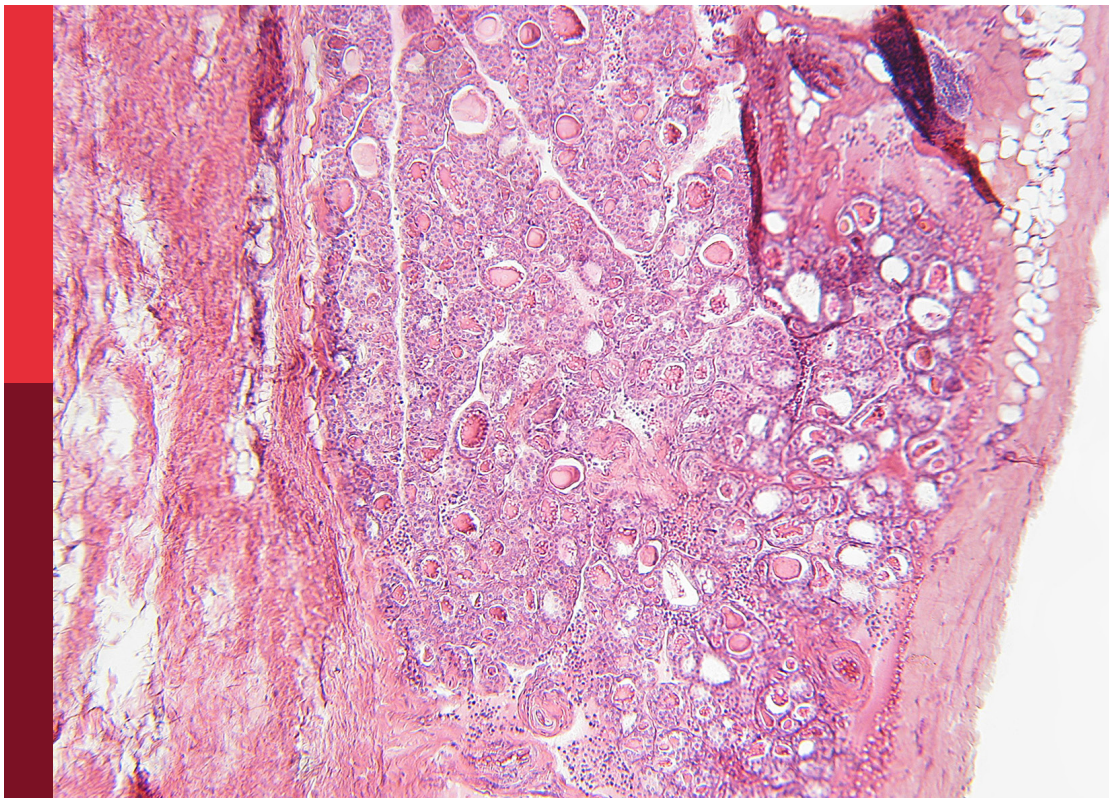
frontiers | Research Topics

Recent advances in endometriosis: from Bench to clinical application

Edited by
Achmad Kemal Harzif, Budi Wiweko and Iveta Yotova

Coordinated by
Mila Maidarti

Published in
Frontiers in Endocrinology



ISSN 1664-8714
ISBN 978-2-8325-6064-8
DOI 10.3389/978-2-8325-6064-8

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Recent advances in endometriosis: from Bench to clinical application

Topic editors
Achmad Kemal Harzif — University of Indonesia, Indonesia
Budi Wiweko — University of Indonesia, Indonesia
Iveta Yotova — Medical University of Vienna, Austria

Topic coordinator
Mila Maidarti — University of Indonesia, Indonesia

Citation
Harzif, A. K., Wiweko, B., Yotova, I., Maidarti, M., eds. (2025). *Recent advances in endometriosis: from Bench to clinical application*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-6064-8

Table of contents

frontiers | Frontiers in Endocrinology

TYPE Editorial
PUBLISHED 19 February 2025
DOI 10.3389/fendo.2025.1570284

OPEN ACCESS

EDITED AND REVIEWED BY
Richard Ivell,
University of Nottingham, United Kingdom

*CORRESPONDENCE
Iveta Yotova
iveta.yotova@meduniwien.ac.at
Achmad Kemal Harzif
kemal.achmad@gmail.com

RECEIVED 03 February 2025
ACCEPTED 10 February 2025
PUBLISHED 19 February 2025

CITATION
Yotova I and Harzif AK (2025) Editorial: Recent advances in endometriosis: from Bench to clinical application.
Front. Endocrinol. 16:1570284.
doi: 10.3389/fendo.2025.1570284

Editorial: Recent advances in endometriosis: from Bench to clinical application

Iveta Yotova[1]* and Achmad Kemal Harzif[2]*

[1]Department of Obstetrics and Gynecology, Medical University of Vienna, Vienna, Austria, [2]Department of Obstetrics and Gynecology, Faculty of Medicine Universitas Indonesia, Dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia

