Garcia-Velasco and Arici, 1999). Indeed, some studies found no significant differences among oocyte/embryo quality and fertilization or implantation and pregnancy rates in women with endometriosis (Suwajanakorn et al., 2001; Suzuki et al., 2005; Filippi et al., 2014). In this regard, an in-depth study that allows describing the altered molecular mechanisms in oocytes developed in endometriosis-affected ovaries should be conducted.

Following recruitment, oocytes undergo final growth and maturation that comprises an accumulation and remodelling of mRNA, which will support the initial stages of embryo development after ovulation and fertilization. Global transcriptomics techniques, such as RNA sequencing (RNA-seq), have recently allowed researchers to determine the whole transcriptome even from single cells (Hwang et al., 2018). RNA-seq has already successfully been applied to macaque (Chitwood et al., 2017) and human oocytes (Zhao et al., 2019). However, no previous research has investigated the complete transcriptomic profile of oocytes from endometriosis patients compared to healthy controls.

We hypothesized that oocytes from patients with endometriosis have a differential gene expression pattern, with deregulation involved in abnormal oocyte and embryo development and thereby the low implantation and pregnancy rates in endometriosis patients. Thus, our aim in this study was to compare the whole transcriptomic profile of oocytes from endometriosis patients (both affected and non-affected ovaries) and healthy controls by single-cell RNA-seq (scRNA-seq).

Materials and Methods

Ethical approval

This study was approved by the ethical committee of clinical investigation of the Instituto Valenciano de Infertilidad (1505-FIVI-040-CS), and all participants provided informed consent.

Sample collection

Metaphase II (MII) oocytes from both healthy egg donors and OE women were collected from apparently healthy women who wanted to participate in our oocyte donation program and were subjected to

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Table I Patient characteristics.

Patient	Group	Endometrioma	Age	BMI
Donor 1	Healthy	-	18	21.4
Donor 2	Healthy	-	22	18.6
Donor 3	Healthy	-	27	27.7
Donor 4	Healthy	-	31	22.4
Donor 5	Healthy	-	24	21.8
MEAN ± SD			**24.4 ± 4.9 (NS)**	**22.4 ± 3.3 (NS)**
Patient 1	Ovarian endometriosis	Bilateral	28	18.7
Patient 2	Ovarian endometriosis	Unilateral	29	31.2
Patient 3	Ovarian endometriosis	Unilateral	24	23.7
Patient 4	Ovarian endometriosis	Unilateral	32	25.5
Patient 5	Ovarian endometriosis	Unilateral	20	25.6
Patient 6	Ovarian endometriosis	Unilateral	30	24.2
Patient 7	Ovarian endometriosis	Unilateral	34	20.3
MEAN ± SD			**28.1 ± 4.7 (NS)**	**24.2 ± 4.0 (NS)**

BMI = body mass index; SD = Standard Deviation; NS = No Significant.

ovarian stimulation during the period January 2017 to February 2018. The participants have an average age of 24.4 (healthy donors) and 28.1 (OE) years, body mass index (BMI) of <30 and >6 pre-antral follicles. Stimulated women who did not show signs of endometriosis or any other infertility problems and met the inclusion criteria were included in this study, 3–4 MII oocytes per donor were collected for our study, while the rest of the donor's oocytes were used for reproductive treatments. However, those stimulated women whose endometrioma was detected during their ovarian stimulation were placed in the endometriosis group. After these women were included in the study as the OE group, endometriosis was corroborated based on evidence of OE by laparoscopy and vaginal ultrasound, showing compatible images of endometrioma in one or both ovaries. Women with endometrial cancer, previous endometrial neoplasia or other anatomical endometrial pathologies were excluded. Both healthy egg donors and women with OE were not subjected to prior surgeries or prior medical therapies.

Using these criteria, 33 donated MII oocytes were recruited between December 2016 and February 2018 from five healthy donors and seven patients with endometriosis undergoing assisted reproduction at IVI-RMA Valencia and Vigo clinics. Among the 33 total oocytes obtained, 17 MII oocytes were from healthy ovaries and 16 MII oocytes were from OE patients (8 oocytes from endometriosis-affected ovaries and 8 oocytes from unaffected ovaries). Patient characteristics are presented in Table I.

Stimulation protocol and oocyte collection

All patients included in the study (healthy donors and endometriosis patients) underwent the same controlled ovarian stimulation. After checking ovarian quiescence on menstruation day by ultrasonography, 150–225 IU of recombinant FSH (Gonal F, Merck-Serono or Puregon, MSD) and 75 IU of highly purified HMG (Menopur, Ferring Pharmaceuticals) were daily administered. Once the leading follicle reached a mean diameter of 14 mm daily GnRH antagonist (0.25 mg Ganirelix: Orgalutran, MSD, Spain) was added. A single dose of GnRH agonist (0.1 mg triptorelin, Ipsen Pharma, Spain) was administered to trigger final oocyte maturation when at least three follicles measuring >17.5 mm or one follicle measuring >20 mm was observed, and oocyte retrieval was scheduled 36 hours later. Follicles were aspirated, and oocytes were washed in gamete medium (Cook Medical, IN, USA). After washing, oocytes were cultured in fertilization medium (cleavage medium, Cook Medical, IN, USA) at 5.5% CO_2 in air at 37°C for 2 hours before oocyte denudation. Oocyte denudation was carried out by mechanically pipetting 40 IU/mL of hyaluronidase in the same medium. Retrieved MII oocytes were vitrified after oocyte denudation.

