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C/EBP-β and SIRT1 regulate IL-18 expression in the proliferative phase endometria of polycystic ovary syndrome patients

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A B S T R A C T

Background: Previous studies have shown that polycystic ovary syndrome (PCOS) patients suffer from low-grade chronic inflammation. Furthermore, our previous studies have confirmed that IL-18 is highly expressed in the serum and endometria of PCOS patients. Nevertheless, the mechanisms underlying IL-18 elevation remain unclear. This study aims to explore the signaling pathways that lead to IL-18 upregulation in the endometria of PCOS patients.
Methods: Using the TF-Search tool, we predicted that C/EBP-β may be a transcription factor for IL-18 and that deacetylase SIRT1 could be involved in its regulation. Subsequently, we analyzed SIRT1 and C/EBP-β in the proliferative endometria of PCOS patients and healthy control using immunohistochemistry, real-time quantitative PCR and western blot; PCOS diagnosis was based on the 2003 Rotterdam ESHRE/ASRM criteria. We further verified the interaction between C/EBP-β and IL-18 using a double luciferase assay.
Results: SIRT1 and C/EBP-β gene and protein levels in the proliferative endometria of PCOS patients were significantly higher than the control group. Immunohistochemical experiments confirmed that SIRT1 was primarily expressed in the endometrial nucleus, while C/EBP-β was predominantly expressed in the endometrial nucleus and cytoplasm. Double luciferase assay verified the interaction between C/EBP-β and IL-18.
Conclusion: SIRT1 and C/EBP-β are probable IL-18 regulatory mechanism in the endometria of PCOS patients.

1. Background

Polycystic ovary syndrome (PCOS) is a multisystem reproductive-metabolic disorder, characterized by polycystic-appearing ovaries, hyperandrogenism, and irregular menstruation, ultimately leading to infertility.[1] The associated metabolic dysfunctions include insulin resistance, dyslipidemia, and obesity.[2] While it is generally believed that PCOS is related to hypothalamic-pituitary-ovarian axis dysfunction, adrenal dysfunction, metabolism, and hereditary factors, its exact pathogenesis remains unclear. Recent studies have indicated that patients with PCOS suffer from chronic inflammation, which may be correlated with the pathogenesis of the disease.[3,4] Although the main causes of infertility in patients with PCOS are anovulation and impaired oocyte maturation, endometrial dysfunction in PCOS likely contributes to reduced endometrial receptivity, subfertility, and poor pregnancy outcomes.[5,6]

Inflammatory factor interleukin 18 (IL-18) is a proinflammatory cytokine primarily produced by mononuclear macrophages and a member of the IL-1 family, with a role in inflammation similar to IL-1β. IL-18 participates in various biological activities and is a growth and differentiation factor of Th-1 cells. It can induce the production of interferon-gamma (IFN-gamma) by B cells, T cells, and NK cells and participates in the body's anti-infective immunity and inflammatory responses as a pro-inflammatory cytokine.

IL-18 is increased both in the serum and endometria of patients with

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PCOS.[7–9] CCAAT enhancer-binding protein beta (C/EBP-β) is an important transcription factor involved in cell proliferation and differentiation.[10] Fields and Ghorpade[11] reported that C/EBP-β regulates multiple IL-1β-induced human astrocyte inflammatory genes. Moreover, Kang et al.[12] determined that loss of *P38α* in macrophages results in decreased IL-18 expression and an inhibition of lipopolysaccharide induced C/EBP-β activation. Accordingly, C/EBP-β may regulate the expression of IL-18. Furthermore, C/EBP-β exhibits polylysine acetylation,[13] which may be related to SIRT1: a critical enzyme involved in acetylation. However, the regulatory effects of C/EBP-β on IL-18 and the signaling pathway mediating these effects have yet to be examined. Herein, we explore the expression of C/EBP-β and SIRT1 in proliferative phase endometria in women with and without PCOS. We then investigate whether C/EBP-β regulates IL-18 expression and explore the signaling mechanisms underlying IL-18 upregulation in the endometria of PCOS patients.