KEYWORDS

endometriosis, diagnosis, pathogenesis, treatment, disease models

Editorial on the Research Topic
Recent advances in endometriosis: from Bench to clinical application

Endometriosis is a chronic, estrogen-dependent disease denoted by endometrial-like growths outside of the uterus, usually on organs in the peritoneal cavity. Endometriosis is often associated with chronic gynecological or pelvic pain, and an increased likelihood of reduced fertility, with reports indicating that up to 10% of reproductive age women are affected by the disease (1). Endometriosis pathogenesis involves complex molecular mechanisms, including genetic, epigenetic, hormonal and immunological dysregulation, oxidative stress and fibrogenesis that remain to be fully deciphered (2). Patients have heterogeneous, non-specific symptoms making definite diagnosis difficult without explorative laparoscopic surgery, although non-invasive imaging techniques, such as magnetic resonance imaging (MRI) and ultrasound, may in some cases facilitate diagnosis. This difficulty in diagnosis can lead to delays in the start of treatment, illustrating the need for improved methods for diagnosis (1). Therefore, studies that hold promise for non-invasive biomarkers for diagnosing endometriosis and/or progression, such as epigenetic regulators, gene profiles or specific proteins could eventually complement imaging in enabling a comprehensive, non-invasive diagnostic approach. Treatment of the disease is largely limited to laparoscopic surgery to remove lesions, and hormonal and pain relief medications (3). Improvements to diagnosis and treatments may be aided by a better understanding of the pathogenesis of the disease, ensuring a high quality of personalized patient care in the future.

In furtherance of this vision, this Research Topic brings together a unique blend of original research, study protocols and review articles, drawing attention to the latest advances in understanding endometriosis pathogenesis, disease-associated comorbidities, and progress made toward non-invasive diagnosis and treatment.

Since their discovery in the early 1980s, exosomes have been indicated to be a universal form of cell-to cell communication, with high potential as therapy delivery systems and biomarkers in wide range of diseases including endometriosis (4). The comprehensive review by Wang et al. provides an overview of the role of exosomal long-noncoding RNAs in the regulation of different aspects of endometriosis pathogenesis, lesion neovascularization and

endometriosis-associated infertility, and discusses their potential as non-invasive diagnostic biomarker for the disease.

Together with chronic pain, endometriosis associated infertility is one of the main factors affecting the quality of life of affected women. Possible causes of infertility are molecular changes in endometrium, chronic intraperitoneal inflammation, progesterone resistance, disturbed folliculogenesis, reduced ovarian reserve, dysfunctional uterotubal motility, hormonal and immunological changes affecting embryo implantation, oogenesis and endometrial decidualization. In their comprehensive review article, Fan et al. summarize the current knowledge of the role of impaired granulosa cell function on oocyte quality in women with endometriosis. One of the ovulatory disfunction subtypes considered to be a cause of endometriosis-associated infertility is so-called luteinized unruptured follicle syndrome (LUFs) (5). However, the precise underlying reasons for association of LUFs with endometriosis remain unknown. In their original work, Geng et al. used an endometriosis mouse model to uncover molecular basis of LUFs in endometriosis. Using a range of *in vivo* and *in vitro* experiments they provide evidence that attenuation of LHCGR in granulosa cells is involved in the increased incident of LUFs in endometriosis via mechanisms leading to sustained COX-2 expression. The original work of Pei et al. links impaired endometrial stroma cells decidualization in women with endometriosis with the deregulation of autophagy associated with an increased Hippo/YAP and reduced mTOR signaling. An interesting study by Li et al. reports the negative influence of oviductal obtained vesicles (oEV) from women with endometriosis on early embryo development *in vitro*. The authors show significant changes in blastocyst transcriptome, with a major reduction of oxidative phosphorylation in murine blastocysts co-cultured with oEVs of women with the disease associated with increased cell death. Conceptually innovative, the work of Xiang et al. evaluates the changes in secretory phase eutopic endometrial transcriptome after surgical removal of lesions from women with severe endometriosis. Based on *in silico* modeling, the authors speculate that the postsurgical changes in the endometrial transcriptome may cause functional and immunological changes that improve endometrial receptivity.