Vitrification/devitrification

MII oocytes included in the study were denuded 2 hours after oocyte retrieval and vitrified immediately after nuclear maturity evaluation to identify the presence of the first polar body. All materials and tools for vitrification were obtained from Kitazato (Japan). We followed the Cryotop method for oocyte vitrification, which has been described elsewhere (Kuwayama *et al.*, 2005; Coello *et al.*, 2016). Specifically, after 12 minutes of stepwise equilibration in a mixture of 15% (v/v) ethylene glycol and dimethylsulfoxide in buffer media supplemented with hydroxypropyl cellulose (Coello *et al.*, 2016), oocytes were exposed to a vitrification solution by maintaining the same mixture of cryoprotectants at double concentration (30% v/v) for 50–60 seconds. Within the next 10 seconds, oocytes were placed on a loading device in a minimum volume and immediately plunged into liquid nitrogen to induce vitrification. To determine the ovary origin of each oocyte, an oocyte per Cryotop was individually loaded and stored in vapour tanks (V1500-AB isothermal freezer, Custom BioGenic Systems, MI, USA). During warming, cryoprotectants were diluted by subjecting oocytes to a hyperosmolar solution that contained 1.0 mol/L trehalose at 37°C (Coello *et al.*, 2016). Dilution continued for 3 minutes at room temperature in a half-diluted solution containing

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the same sugar. The warming procedure was completed with two washes (one 5-minute wash; one 1-minute wash) in buffer solution at room temperature.

Library construction and RNA sequencing

Whole oocyte total RNA was extracted from 33 oocytes—16 OE MII oocytes (8 from endometriosis-affected ovaries and 8 from non-affected ovaries) and 17 healthy oocytes—using a SMART-Seq V4 ultra-low input RNA sequencing kit (Clontech, CA, USA) that uses Oligo(dT) priming. Oocytes were lysed, and cDNA was synthesized with 3'SMART-Seq CDS primer II (Clontech, CA, USA). cDNA was PCR-amplified (17 cycles) from 10 pg of RNA following manufacturer's instructions. Amplified cDNA was purified using AMPure XP magnetic beads (Illumina, CA, USA). After confirming cDNA integrity on a 2100 Bioanalyzer (Agilent Technologies, CA, USA), 50 ng of cDNA per sample were fragmented, and libraries were constructed using NexteraXT DNA sample preparation (Illumina, CA, USA) according to manufacturer's instructions. Samples were quantified using 2100 Bioanalyzer (Agilent Technologies, CA, USA), and an RNA pool was generated using equal concentration (4 nM) of RNA per sample. Barcodes and adapters were included in the library to sequence and identify all samples. Finally, libraries were sequenced in two runs using an Illumina NextSeq 500 (Illumina, CA, USA) with a 300-nt read length in a paired-end design (150-bp fragments). To control for sequencing reproducibility, one single-oocyte library was technically replicated during library preparation, and each replicate was submitted equally in each sequencing run.

Processing of scRNA-seq data

Sequencing reads were aligned against human genome reference HG38 using STAR_2.5.2 mapping software with default settings (Dobin *et al.*, 2013). For gene expression quantification, uniquely mapping reads were submitted to *rpkmforgenes* algorithm (Ramsköld *et al.*, 2009) to calculate counts of fragments per kilobase of exon and per million mapped reads (FPKMs). Genome reference annotation was extracted from Gencode 24 (Ensembl version 84). Sequencing samples yielded a total of 789 million reads, with an average of 23 million reads per sample. All raw sequencing data are submitted in Gene Expression Omnibus (GEO) under accession number (PRJNA514416), as a probe of scientific quality of the transcriptomic analysis.

Quality control, data filtering and normalization

A total of 34 scRNA-seq data libraries were processed and subjected to statistical analysis within the R/Bioconductor (3.5.0) computing environment, applying generic statistical functions as well as plotting functions from *ggplot2* package and specific tools from *SingleCellExperiment*, *scater* and *scde* packages. Quality control of single-cell libraries was assessed based on distributions of the following quality control metrics: (i) number of total mapped reads, (ii) rate of uniquely mapping reads and multi-mapping reads, (iii) rate of mismatches or single-nucleotide insertions and deletions (indels), (iv) rate of reads spanning over splice junctions, (v) number of normalized reads and (vi) gene detection rate. An average of 90% of reads per sample were mapped to unique locations in the reference genome (Supplementary Figure S1A). One library (HD8_S8) resulted in a low gene detection level (Supplementary Figure S1B), so it was considered unsatisfactory and filtered out from downstream processing.

To analyse sample clustering, an initial selection of highly variant genes was performed following Brennecke's standard pipeline (Brennecke *et al.*, 2013; Lun *et al.*, 2016), with winsorization to reduce effects of the most extreme values. This gave us 965 variable genes that were used for further principal component analysis and T-distributed stochastic neighbouring entities (t-SNE) clustering.