2. Materials and Methods

2.1. Patients and endometrial sample collection

Tissues were obtained from the endometria of PCOS patients (n = 18) and a normal control group (n = 18) who underwent hysteroscopy for Assisted Reproductive Technology (ART) at the Division of Reproductive Medicine Center, Peking University Third Hospital. The PCOS diagnosis was based on the 2003 Rotterdam ESHRE/ASRM criteria, and was finalized if any two of the following three criteria were met and other causes were ruled out[1]: oligo-and/or anovulation,[2] clinical hyperandrogenism, or[3] polycystic ovaries. Healthy control women were selected from patients attending the clinic on account of male azoospermia, with regular menstrual cycles and normal ovarian morphology. Women who received any hormonal treatment within 3 months prior to the start of the study, as well as patients with pelvic inflammatory disease, genital tract infection, chromosome abnormality, or endometriosis, were excluded from the study. Written informed consent was obtained from all patients before study participation and ethics approval was obtained from the Research Ethics Committees of the Reproductive Center, Peking University Third Hospital.

Endometrial tissues were divided into three equal pieces. Two pieces of each sample were frozen in liquid nitrogen and maintained at – 80 °C for quantitative real-time polymerase chain reaction and western blot analyses. One-piece was used for histological and immunohistochemical examinations. Endometria were obtained during the proliferative phase of the menstrual cycle (cycle days 5–11).

2.2. Histology and immunohistochemistry

Five-micrometer-thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks and placed on coated slides, dewaxed in xylene, and then rehydrated in descending grades of ethanol (100–70%). Half of the sections were stained with hematoxylin and eosin. Antigen retrieval was performed using citric acid buffer (0.1 M, pH 6.0) and 10 min microwaving on high power. After cooling to room temperature and washing three times in phosphate-buffered saline (PBS), endogenous peroxidase was quenched using 3% hydrogen peroxide for 10 min. After washing three times with PBS, the sections were incubated with anti-C/EBP-β antibodies (diluted 1:50, ab32358, Abcam) and anti-SIRT1 antibodies (diluted 1:50, ab32441, Abcam) then diluted in PBS for 2 h at 37 °C in a humidified chamber. The negative controls were incubated with a solution devoid of any primary antibody. The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (1:250; Beijing Zhongshan Biotechnology Co., Beijing, China). After incubation with the secondary antibodies for 1 h at 37 °C in a humidified chamber, signals were viewed under an Axiokop2 microscope (Carl Zeiss, Thornwood, New York, NY, USA).

2.3. Dual-luciferase assay

HEK293T cells (Beijing qualityard and biotechnology Co., Ltd, Peking, China) in the logarithmic phase were cultured in cell suspension, counted, and inoculated in 24-well plates (the number of cells was ~105, depending on the size of cells) in an incubator at 37 °C and 5% CO_2 until the degree of cell fusion reached ~60%. ROCHE: X-tremegene HP transfection reagent was used for plasmid transfection. The expression of fluorescently-labeled genes were observed 24–48 h after transfection to determine transfection efficiency. As a control, equal amounts of GFP plasmid were separately transfected from the target plasmid. Luciferase was detected 48 h after transfection. The culture medium in 24-well plates was sucked out and 300 mL Passive Lysis Buffer was added to the plate. Reactions were continued at 4 °C for 20 min before cell lysis.

The cells were then added into Lockwell maxisorp detection board, and Luciferase Assay Reagent was applied. Immediately after shaking and mixing, firefly luminescence was detected by an enzyme-labeled instrument. Firefly luminescence detection, 20 μL Stop & Glo Reagent was added to each well. Renilla luminescence was detected via an enzyme-labeled instrument after shaking and mixing for 3 min. Relative fluorescence values of fluorescence intensity/renilla luminescence intensity were used as indicators to determine the difference among groups.

2.4. Quantitative RT-PCR

Quantitative RT-PCR was performed following a previously reported method.[14] Dissociation curves for both the target and housekeeping genes were utilized to ensure the absence of primer-dimers and other non-specific amplification. PCR and real-time measurements of fluorescence were performed in the 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) in at least triplicates, using the SYBR Select Master Mix (Applied Biosystems). Primers: 5′-CCAAGAAGACCGTGGACAAG-3′(forward) and 5′-TTGCGCATCTTGGCCTT-3′(reverse) for *C/EBP-β*; 5′-AGAACCCATGGAGGATGAAAG-3′(forward) and 5′-TCATCTCCATCAGTCCCAAATC-3’ (reverse) for *SIRT1*. The comparative ΔΔCt method was performed to measure relative gene expression (ABI User Bulletin 2). All 36 patients were sampled in the q-PCR experiments.