The interplay between the gut microbiome and estrogen in the pathogenesis of endometriosis was evaluated by Alghetaa et al. Using a mouse model of endometriosis, the authors showed that significant changes in immune cell response in peritoneal cavity of endometriosis mice are strongly associated with changes in the gut microbiota, with an increase in bacterial species producing less short chain fatty acids metabolites compared to respective controls. These changes were associated with an accelerated metabolic rate in peritoneal inflammatory immune cells, suggesting that modulating the gut microbiota in women with endometriosis might be a powerful therapeutical strategy for treatment of endometriosis associated pain and inflammation. The advantage of in silico modeling of existing expression profiling data, followed by *in vitro* validation to aid understanding of the immune responses in endometriosis is demonstrated by the work of (Lv et al.). Here the authors identified and validated 10 central genes in an endometriosis co-expression network analysis, so-called "hub" genes, which were significantly correlated with specific disease-related immune cell infiltration and/or immune-related pathways. A similar experimental strategy was used by Pei et al. to study the molecular mechanisms of regulation of autophagy in endometrial endometriosis stroma cells. Autophagy plays an essential role for cellular response to stress conditions and ensures tissues repair and survival in health and disease (6). Hypoxia is the main stress factor that endometrial cells face in the peritoneal cavity during menstruation, and therefore, tight regulation of autophagy is an essential mechanism for endometriosis lesion development in women with endometriosis. However, the role of dysregulated autophagy in impaired endometrial receptivity in women with endometriosis is still largely unknown. In their paper, Pei et al. reported the association of Yes-associated protein (YAP) with the regulation of mTOR signaling pathway in eutopic endometrial stroma cells of women with endometriosis, and showed that YAP-mediated suppression of mTOR autophagy signaling leads to improved decidualization of eutopic endometriosis stroma cells.

Hypoxic conditions are known to favor the increase of Reactive Oxygen Species (ROS) and oxidative stress, both recognized as important pro-inflammatory mediators in endometriosis associated neurogenic pain (7). Therefore, antioxidant therapies for effective management of ROS and inflammation are of particular interest for treatment of endometriosis-associated pain and inflammation. In their randomized, triple-blind, placebo-controlled clinical study Rostami et al. tested the therapeutical potential of the strong antioxidant astaxanthin (AST) in women with the disease. The study showed that AST supplementation significantly improves oxidative stress serum markers and reduces the levels of pro-inflammatory cytokines in follicular fluid of women with endometriosis, followed by an increase in the quality and the number of oocytes, leading to improvement of assisted reproductive treatment (ART) outcomes. These findings indicate that AST pretreatment may be a suitable therapy for infertile endometriosis patients undergoing ART.

Since high fat diet (HFD) can induce chronic pain and inflammation (8), in their study the group of Herup-Wheeler et al. asked whether and how unhealthy HFD can influence endometriosis-associated pelvic pain and inflammation. Using a diet-induced obesity mouse model of endometriosis, the authors showed that HFD alone might not establish a local inflammatory environment in the pelvic cavity, but can contribute to existing endometriosis-related chronic inflammation. This leads to the aggravation of endometriosis-associated abdominal hyperalgesia due to significant increase in pro-inflammatory cytokine levels, macrophages in the peritoneal cavity, neuromodulators in the root ganglia, and dysbiosis of gut microbiota of endometriosis HFD mice, compared to obese mice without endometriosis.

Recent experimental evidence suggest that changes in glucose, lipid, amino acid and nucleotide metabolism and their molecular regulators on cellular and systemic levels are closely related to the development of endometriosis and/or with an increased risk for the disease (9). The population- based study of Liu et al. evaluated the association of the triglyceride-glucose (TyG) index with susceptibility to endometriosis in a cohort of 1590 eligible participants and found that a higher TyG index is significantly associated with increased endometriosis risk. This indicates that TyG index can potentially be used as risk assessment molecular biomarker for endometriosis and may serve as a guide to develop

future prevention strategies. The studies by Wójtowicz et al. and Zyguta et al. assessed the potential of adipokines as diagnostic markers for endometriosis. These studies indicated that an integrated multi-body fluid approach on large study cohorts is needed for successful identification and validation of clinically applicable non-invasive diagnostic biomarkers for endometriosis.

An intriguing study by the group of Bai et al. sheds a light on the causal relationship between leukocyte telomere length (LTL) and endometriosis. The authors performed Mendelian randomization study and showed that longer LTL are associated with an increased risk of endometriosis. This novel finding opens potential new aspect for investigating the genetic risk factors of the disease.

To conclude, this Research Topic represents a series of papers ranging from basic research to clinical studies that provide a valuable addition to the expanding knowledge of endometriosis.

Author contributions

IY: Conceptualization, Writing – review & editing, Writing – original draft. AH: Writing – original draft.