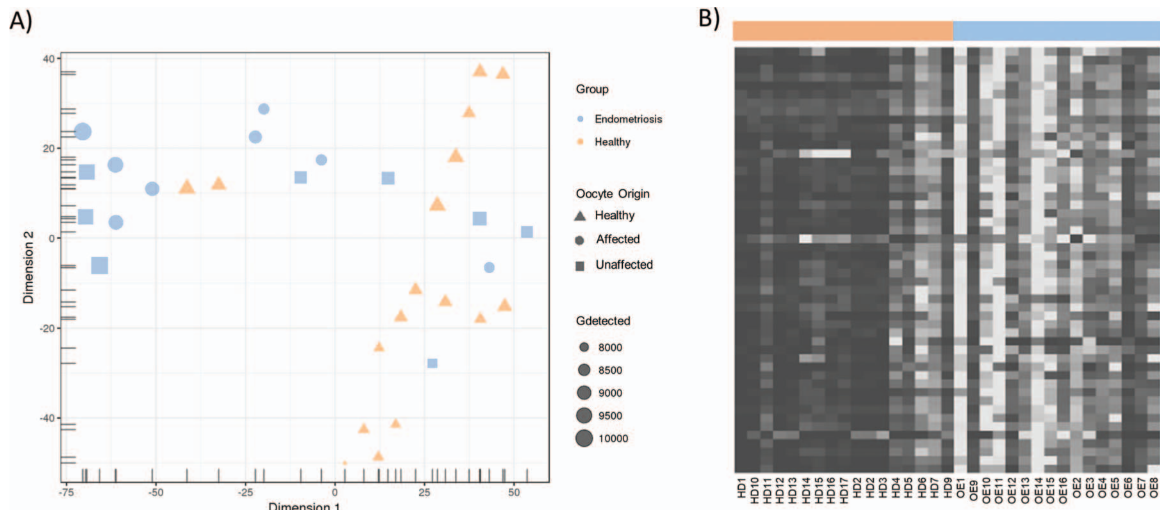

Figure 1 Global transcriptome behaviour in oocytes from OE. t-SNE of global transcriptome in oocytes from women with OE (blue) and healthy donors (orange). Dimensions represent % variance (**A**). Gdetected indicates the number of genes detected. Heatmap of all samples from OE (blue) and healthy donors (HD) (orange) based on the 50 most highly variant genes (**B**).

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Differentially expressed genes and functional enrichment analysis

After filtering of the outlier sample (HD8_S8) and technical replicate, the following analysis was carried out with 32 oocytes. Differential gene expression analysis with *scde* package was performed for four different comparisons: (i) endometriosis ($n = 16$) versus healthy donors ($n = 16$); (ii) affected ovaries ($n = 8$) versus healthy donors ($n = 16$); (iii) unaffected ovaries ($n = 8$) versus healthy donors ($n = 16$); and (iv) affected ovaries ($n = 8$) versus unaffected ovaries ($n = 8$). Genes were considered significant when the adjusted *P*-value was <0.05. Functional enrichment clustering analysis of each list of differentially expressed genes (DEGs) was performed with the DAVID web tool (Huang *et al.*, 2009).

Validation

To corroborate gene expression from scRNA-seq analysis, cDNA from OE ($n = 16$) and healthy donor ($n = 16$) MII oocytes was analysed by quantitative real-time PCR (qRT-PCR) on a StepOnePlus system (Applied Biosystems, CA, USA) to determine gene expression levels for selected genes using Power-Up SYBR Green (ThermoFisher, MA, USA): apolipoprotein E (*APOE*), dual specificity phosphatase 1 (*DUSP1*), microsomal glutathione S-transferase 1 (*MGST1*), G0/G1 switch 2 (*G0S2*), inhibitor of DNA binding 4 (*ID4*), WEE1 G2 checkpoint kinase (*WEE1*), PX Domain Containing Serine/Threonine Kinase Like (*PXK*) and H2A histone family member Z (*H2AFZ*), primers description in Supplementary Table SI. Subsequently, gene expression levels were normalized with housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), quantified by the ΔΔCt method. Subsequently, fold change was calculated as the normalized gene expression (2^ (- ΔCT)) in OE group divided by the normalized gene expression (2^ (- ΔCT)) in healthy donors (reference group). Qiagen data analysis software was used to calculate fold regulation.

Statistical analyses

All statistical analyses and plotting of scRNA-seq data were performed using R software (The R Foundation, https://www.r-project.org). Graphics were made using the *R core* package and packages *gplots*, *ggplot2*, *RColorBrewer* and *GraphPad Prism 6.0*. Gene expression and validation analyses were carried out with Qiagen data analysis software applying Student's *t*-test. The *P*-values were adjusted for multiple comparisons using the Benjamini–Hochberg algorithm (Benjamini and Hochberg, 1995) and adjusted *P*-values < 0.05 was considered statistically significant.

Results

Global transcriptome behaviour in oocytes from OE

Oocytes from OE patients and healthy donors had similar percentages of reads mapping in unique or multiple locations and number of genes detected at different expression levels (Supplementary Figure S1A, S1B). As a biological control test, we selected 10 genes typically expressed in oocytes (*DAZL*, *KIT*, *BMP15*, *ZP1*, *ZP2*, *ZP3*, *BUB3*, *NOBOX*, *NLRP5* and *CCNB1*) (Virant-Klun *et al.*, 2013) and analysed FPKMs of these genes in the 32 single-cell oocyte libraries. Results demonstrated that FPKMs of these genes were similar between oocytes from healthy donors versus oocytes from the affected or unaffected ovary of endometriosis patients (Supplementary Figure S1C).

Clustering the 32 oocytes by gene expression using t-SNE showed that there was not a tendency to separate populations by age, body mass index, donor or mitochondrial gene content (Supplementary Figure S2A–D), suggesting that these parameters did not disturb our model. In addition, reproducibility of the sequencing runs was confirmed by clustering the runs by t-SNE and comparing the top expressed genes of each replicated sample against the complete sample set (Supplementary Figure S3A, B).

However, clustering endometriosis versus healthy donors showed an effect of endometriosis on global transcriptome behaviour in the analysed oocytes by t-SNE analysis (Fig. 1A). Although most endometriosis oocytes were clearly differentiated from healthy donors, some endometriosis oocytes behaved similarly to healthy oocytes. This tendency was also observed by a heatmap of the most highly variant genes (Fig. 1B), where expression of most genes discriminated analysed populations.

Differential gene expression in oocytes from endometriosis patients compared to healthy donors

ScRNA-seq of the 32 individual human oocytes included in this study demonstrated that among the 46 696 genes referenced in the genome, an average of 10 000 genes (35% of the total reference) were detected in our samples (threshold: FPKM > 1) (Supplementary Figure S1B). DEGs were identified through all possible pairwise comparisons between sample groups. The most prominent DEGs were found between oocytes from OE compared to oocytes from healthy donors, with 520 DEGs (394 upregulated and 126 downregulated) (Fig. 2A–C; Supplementary Table SII). Among the top 20 DEGs in oocytes from endometriosis patients (independently if from an affected or unaffected ovary) compared to healthy donors, most genes were upregulated, including *APOE*, *DUSP1*, *MGST1*, *G0S2*, *ID4*, *WEE1* and *H2AFZ* (Fig. 2D, E). *PXK* was the only downregulated gene in the top 20 DEGs (Fig. 2D, E).