2.5. Western blot

Western blotting was performed to detect C/EBP-β according to previously described methodology.[14] Briefly, 10 endometria samples from the PCOS group and 10 endometrium samples from normal control groups, each containing 60 μg of protein, were electrophoresed on 10% polyacrylamide gels. After which, they were denatured for 5 min at 100 °C and transferred to PVDF (polyvinylidene fluoride) membranes. The membranes were blocked in Tris-buffered saline solution with 0.1% Tween 20 and 5% nonfat milk for 1 h at room temperature. Primary antibodies were anti-C/EBP-β (diluted 1:500, ab32358, Abcam) and anti-SIRT1 (diluted 1:50, ab32441, Abcam), and blots were incubated with primary antibodies overnight at 4 °C. After washing three times in Tris-buffered saline, the membranes were incubated for 1 h at room temperature with 1:500 horseradish peroxidase-conjugated secondary antibodies. Last, the membranes were processed and visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA), with the relative band density (normalized to β-actin) determined from light scans of the resulting films.

Table 1
Clinical characteristic of patients with and without PCOS.

	PCOS group (n = 18)	Control group (n = 18)	P
Age(years)	30.6 ± 4.74	29.87 ± 4.79	0.677
BMI(kg/m^2)	23.47 ± 2.91	22.64 ± 3.76	0.503
FSH(mIU/mL)	6.11 ± 1.82	6.67 ± 1.98	0.433
LH(mIU/mL)	8.44 ± 4.84	2.91 ± 1.40	0.001[a]
E2(pmol/L)	201.56 ± 77.22	184.47 ± 36.65	0.445
A(nmol/L)	11.56 ± 4.98	8.04 ± 4.96	0.068
LH/FSH	1.48 ± 0.78	0.46 ± 0.25	0.001[a]

Note: Data were analyzed by t-tests.
BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone.
E2: estradiol; A: androgen.
[a] Significant difference between the control and PCOS groups (P <0.05).

2.6. Statistical analysis

Statistical analyses were performed using SPSS 18.0 (Chicago, IL, USA). The Shapiro–Wilk test was performed to determine whether the continuous variables were normally distributed. All error bars in figures indicate standard errors (SE). Data were analyzed by *t*-tests and Mann–Whitney U tests. All experiments were performed in at least in triplicate, with analogous results obtained in each repetition. A P-value of < 0.05 was considered statistically significant.

3. Results

3.1. Clinical characteristics

The general conditions of 18 PCOS patients and 18 healthy controls are described in Table 1. All women with PCOS displayed oligomenorrhea and polycystic ovaries. The PCOS and control groups were similar with respect to age and body mass index (BMI). There were no significant differences in basic follicle-stimulating hormone (FSH), estradiol (E2), or androgen (A) between the PCOS and normal control groups. However, luteinizing hormone (LH) levels and LH/FSH ration in PCOS patients were statistically higher than in the control group.

3.2. C/EBP-β and SIRT1 staining in endometria samples

Immunochemistry detected C/EBP-β and SIRT1 in the endometrial samples of both PCOS patients and normal women. We observed strong, dense immunostaining of C/EBP-β in the nuclei of endometrial cells and faint immunostaining in the cytoplasm. We also observed dense SIRT1 expression in endometrial nuclei (Fig. 1).