Acknowledgments

The authors would like to thank Dr. Quanah J. Hudson for his contribution to the final revision of the editorial draft.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Giudice LC. CLINICAL PRACTICE: endometriosis. *N Engl J Med.* (2010) 362:2389–98. doi: 10.1056/NEJMcp1000274

2. Hogg C, Horne AW, Greaves E. Endometriosis-associated macrophages: origin, phenotype, and function. *Front Endocrinol (Lausanne).* (2020) 11:1–15. doi: 10.3389/fendo.2020.00007

3. Allaire C, Bedaiwy MA, Yong PJ. Diagnosis and management of endometriosis. *C Can Med Assoc J.* (2023) 195:E363–71. doi: 10.1503/cmaj.220637

4. Barile L, Vassalli G. Exosomes: Therapy delivery tools and biomarkers of diseases. *Pharmacol Ther.* (2017) 174:63–78. doi: 10.1016/j.pharmthera.2017.02.020

5. Mio Y, Toda T, Harada T, Terakawa N. Luteinized unruptured follicle in the early stages of endometriosis as a cause of unexplained infertility. *Am J Obstet Gynecol.* (1992) 167:271–3. doi: 10.1016/S0002-9378(11)91673-1

6. Saha S, Panigrahi DP, Patil S, Bhutia SK. Autophagy in health and disease: A comprehensive review. *BioMed Pharmacother.* (2018) 104:485–95. doi: 10.1016/j.biopha.2018.05.007

7. Clower L, Fleshman T, Geldenhuys WJ, Santanam N. Targeting oxidative stress involved in endometriosis and its pain. *Biomolecules.* (2022) 12:1–22. doi: 10.3390/biom12081055

8. Duan Y, Zeng L, Zheng C, Song B, Li F, Kong X, et al. Inflammatory links between high fat diets and diseases. *Front Immunol.* (2018) 9:1–10. doi: 10.3389/fimmu.2018.02649

9. Lu J, Ling X, Liu L, Jiang A, Ren C, Lu C, et al. Emerging hallmarks of endometriosis metabolism: A promising target for the treatment of endometriosis. *Biochim Biophys Acta Mol Cell Res.* (2023) 1870:119381. doi: 10.1016/j.bbamcr.2022.119381

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 31 January 2022
doi: 10.3389/fendo.2022.813165

Increased Expression of YAP Inhibited the Autophagy Level by Upregulating mTOR Signal in the Eutopic ESCs of Endometriosis

Tianjiao Pei[1,2†], *Bin Luo*[1,2†], *Wei Huang*[1,2], *Dong Liu*[2,3], *Yujing Li*[1,2], *Li Xiao*[1,2], *Xin Huang*[1,2], *Yunwei Ouyang*[2,3] *and Huili Zhu*[1,2*]

[1] *Department of Reproductive Medicine, West China Second University Hospital of Sichuan University, Chengdu, China,* [2] *Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, West China Second University Hospital of Sichuan University, Chengdu, China,* [3] *Department of Reproductive Endocrinology, West China Second University Hospital of Sichuan University, Chengdu, China*

OPEN ACCESS

Edited by:
Abdel Halim Harrath, King Saud University, Saudi Arabia

Reviewed by:
Muhammad Farooq, King Saud University, Saudi Arabia
Abdulsamed Kükürt, Kafkas University, Turkey

***Correspondence:**
Huili Zhu
hlzhu78@139.com

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *11 November 2021*
Accepted: *03 January 2022*
Published: *31 January 2022*

Citation:
Pei T, Luo B, Huang W, Liu D, Li Y, Xiao L, Huang X, Ouyang Y and Zhu H (2022) Increased Expression of YAP Inhibited the Autophagy Level by Upregulating mTOR Signal in the Eutopic ESCs of Endometriosis. Front. Endocrinol. 13:813165. doi: 10.3389/fendo.2022.813165

We first reported that the Hippo-YAP signaling pathway plays a critical role in the pathogenesis of endometriosis (EMS). Autophagy is also related to the invasion ability of endometrial cells and is involved in the pathogenesis of EMS through multi-levels. However, the precise regulatory mechanism of YAP on autophagy in the eutopic endometrial stromal cells (ESCs) is still unclear. Primary eutopic ESCs of EMS patients (n = 12) and control patients without EMS (n = 9) were isolated and cultured to investigate the expressions of YAP and mTOR, the role of YAP in autophagy, and the effect of the YAP-autophagy signal on the decidualization of the eutopic ESCs. Endometriosis-related sequencing data (GSE51981) in the GEO database were used to find the genes significantly correlated with YAP. We found 155 genes with significant differences in the interaction with YAP in EMS from the dataset, and the autophagy pathway was significantly enriched. Following on from our previous studies of YAP knockdown, overexpression of YAP resulted in an increased expression of mTOR and decreased ratio of LC3-II/LC3-I and autophagy markers, in the eutopic ESCs; transmission electron microscope observation also showed fewer autophagosomes compared with the control cells. Furthermore, ESCs of the Rapamycin-treated group showed significant decidual-like changes with significantly increased decidual prolactin level at 72 h after *in vitro* decidualization. These results demonstrate that the increased YAP inhibited the level of autophagy by upregulating the mTOR signal in the eutopic ESCs of endometriosis. The YAP-autophagy signal plays an important role in the pathogenesis of endometriosis-associated infertility.