When the transcriptome of oocytes from the affected ovary of endometriosis patients was compared to oocytes from healthy donors, we found 103 DEGs (97 upregulated and 6 downregulated) (Fig. 2A–C; Supplementary Table SIII). In this comparison, all top 20 DEGs, such as *CYR61*, *DUSP1*, *FILIP1L*, *FLNA*, *G0S2*, were upregulated (Fig. 2D). In addition, comparison of oocytes from the unaffected ovary with oocytes from healthy donors revealed 71 DEGs (62 upregulated and 9 downregulated) (Fig. 2A–C; Supplementary Table SIV). As observed before, most genes were significantly upregulated, such as *CORO1A*, *CTGF*, *ELANE*, *HES6* and *ID4*, while the minority were downregulated (*COPB2*, *PXK*). Interestingly, in these three comparisons we found some upregulated genes (*DUSP1*, *APOE*, *MGST1*) and a downregulated gene (*PXK*) in common. Finally, when we compared oocytes from endometriosis patients (affected versus unaffected ovaries), we found only one downregulated gene, *MT1G*.

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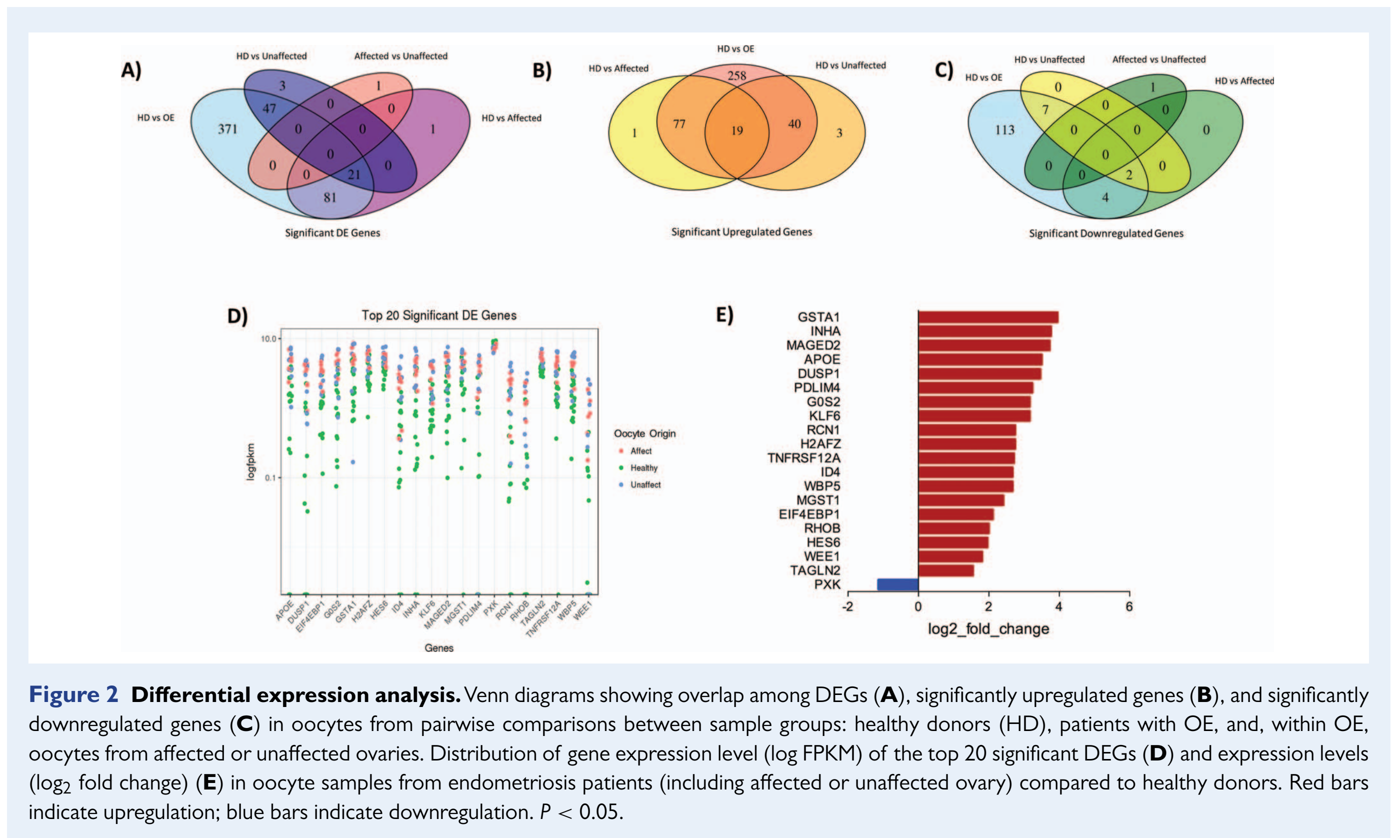

Figure 2 Differential expression analysis. Venn diagrams showing overlap among DEGs (**A**), significantly upregulated genes (**B**), and significantly downregulated genes (**C**) in oocytes from pairwise comparisons between sample groups: healthy donors (HD), patients with OE, and, within OE, oocytes from affected or unaffected ovaries. Distribution of gene expression level (log FPKM) of the top 20 significant DEGs (**D**) and expression levels ($\log_2$ fold change) (**E**) in oocyte samples from endometriosis patients (including affected or unaffected ovary) compared to healthy donors. Red bars indicate upregulation; blue bars indicate downregulation. $P < 0.05$.