3.3. C/EBP-β enhances IL-18 mRNA transcription in the endometrium

We next determined whether C/EBP-β promoted IL-18 secretion. First, we sequenced the recombinant plasmids, pGL4.10-IL-18 and pEnter-C/EBP-β, and determined their relative luciferase activity via luciferase reporter assay. Subsequent BLAST search indicated that the target sequences of IL-18 and C/EBP-β had been successfully cloned into the

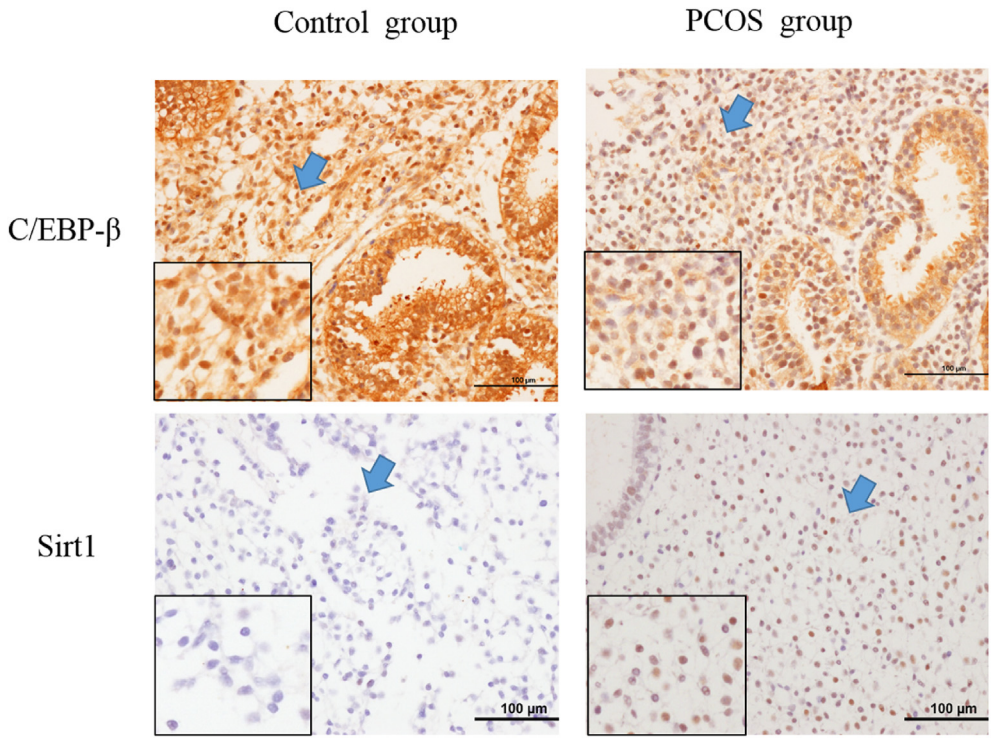

Fig. 1. Immunohistochemical analysis of C/EBP-β and SIRT1 protein expression in the proliferative endometria phase of patients with and without PCOS. C/EBP-β protein expression was dense in the nuclei of endometrial stroma cells and faint in the cytoplasm. SIRT1 protein was dense in the nuclei of endometrial stroma cells. The blue arrow points to the area which was enlarged showed in the lower left corner. *Note: The part in the lower left corner of the figure was enlarged 4-fold.*

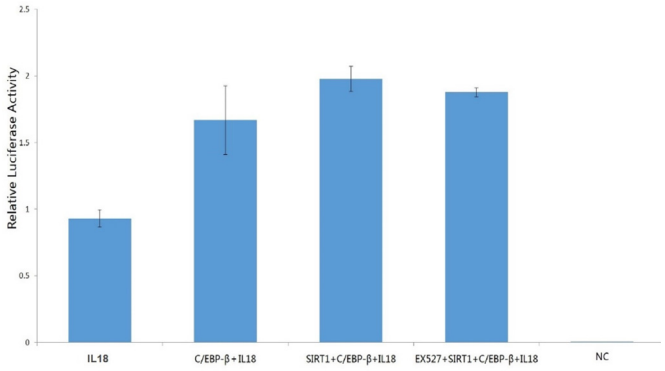

Fig. 2. ***C/EBP-β*** **increased the luciferase activity of** ***pGL4.10-IL18*****;** ***SIRT1*** **increased the luciferase activity of** ***C/EBP-β-IL18*****, EX527 decreased the luciferase activity of** ***SIRT1- C/EBP-β-IL18*****.** *Note: NC: negative control only with culture medium. n = 3.*

Table 2
C/EBP-β and SIRT1 mRNA levels in the endometria of PCOS patients and controls (median: p25, p75).