Keywords: endometriosis, Yes-associated protein (YAP), autophagy, eutopic endometrial stromal cells (eutopic ESCs), mammalian target of rapamycin (mTOR), decidualization

INTRODUCTION

Endometriosis (EMS) is a chronic inflammatory hormone-dependent disease, characterized by the growth of the endometrium outside the uterus, affecting more than 190 million women worldwide and up to 10% of women of reproductive age (1–3). Chronic pelvic pain and infertility caused by the disease seriously affect women's reproductive health and quality of life. According to statistics, about 25%–50% of infertile women suffer from EMS, while 30%–50% of patients with EMS suffer from infertility (4–6). Before a definitive diagnosis is made, women often endure symptoms for years with negative effects on wellbeing and quality of life (3). EMS is invasive and recurrent, so effective and thorough treatment and reduction of disease recurrence have become one of the most urgent and difficult problems in clinical practice.

The influence of EMS on women's fertility is mainly related to the abnormalities of pelvic anatomical structure, changes of abdominal microenvironment, ovarian function abnormalities, and endometrial receptivity abnormalities (7). The receptivity of endometrium is one of the important factors affecting women's fecundity. The endometrium is a complex tissue with periodic changes, which is regulated by ovarian steroids, autocrine/paracrine, and signaling pathways. During the secretion period, endometrial stromal cells undergo decidualization under the action of estrogens, which plays a crucial role in the establishment and maintenance of pregnancy. At present, endometrial receptivity abnormalities in EMS are considered to include defects in the proliferative phase, reduction of integrin αvβ3 and its direct transcriptional regulator HOXA10, and progesterone resistance (8–10). In addition, many studies have found that some signaling pathways play an important role in endometriosis, such as the over-activated MAPK pathway and PI3K/AKT pathway, which affect the role of progesterone and block the decidualization of endometrial stromal cells (ESCs) (11). In a word, abnormal regulation of endometrial signaling pathways, local inflammation, stromal differentiation, and improper endometrial reconstruction in EMS may lead to a condition of endometrium that is unacceptable for implanting embryos (8, 12).

As one of the core effector components of the Hippo signaling pathway, Yes-associated protein (YAP) is a molecule closely related to organ formation and malignancy. When the phosphorylation of YAP occurs because of intracellular and extracellular signals mediated by upstream regulatory molecules and core molecules, the phosphorylated YAP protein accumulates in the cytoplasm or degrades through the ubiquitination pathway, at which time the regulatory function of the Hippo pathway is inhibited, whereas when YAP protein is not phosphorylated, it will enter the nucleus and bind to TEA domain transcription factor, jointly regulating the expression of downstream target genes, cell proliferation, migration, and survival (13). It is currently believed that the Hippo pathway can integrate the functions of multiple signaling pathways to form a complex signaling network. YAP and its downstream transcription factors determine cell behavior in a coordinated manner and play an important role in organ development, tumor genesis and development, epithelial–mesenchymal transformation, and other cell biological behaviors (14). Abnormal programmed cell apoptosis and reduced apoptotic susceptibility play a key role in the development and invasion of EMS. Our group first reported in 2016 (15) that YAP knockdown in the eutopic ESCs decreased cell proliferation and enhanced cell apoptosis, while overexpression of YAP resulted in increased proliferation and decreased apoptosis of ESCs. We also found that after treatment with Verteporfin in the EMS animal model of nude mice, the size of endometriotic lesions was significantly reduced. It hints that the Hippo-YAP signaling pathway plays a critical role in the pathogenesis of EMS.

Autophagy, as a highly effective subcellular degradation pathway, can remove chromosomes with gene mutation damage and abnormal structure and aging or damaged organelles, carry out subcellular level reconstruction of cells, provide energy for cells, maintain intracellular material anabolism, and maintain homeostasis of the intracellular environment. In the menstrual cycle, spontaneous and periodic apoptosis of normal endometrium is an important factor to maintain its normal structure and function, and autophagy plays a key regulatory role in the apoptosis of endometrium cells in different phases of the human endometrium cycle (16).

In recent years, basic studies on EMS have shown that abnormal regulation of signaling pathways plays an important role in the occurrence and development of EMS (17). The mammalian target of rapamycin (mTOR) in the PI3K/AKT/mTOR pathway is a serine/threonine protein kinase, which is also the confluence point of the upstream pathway to regulate cell growth, proliferation, movement, survival, etc. Currently, it has been clarified that mTOR is a negative regulator of autophagy and participates in the regulatory mechanism of autophagy. Oncology studies have found that mTOR, as the junction point of the signal pathway, regulates the phosphorylation of YAP so that phosphorylated YAP remains in the cytoplasm and cannot bind to TEAD in the nucleus, thus participating in the regulation of cell metabolism and autophagy (18). Other studies have reported that MST1/2 (Hippo key enzyme) maintains autophagy through autophagy marker protein (LC3), suggesting that there may be a precise dialogue between Hippo signal and autophagy (19).