Functional implications of endometriotic oocytes

Functional enrichment analysis was carried out to identify pathways significantly deregulated in oocytes from endometriosis patients (independently if from an affected or unaffected ovary) compared to healthy donors. This analysis showed 31 enriched functions deregulated in endometriosis patients (Benjamini $P < 0.1$), being 16 significant enriched functions considering Benjamini $P < 0.05$ (Fig. 3A; Supplementary Table SV), which were involved in different pathways, biological processes, molecular functions and/or cellular components. Interestingly, most genes involved in these enriched functions were upregulated in endometriotic oocytes compared to healthy donors (Fig. 3B). Relevant enriched functions related to biological processes and molecular functions were assigned to different functional groups, such as steroid metabolism, response to oxidative stress and cell growth regulation (Fig. 3C). In addition, mitochondrion was enriched in our functional analysis, which is an important cellular component in oocyte development. Other functions important in embryo development, such as angiogenesis and methylation, were also included in the significantly enriched functions (Fig. 3C).

Validation

To validate scRNA-seq results, eight genes were selected among the top 20 DEGs based on their significance and their inclusion within significantly enriched functions: *APOE*, *DUSP1*, *MGST1*, *G0S2*, *ID4*, *WEE*, *H2AFZ* and *PXK*. These genes were related to steroid metabolism (*APOE*), response to oxidative stress (*APOE*, *DUSP1*, *MGST1*), mitochondrion (*G0S2*, *MGST1*), and cell growth regulation (*G0S2*, *ID4*, *H2AFZ*, *WEE1*) (Supplementary Figure S4). *PXK* was selected as only downregulated gene. All genes were validated by qRT-PCR from the same cDNA samples used in scRNA-seq. qRT-PCR results corroborated the differential gene expression observed by scRNA-seq in oocytes from women with OE compared to healthy donors, although this gene expression was not statistically significant in most of them due to the high variability between samples: *APOE* (fold change = 12.58; $P = 0.67$), *DUSP1* (fold change = 13.44; $P = 0.09$), *MGST1* (fold change = 3.98; $P = 0.66$), *G0S2* (fold change = 1.92; $P = 0.02$), *ID4* (fold change = 15.83; $P = 0.05$), *WEE1* (fold change = 256.55; $P = 0.003$), *PXK* (fold change = 0.14; $P = 0.01$), *H2AFZ* (fold change = 1.69; $P = 0.05$) (Fig. 4A–H).

Discussion

Our scRNA-seq results show an effect of endometriosis on global transcriptome behaviour in oocytes from endometriosis patients. Several studies have postulated that the negative effects of OE during oocyte development, embryogenesis and implantation (Rossi and Prefumo, 2016) could be due to different deregulated mechanisms in these women, such as impaired folliculogenesis; exposure to a hostile environment of macrophages, cytokines and vasoactive substances in the peritoneal fluid; and anatomical dysfunction of the fallopian tube and ovary (Harb *et al.*, 2013). However, there is controversy about whether OE affects oocyte quality and, consequently, embryo implantation. Therefore, our study evaluated the whole transcriptomic profile of oocytes from endometriosis patients compared to healthy oocytes by scRNA-seq.

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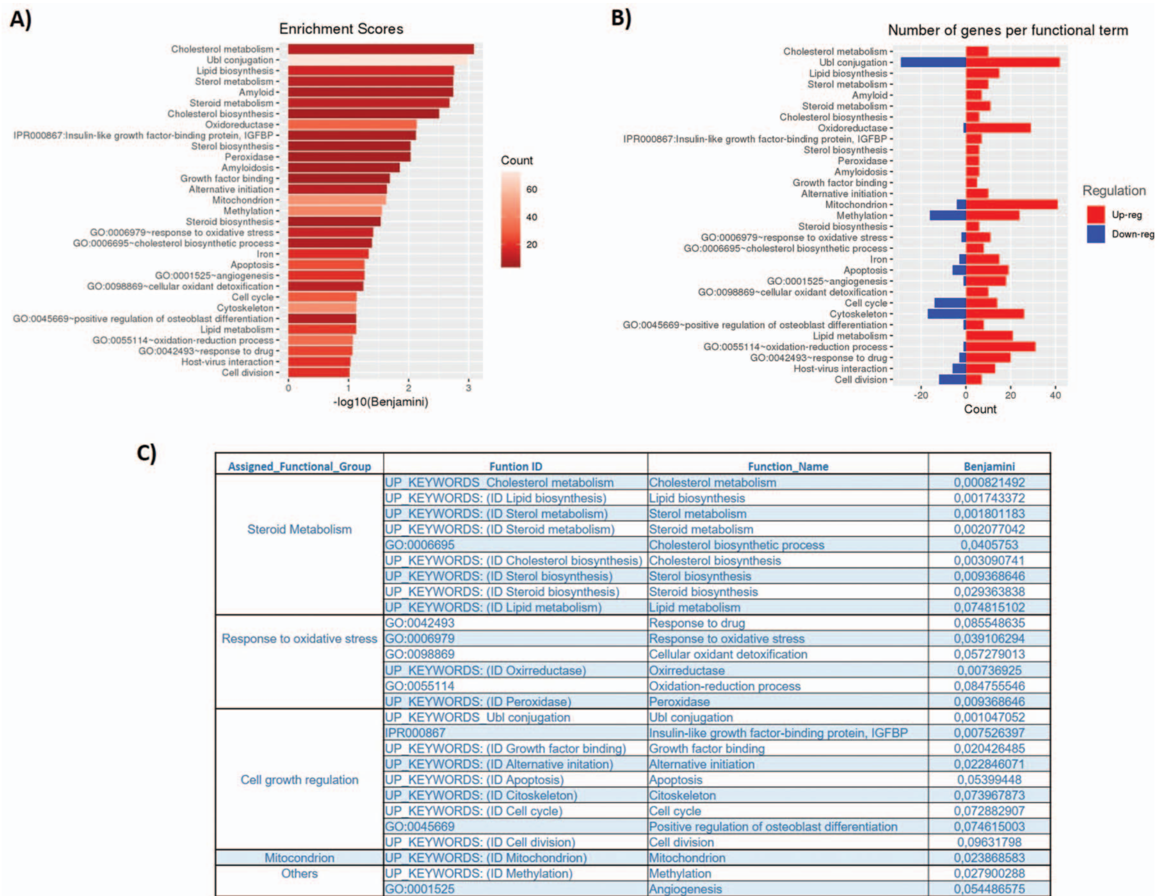