	Control group (n = 10)	PCOS group (n = 10)	P
Sirt1	0.676 (0.529,1.000)	1.216 (0.770,2.802)	0.024[a]
C/EBP-β	0.503 (0.058,0.989)	1.062 (0.365,1.387)	0.018[a]

Note: Data were analyzed by Mann–Whitney U tests.
[a] Significant difference between the control group and PCOS group (P <0.05).

dual-luciferase reporter vector and could be used for luciferase detection. A dual luciferase assay then revealed that C/EBP-β significantly increased luciferase activity in pGL4.10-IL-18 and that this effect was dependent on the IL-18 promoter sequence (Fig. 2).

3.4. SIRT1 and C/EBP-β are overexpressed in PCOS patients

We then performed qPCR to further characterize C/EBP-β and SIRT1 levels in the endometria of women with and without PCOS. C/EBP-β mRNA expression was significantly higher in the endometria of PCOS patients compared to healthy women (P = 0.018; Table 2). SIRT1 mRNA expression was also significantly higher in the endometria of PCOS patients compared to normal women (P = 0.024; Table 1). We used western blots to further verify the high expressions of C/EBP-β and SIRT1 in PCOS endometria. These results confirmed that SIRT1 and C/EBP-β expression were significantly higher in PCOS endometrial samples compared to the controls (Fig. 3).

4. Discussion

Previous studies have demonstrated that IL-18 is upregulated in the serum and endometrium of PCOS patients.[9,15] Accordingly, we postulated that IL-18 overexpression in women with PCOS might result in reduced endometrial receptivity. However, why IL-18 is upregulated in PCOS patients remained unknown, as are the regulatory pathways that mediate this relationship.

Bioinformatics analysis has implicated C/EBP-β as a potential target of the IL-18 promoter. C/EBPs encompass a family of six proteins, of which the C/EBP-α and C/EBP-β isoforms are the most widely expressed. C/EBP-β was initially identified as a transcription factor highly expressed in liver, adipose, and lung tissues, and linked to cell proliferation and differentiation.[16] C/EBP-β has also been shown to regulate multiple IL-1β-induced human astrocyte inflammatory genes. IL-18 and IL-1β function via similar mechanisms in proinflammatory processes,[17] and previous studies have found similarities in IL-18 and C/EBP-β regulation.[18]

In our study, we found that C/EBP-β mRNA expression in the endometria of PCOS patients was 2.2-fold that of healthy controls, paralleling changes in IL-18 mRNA levels. Accordingly, we also found that C/EBP-β protein levels were increased 1.2-fold over the controls in the proliferative endometria of PCOS patients. These results suggest that C/EBP-β participates in endometrial IL-18 regulation. To further evaluate this

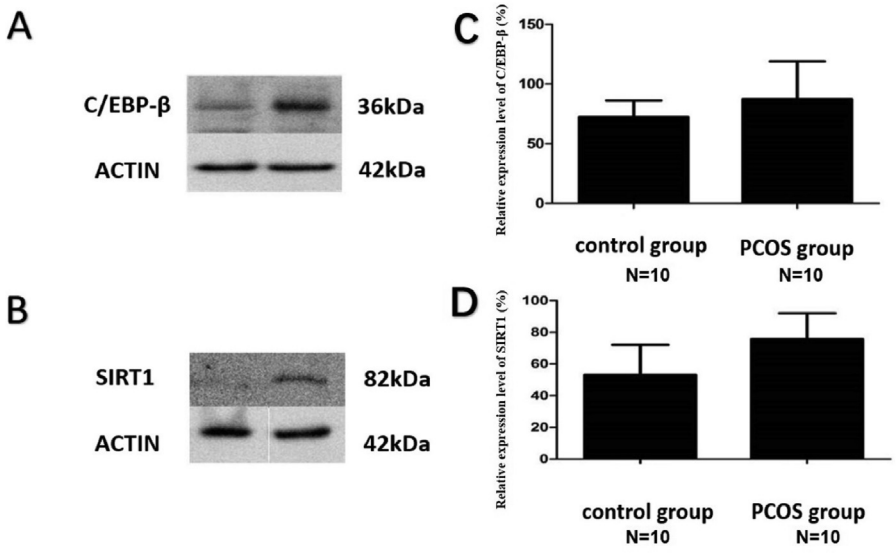

Fig. 3. C/EBP-β and SIRT1 protein expression were both increased in the endometria of PCOS patients compared to healthy women. Note: Results are expressed by relative value. Vertical axis represents relative gray value. Each band was quantified by Image J, and target protein bands are normalized by actin blotted from same electrophoresis lane.