Many studies have shown that autophagy achieves the regulation of EMS through the interference of multiple pathways at multiple levels, and promotes the occurrence, development, and invasion of EMS (20–22). In 2015, Zhang et al. (15) found that autophagy gene Beclin-1 mRNA and protein expression in ESCs in EMS diseases decreased and were negatively correlated with CA125 level and pain. Choi et al. (23) reported that autophagy and apoptosis are simultaneously involved in the pathological process of EMS, and this process is mediated by mTOR, and the abnormal mTOR activity affects the change of autophagy activity. Rat model studies have shown that there is an autophagy downregulation in both the eutopic and ectopic of endometrium, and the autophagy flow inhibitor hydroxychloroquine (HCQ) can

effectively shrink and destroy the ectopic lesions, which is expected to be a new target for the treatment of EMS (21). Recently, we reported that increased expression of YAP is associated with decreased cell autophagy in the eutopic ESCs of EMS (24). Although there is a downregulation of autophagy in EMS, the precise regulation of autophagy in endometrial stromal cells in EMS remains unclear, which may involve multiple signaling pathways.

In recent years, studies on the interaction and regulation of YAP and mTOR/autophagy have been one of the hotspots in the study of tumor cell mechanisms. In 2019, it was reported (25) that YAP is highly expressed in ovarian cancer, and silencing YAP may significantly inhibit the malignant behavior of ovarian cancer cells by regulating the PI3K/Akt/mTOR pathway. Zhou et al. (26) found that YAP promoted multi-drug resistance of liver cancer cells and inhibited autophagy-related cell death. However, there have been no reports on the regulatory relationship between YAP and autophagy, the role of YAP and autophagy in the pathogenesis of EMS, and their effects on endometrial receptivity. Therefore, the objective of this study is to explore the role of YAP in the regulation of cell autophagy in the eutopic ESCs from a subset of women with endometriosis and to understand the effect of the YAP-autophagy signal on the decidualization of the eutopic ESCs.

MATERIALS AND METHODS

Participants

This study was approved by the Ethics Committee of West China Second University Hospital of Sichuan University. Written informed consent was obtained from each patient. All participants aged 20–35 years, with regular menstrual cycles and no history of hormonal treatment for at least 3 months before surgery, were included in the study between September 2017 and August 2018. Those who suffered from infertility associated with factors of fallopian, tube, ovary or uterine, or abnormal semen were excluded from the study. Endometrial samples were collected during hysteroscopy and determined by endometrial pathological dating to be in the mid-secretory phase. Twelve women were laparoscopically diagnosed with endometriosis. Another 9 women with hysteroscopic normal uterine cavity and who were laparoscopically endometriosis-free were treated as controls. Samples of the eutopic endometrium that showed endometrial lesions through pathological examinations were excluded. All samples were immediately transferred to the laboratory for primary cell culture or stored in nitrogen for further analysis.

Data Collection

The gene expression profile of GSE51981 was downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). We selected 111 samples from the GSE51981 dataset, containing a total of 77 endometriosis samples (different menstrual cycle phases and different stages of disease) and 34 non-endometriosis samples without uterine pelvic pathology (27). To find gene sets and pathways significantly correlated with YAP in this research and to understand the signal pathways and functional modules related to YAP in EMS, we used the processed data to filter differentially expressed genes (DEGs) and conducted gene enrichment analysis.

Differentially Expressed Genes

Gene differential analysis was conducted using the limma R package between EMS samples and non-EMS/normal uterus samples. We considered DEGs as $|\log2\text{fold change (FC)}| > 1$ and adjusted p-value < 0.05. Heatmap and Volcano Plot were generated by pheatmap package and ggplot package, respectively.

PPI Network Construction

Protein–protein interaction network analysis provides much valuable information for researchers to understand cell function and biological processes. Many studies have shown that neighboring proteins always have some common characteristics in PPI networks (28). We used Search Tool for the Retrieval of Interacting Genes Database (STRING) [https://www.string-db.org/, (9606.protein.links.v9.1) (29)] to assess PPI information (30). To explore the possible protein interaction and relationship between DEGs and YAP in EMS, we used the STRING analysis and converted the results visually by using Cytoscape software. Protein–protein interaction score > 0.4 was set as significant (30).