Assigned_Functional_Group	Funtion ID	Function_Name	Benjamini
Steroid Metabolism	UP_KEYWORDS_Cholesterol metabolism	Cholesterol metabolism	0,000821492
	UP_KEYWORDS: (ID Lipid biosynthesis)	Lipid biosynthesis	0,001743372
	UP_KEYWORDS: (ID Sterol metabolism)	Sterol metabolism	0,001801183
	UP_KEYWORDS: (ID Steroid metabolism)	Steroid metabolism	0,002077042
	GO:0006695	Cholesterol biosynthetic process	0,0405753
	UP_KEYWORDS: (ID Cholesterol biosynthesis)	Cholesterol biosynthesis	0,003090741
	UP_KEYWORDS: (ID Sterol biosynthesis)	Sterol biosynthesis	0,009368646
	UP_KEYWORDS: (ID Steroid biosynthesis)	Steroid biosynthesis	0,029363838
	UP_KEYWORDS: (ID Lipid metabolism)	Lipid metabolism	0,074815102
Response to oxidative stress	GO:0042493	Response to drug	0,085548635
	GO:0006979	Response to oxidative stress	0,039106294
	GO:0098869	Cellular oxidant detoxification	0,057279013
	UP_KEYWORDS: (ID Oxirreductase)	Oxirreductase	0,00736925
	GO:0055114	Oxidation-reduction process	0,084755546
	UP_KEYWORDS: (ID Peroxidase)	Peroxidase	0,009368646
Cell growth regulation	UP_KEYWORDS_Ubl conjugation	Ubl conjugation	0,001047052
	IPR000867	Insulin-like growth factor-binding protein, IGFBP	0,007526397
	UP_KEYWORDS: (ID Growth factor binding)	Growth factor binding	0,020426485
	UP_KEYWORDS: (ID Alternative initation)	Alternative initiation	0,022846071
	UP_KEYWORDS: (ID Apoptosis)	Apoptosis	0,05399448
	UP_KEYWORDS: (ID Citoskeleton)	Citoskeleton	0,073967873
	UP_KEYWORDS: (ID Cell cycle)	Cell cycle	0,072882907
	GO:0045669	Positive regulation of osteoblast differentiation	0,074615003
	UP_KEYWORDS: (ID Cell division)	Cell division	0,09631798
Mitocondrion	UP_KEYWORDS: (ID Mitochondrion)	Mitochondrion	0,023868583
Others	UP_KEYWORDS: (ID Methylation)	Methylation	0,027900288
	GO:0001525	Angiogenesis	0,054486575

Figure 3 **Functional enrichment analysis.** Deregulated significantly enriched functions (**A**) and number of upregulated (red) and downregulated (blue) genes related to these enriched functions (**B**) in oocytes from endometriosis patients compared to healthy donors. Relevant enriched functions were classified into different functional groups (**C**). Functional enrichment analysis was performed using the functional annotation tool DAVID. Benjamini $P < 0.1$ were included as enriched function, considering statistically significant Benjamini $P < 0.05$.

The highest number of DEGs was found when oocytes from women with endometriosis were compared to oocytes from healthy donors, independently if oocytes came from an affected or unaffected ovary. Surprisingly, only one DEG was found when oocytes from an affected were compared to an unaffected ovary. These findings demonstrate that oocytes from women with OE have similar gene expression patterns independently of whether they come from affected or unaffected ovaries, suggesting that OE exhibits a global effect on oocyte quality and thus, less potential to implant once it is fertilized.

Subsequently, functional enrichment analysis identified functions involved in different pathways, biological processes and molecular functions that could be functionally grouped in steroid metabolism, response to oxidative stress and cell growth regulation.

Our results show that functions such as metabolism and biosynthesis of steroids and lipids were enriched in oocytes from women with endometriosis compared to oocytes from healthy donors. Lipid dysregulation in serum (Dutta *et al.*, 2016) and endometrial fluid (Domínguez *et al.*, 2017) has been demonstrated in both mouse models and women with endometriosis, as well as altered steroidogenesis in human granulosa cells (GCs) (Sanchez *et al.*, 2016), suggesting a deregulated lipid profile in these women. Several studies suggest that lipid metabolism is involved in mechanisms related to endometriosis progress in the ovary (Cordeiro *et al.*, 2015), such as oxidative stress, due to its important role in the genesis of reactive oxygen species (Nasiri *et al.*, 2017). In addition, other studies suggest that oxidative stress increases steroid hormone production in GCs from women with endometriosis (Appasamy *et al.*, 2008).

Our differential expression analysis showed that *APOE* was upregulated in oocytes from women with endometriosis compared to healthy donors. Several studies report that women with endometriosis have a deregulated low-density lipoprotein lipid profile and increased *APOE* production. *APOE* may have an important role in the initiation of endometriosis as well as progression of the disease because it contributes to endometrial attachment, adhesion or invasion and creates a self-propagating loop (Neyen *et al.*, 2009) due to its important role in lipoprotein metabolism and cellular lipid transport. These findings suggest that the steroidogenic environment present in women with OE promotes upregulation of steroid-related genes, such as *APOE*, that increase lipid metabolism, which, in turn, contributes to oxidative stress in these women.