hypothesis, we performed a dual luciferase assay to verify the relation between C/EBP-β and IL-18. C/EBP-β overexpression resulted in increased pGL4.10-IL-18 luciferase activity and this effect depended on the IL-18 promoter sequence, indicating that C/EBP-β may promote the transcription of pGL4.10-IL-18.

In the endometria of PCOS patients, high C/EBP-β expression upregulated IL-18 expression by activating the IL-18 promoter. Recent studies have shown that C/EBP-β plays an important role in the regulation of reproductive functions in female mice.[19] C/EBP-β is directly involved in the ovulation process, and the lack of C/EBP-β may result in ovulation dysfunction.[20] Mantena et al. demonstrated that C/EBP-β is an essential mediator of steroid responsiveness in the epithelia and stroma in mouse uteri and has an important role in the proliferation and differentiation of endometrial cells.[21] C/EBP-β expression is rapidly induced in pregnant uteri at the time of blastocyst attachment. Plante et al. have reported that in the normal human menstrual cycle C/EBP-β mRNA and protein expression levels also change, with increased nuclear immunostaining in the mid-secretory phase, which highlights the role of C/EBP-β in human endometrial receptivity.[22]

Notably, our findings indicate that C/EBP-β expression increases during the proliferative endometria phase in PCOS patients, as compared to healthy patients, which is not consistent with a PCOS-driven decline in endometrial receptivity. A study by Villavicencio et al.[23] found higher estrogen receptor expression during the proliferative phase; chronic estrogen exposure, or a lack of progesterone due to ovarian dysfunction, can result in endometrial hyperplasia and carcinoma. We hypothesize that the endometria implantation window is moved forward in these PCOS patients; however, there is a lack of evidence for this association. Additional research is needed to characterize endometrial receptivity and explain the poor reproductive performance associated with PCOS.

There are a couple limitations in this study. One is that no differentiation was made between the types of chronic inflammation associated with IL-18 PCOS women: obesity, insulin resistance, and/or hyperandrogenism. Additionally, secretary phase endometria may also play an important role in the embryonic implantation and warrant further investigation.

5. Conclusion

In this study, we identified the upregulation of both C/EBP-β and SIRT1 in the proliferative endometria of PCOS patients at both the gene and protein levels. Our findings indicate that SIRT1 as a key enzyme could regulate C/EBP-β expression in acetylation and activate the SIRT1/C/EBP-β/IL-18 signaling pathway, thereby leading to IL-18 upregulation, which in turn may be related to the endometrial receptivity abnormality of PCOS patients. This study furthers the understanding of C/EBP-β in PCOS and could be a basis for the development of targeted therapies for this disease.

Disclosure statement

The authors have nothing to disclose.

Ethics approval and consent to participate

This study was examined by the Peking University Third Hospital Medical Science Research Ethics Committee. The approval number of ethics examination and approval is: 2014 (083).

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

XL: Wrote the manuscript; contributed to experimental design and research plan; performed all experimental work; HW: Assisted with organization and collection of clinical specimens by gynecologists; contributed to experimental planning and design; critically reviewed manuscript proofs. XZ: provided feedback and assistance on experimental design and execution; critically revised manuscript and approved final version of manuscript; RL: assisted with organization and collection of clinical specimens by gynecologists; critically reviewed manuscript and approved final version of manuscript; YY: contributed to experimental planning and design; critically reviewed manuscript proofs, provided intellectual input on the manuscript and approved final version of manuscript; JQ: assisted with organization and collection of clinical specimens by gynecologists; contributed to experimental planning and design; critically reviewed manuscript proofs, provided intellectual input on the manuscript and approved final version of manuscript.

Authors' information (Optional)

Not applicable.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgments

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