GO Term and KEGG Pathway Enrichment Analyses of DEGs

To explore the signaling pathway and biological function most closely related to YAP in EMS, we conducted GO functional annotation analysis and KEGG pathway enrichment analysis on the DEGs with the most significant interaction with YAP obtained from the previous PPI analysis. KEGG is a comprehensive database that integrates information on genome, chemistry, and system functions. GO is a comprehensive database describing the function of genes, which can be divided into three categories: biological process and cellular component molecular function. The pathway enrichment analysis and functional enrichment analysis of DEGs were analyzed and visualized by Clusterprofiler R package. The standard setting of KEGG pathway enrichment with statistical significance was $p < 0.05$ and enrichment score >2.0. The criteria with statistical significance for GO functional annotation were $p < 0.05$ and enrichment score >1.0.

Immunofluorescence

When ESCs were passaged to the third passage and cell fusion to 90%, cell suspension after passage was added into the 24-well plate according to the cell density of 50% per well and incubated overnight in an incubator (37°C, 5% CO_2). The next day, ESCs on chamber slides were washed two times with PBS and fixed in 4% paraformaldehyde for 10 min and then cells were washed with PBS again two times. Cells were permeabilized with 0.2% Triton X-100 (Sigma) for 30 min at room temperature when individual cells and cell clusters were observed under the microscope. After blocking with 5% BSA for 15 min, slides were incubated overnight at 4°C with monoclonal rabbit

antihuman YAP (1:200, ab52771, Abcam) and monoclonal rabbit antihuman mTOR (1:300, CST#2983S, Abcam). Primary antibodies were detected by incubation with corresponding IgG secondary antibodies conjugated with Alexa Fluor 594 (red) and 488 (green) (1:500, Invitrogen, Eugene, Oregon, USA) for 1 h. Nuclei of the cells were counterstained with 6-diamino-2-phenylindole (DAPI; Sigma-Aldrich, USA).

Cell Culture and Transfection

The ESCs were isolated from the eutopic endometrium of the endometriosis and control group, cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) supplemented with 10% fetal bovine serum (life technologies) and 100 U/ml penicillin/streptomycin at 37°C under a 5% CO_2 condition. Construction and production of YAP-overexpression (OE) plasmid was made by Shandong Vigene Biosciences Co., Ltd. When cells reached 80% confluence, they were digested, seeded at 1×10^5 cells per six-well plate, cultured to 30% to 40% confluency, and then transfected with the YAP-OE plasmid (1 μg) and empty plasmid using Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After transfection for 72 h, cells were harvested for further analysis. The transfection efficiency of YAP was measured by qPCR and Western blotting.

RNA Isolation, cDNA Synthesis, and Real-Time PCR

Total RNA was isolated from all ESCs using the TRIzol (Life Technologies, Carlsbad, CA) reagent according to the manufacturer's protocol. RNA quantification and purification were performed using a NanoVue Plus spectrophotometer (Healthcare Bio-Science AB, Uppsala, Sweden). The nucleotide: protein ratios (A260:A280) of all the samples were within the range 1.9–2.1. cDNA was synthesized using a PrimeScript RT reagent kit (Takara Biomedical Technology Co., Ltd., Beijing, China) and was diluted 8-fold for PCR amplification. Amplification and detection *via* qPCR were performed in a total reaction volume of 10 μl, consisting of diluted cDNA (3 μl), SYBR Green real-time PCR Master Mix (Applied Biosystems, Carlsbad, CA) (5 μl), forward primer (1 μl), and reverse primer (1 μl), using a CFX96 Realtime PCR system (Bio-Rad Laboratories), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The PCR cycle was 20 s at 95°C, then 40 cycles of 10 s at 95°C and 20 s at 60°C. The specificity of PCR products was confirmed by analysis of the dissociation curve. Relative gene expression was calculated using the $2^{-\Delta\Delta}$CT method. The sequence-specific primers used to amplify the gene products are shown in **Table 1**. Samples were examined in triplicate, and all experiments were repeated three times.

TABLE 1 | Primer sequences used in quantitative real-time PCR.

Gene	The sequence-specific primers
YAP1	Forward primer: 5′-CACAGCATGTTCGAGCTCAT-3′ Reverse primer: 5′-GATGCTGAGCTGTGGGTGTA-3′
GAPDH	Forward primer: 5′-TGCACCACCAACTGCTTAGC-3′ Reverse primer: 5′-GGCATGGACTGTGGTCATGAG-3′