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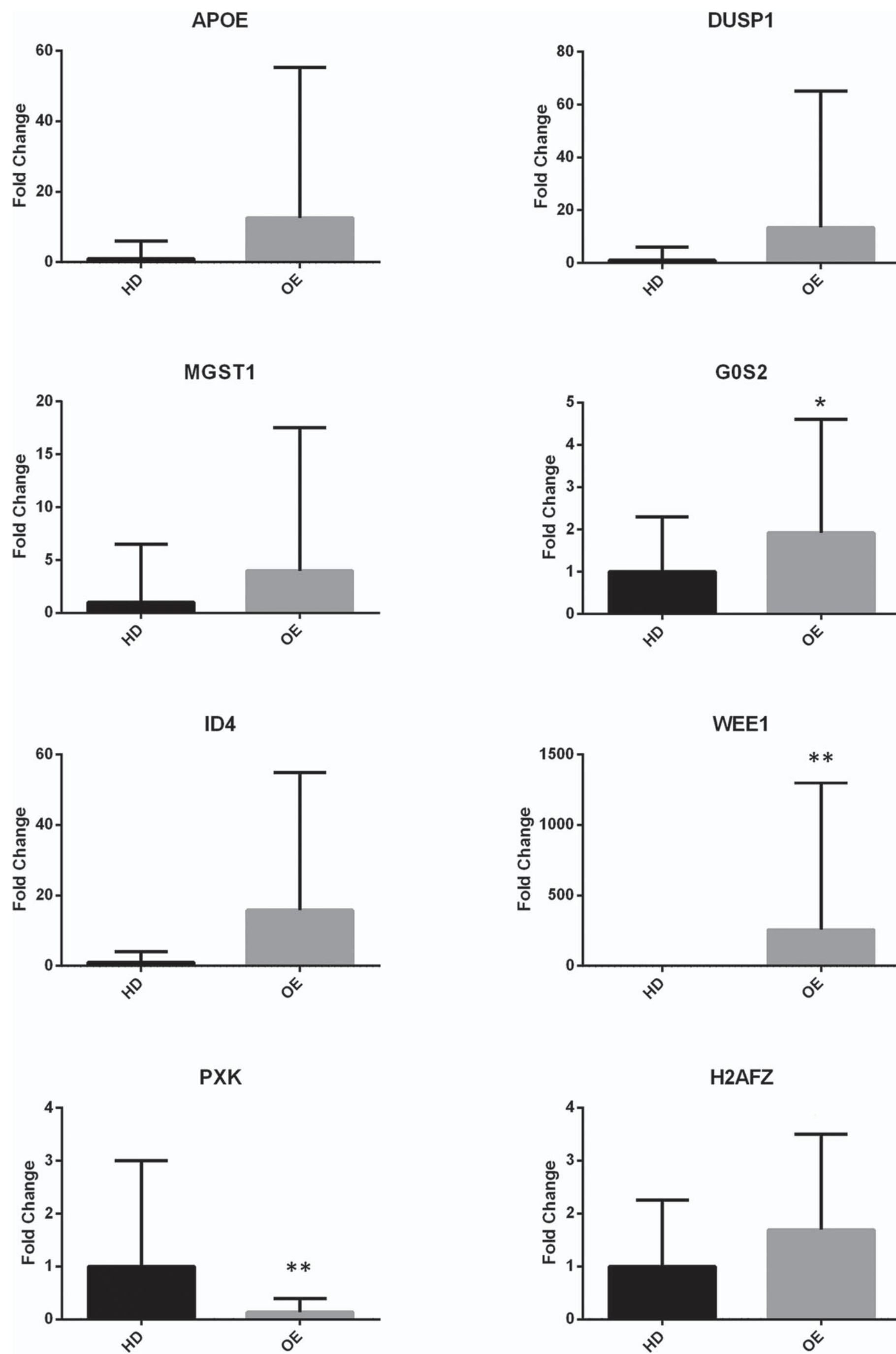

Figure 4 Validation of scRNA-seq data of genes in oocytes from endometriosis and healthy donors. Expression levels of genes related to functional groups of steroid metabolism, response to oxidative stress, mitochondrion and cell growth regulation in oocytes from OE patients (n = 16) and healthy donors (n = 16): *APOE*, *DUSP1*, *MGST1*, *G0S2*, *ID4*, *WEE1*, *PXK*, *H2AFZ*. Genes were validated by qRT-PCR, quantified by the $\Delta\Delta$Ct method and expressed as fold regulation. $*P < 0.05$; $**P < 0.01$ were considered significant.

Interestingly, several studies also report that women with endometriosis have high levels of oxidative stress markers and low levels of antioxidant markers in serum (Lambrinoudaki *et al.*, 2009; Prieto *et al.*, 2012) and follicular fluid (Singh *et al.*, 2013; Nasiri *et al.*, 2017), suggesting increased oxidative stress in these patients (Nakagawa *et al.*, 2016). Recently, altered oxidative stress in the follicular fluid of women with endometriosis was proposed to have a negative impact in folliculogenesis, affecting apoptosis, damaging DNA in GCs and cumulus cells (Goud *et al.*, 2014; Donabela *et al.*, 2015), and potentially altering oocyte development. Accordingly, follicular fluid from women with endometriosis, when applied to bovine and murine oocytes, promotes meiotic abnormalities such as chromosomic instability and

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spindle abnormalities (Choi *et al.*, 2007; Mansour *et al.*, 2010; Da Broi *et al.*, 2014), implying a connection between oxidative stress and cell cycle. However, little is known about if this increased oxidative stress in endometriosis patients affects human oocyte quality.

This study showed that functions related to oxidative stress response were significantly enriched in human oocytes from women with endometriosis compared to oocytes from healthy donors, suggesting that the increased oxidative stress described in serum and follicular fluid of women with endometriosis could affect their oocyte quality. In addition, in our study, most oxidative stress-related genes were upregulated in endometriotic oocytes. Among them, *DUSP1* plays an important role in the human cellular response to environmental stress as well as negative regulation of cellular proliferation by inhibiting MAPK activity (Ferguson *et al.*, 2016), which, in turn, plays a role in bovine oocyte maturation, developmental competence, spindle morphology and chromosome alignment (Gordo *et al.*, 2001). These findings suggest that *DUSP1* upregulation could be involved in decreased oocyte quality, suggesting this gene as a potential biomarker associated with decreased oocyte competence due to its implication in oxidative stress and cell growth regulation.

Oxidative stress and increased levels of inflammatory response molecules, such as IL-1, IL-6 or TNF-alpha, also play an important role in the damage of microtubules, spindles and chromosomes that induces cell cycle impairment in endometriosis patients, suggesting cell growth dysregulation in these women (Toya *et al.*, 2000; Mansour *et al.*, 2009; Banerjee *et al.*, 2012). In addition, women with endometriosis exhibit increased apoptosis of cumulus cells surrounding the oocyte, probably leading to reduced oocyte quality (Díaz-Fontdevila *et al.*, 2009). Accordingly, our scRNA-seq results show that biological processes and molecular functions related to cell growth regulation, such as cell cycle, apoptosis, cytoskeleton, cell growth and differentiation, were enriched in oocytes from women with OE compared to oocytes from healthy donors. OE patients reportedly have a lower percentage of GCs in G2/M-phase compared to other infertile patients without ovarian lesions, probably caused by aberrations in molecules that regulate G2/M-phase, including cyclin B, Cdc2, cdc2s and WEEl kinase (Toya *et al.*, 2000).

Our differential expression analysis showed that *WEE1* was upregulated in oocytes from women with OE compared to healthy donors. WEE1 kinase is a key regulator of the G2/M cell cycle checkpoint through inhibitory phosphorylation of Cdc2 (CDK1), to halt cell cycle progression in response to DNA damage during meiosis (Nakanishi *et al.*, 2000; Mills *et al.*, 2017). Based on these findings, we suggest for the first time that *WEE1* upregulation may potentially promote cell cycle arrest in G2/M-phase in oocytes from women with OE, limiting appropriate oocyte development by inhibiting meiotic maturation and thereby compromising oocyte quality. However, further functional studies that demonstrated the important role of *WEE1* in cell cycle regulation in oocytes will be needed to corroborate its implication in oocytes development and thereby, oocyte quality in OE.

In addition, other genes related to cell cycle regulation, such as *ID4* and *G0S2*, were upregulated in oocytes from women with endometriosis. *ID4* promotes cell arrest in S-phase and thereby decreases cell proliferation (Carey *et al.*, 2009). *G0S2* encodes a mitochondrial protein that blocks G1/S transition and interacts with BCL2, promoting apoptosis by preventing the formation of protective BCL2/BAX heterodimers (Welch *et al.*, 2009).

Accordingly, the observed upregulation of genes in oocytes from endometriosis women implicated in cell cycle arrest, apoptosis, spindle morphology and chromosome alignment could explain the lower number of MII oocytes and poor oocyte quality (38% oocytes included in our study with elongated appearance, presence of debris in perivitelline space and fragmentation, none of which occurred in oocytes from healthy egg donor), as well as the slower embryonic development after fertilization. However, further studies using new strategies based in poly-A RNA isolation followed by q-RT-PCR or RNA-seq could be used to identify mRNAs actively translated and thus avoid the possible biases caused by the use of oligo-dT amplified libraries (Martins and Conti, 2018).

Several studies have reported that appropriate chromosome condensation, spindle assembly and chromosome trafficking are essential for generating an oocyte capable of developing into an embryo (Conti and Franciosi, 2018). In this regard, the deregulated functions observed in this study in oocytes from women with endometriosis could be affecting nuclear maturation, as well as cytostatic and growth factor expression, previously described in human and mouse oocytes as essential to oocyte competence (Hassold and Hunt, 2001; Bernhardt *et al.*, 2012), causing developmental arrest or an oocyte uncapable of developing into an embryo. However, further functional studies are needed to clarify the implication of these factors in developmental competence of oocytes from women with endometriosis.

In conclusion, this study shows that endometriosis exhibits a global transcriptomic effect on oocytes, independently if they come from an affected or unaffected ovary and alters key biological processes and molecular functions related to steroid metabolism, response to oxidative stress and cell growth regulation, which reduce oocyte quality. In addition, upregulated genes involved in these functions, such as *APOE*, *DUSP1* and *WEE1*, could be potential biomarkers to diagnose oocyte quality due to their association with decreased oocyte competence. However, further studies of the molecular mechanisms involved in the deregulated functions described in this study would be necessary to confirm our results and develop a possible clinical diagnostic method of oocyte quality.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors’ roles

H.F. was involved in study design, executed experiments and wrote and edited the manuscript. A.C. was involved in experimental execution and wrote the manuscript. A.A. and A.Q. were involved in sample collection after devitrification and single-cell library construction.

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M.C.C.-G. analysed results and executed qRT-PCR to validate gene expression. A.T. and E.T. were involved in sample collection and oocyte vitrification/devitrification. P.A. and E.M. were involved in the selection and recruitment of healthy donors and OE patients. A.P. devised and supervised the study, contributed to data interpretation and drafted the manuscript. F.D. coordinated the study design, contributed to data interpretation and edited the manuscript. All authors reviewed the manuscript and provided critical feedback and discussion.

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Conflict of interest

The authors have no conflicts of interest to declare.

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