Protein Extraction and Western Blotting

Total cellular protein was isolated from ESCs using RIPA buffer with 1% PMSF (Beyotime Biotechnology, Shanghai, China) and protease inhibitors on ice, and protein concentration was determined using the BCA protein assay kit (Beyotime, Biotechnology, Shanghai, China). Equal amounts of protein extracts (50–100 mg) were separated through 6%, 8%, and 15% polyacrylamide gels containing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), respectively, and transferred to 0.45 μm polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA). Membranes were blocked in 5% milk for 1 h at room temperature and then were washed and incubated with primary antibodies: rabbit monoclonal anti-YAP (Abcam, USA; 1:1,000 dilution), rabbit monoclonal anti- mTOR (CST, USA; 1:500 dilution), rabbit polyclonal anti-LC-3B (Abcam, USA; 1:500 dilution), rabbit anti-GAPDH (Sigma-Aldrich, USA; 1:3,000 dilution), and rabbit anti-beta Actin (Abcam, USA; 1:1,000 dilution) (**Table 2**). Following the overnight incubation, membranes were washed thrice with 0.1% Tween in Tris-buffered saline (TBST) and incubated with a DyLight 800-conjugated goat anti-rabbit IgG secondary antibodies (Thermo Scientific, USA; 1:10,000 dilution) for 2 h. Protein bands were imaged on an infrared imaging system using a Double color infrared laser imaging system (Odyssey, LI-COR, USA), and quantified by Quantity One

TABLE 2 | Antibodies used in this study.

Peptide/Protein Target	Antibody Name	Manufacturer, Catalog No.	Species Raised in; Monoclonal or Polyclonal	Dilution Used	RRID
YAP1 Human	YAP1 antibody	Abcam, #ab52771	Rabbit; monoclonal	1:1,000	AB_2219141
mTOR Human, Mouse, Rat,	mTOR (7C10) Rabbit mAb antibody	CST, 2983S	Rabbit; monoclonal	1:500	AB_2105622
LC-3B Mouse, Human	LC3B antibody	Abcam, #ab51520	Rabbit; polyclonal	1:500	AB_881429
GAPDH Rat, Mouse, Human,	GAPDH antibody	Sigma-Aldrich, #G9545	Rabbit; polyclonal	1:3,000	AB_796208
Rabbit IgG	Goat anti-rabbit IgG secondary antibodies	Thermo Fisher Scientific, SA5-10036	Goat; polyclonal	1:10,000	AB_2556616

software (Bio-Rad Laboratories). Protein levels were normalized to that of the internal control GAPDH and β-actin.

Transmission Electron Microscope Observation

To observe the autophagy and ultrastructural changes of the eutopic ESCs after YAP-OE transfection, we performed a TEM observation. Transfected cells were digested and centrifuged, the supernatant was discarded, the precipitate was kept, and fixed in 3% glutaraldehyde. After washing in 0.1 M phosphate buffer, the samples were postfixed with 1% osmium tetroxide in the same buffer for 1 h at 4 °C. Then, the samples were dehydrated with a series of the graded acetone solution, and the samples were next embedded in Epon. Ultrathin sections (~50 nm) were obtained by an ultramicrotome (Leica Ultracut UCT, Germany). Ultrathin sections were double stained with uranyl acetate and lead citrate, and they were examined in a TEM (JEM-1400PLUS, Japan) to detect autophagosomes.

In Vitro Decidualization

To explore the effect of YAP-mTOR signal on the decidualization of the eutopic ESCs, we performed an *in vitro* decidualization induction of the eutopic ESCs after interfering with the YAP function by YAP-TEAD inhibitor Verteporfin (S1786, Selleckchem, USA) (1 μM, 18 h) and blocking the mTOR signal by Rapamycin (S1039, Selleckchem, USA) (100 nM, 4 h) which also can induce autophagy. Verteporfin and Rapamycin treatments were described previously (24). DMSO is the negative control group. Decidualization was induced in ESCs at 70% to 80% confluence after Verteporfin and Rapamycin treatments in 10% charcoal-stripped phenol red-free medium for 24 h. The medium was then replaced, and the cells were cultured with 2% charcoal-stripped phenol red-free medium supplemented with 0.5 mM 8-Br-cAMP (Abcam, Cambridge, MA) and 1 mM medroxyprogesterone acetate (MPA) (Sigma-Aldrich, St. Louis, MO) for 72 h. The culture medium was collected every 24 h, and the supernatants were incubated at −20°C to detect decidual prolactin (dPRL). dPRL protein levels in the supernatants, which are a representative marker of decidual cells, were determined using a commercially available ELISA kit (Cusabio Biotech, Wuhan, China). The ESCs were observed under an inverted microscope every 24 h to study their morphological changes.

Statistical Analysis

All statistical analyses were performed using the software program SAS version 9.2 (SAS, Institute Inc, USA). The normally distributed data were analyzed by Student's *t*-test. Data were represented as the mean ± standard deviation (SD). $p < 0.05$ was considered statistically significant (two-tailed).

RESULTS

Screening of Differentially Expressed Genes

The R package "limma" was used to screen DEGs between EMS and non-endometriosis samples without uterine pelvic pathology in GSE51981, where a total of 829 DEGs associated with YAP were screened under the threshold of |log2fold change (FC)| > 1 and adjusted *p*-value <0.05. All 829 DEGs are listed in **Supplementary Table S1**. The Volcano Plot is shown in **Figure 1A**. Genes that are upregulated are in red, those that are downregulated are in blue, and those that are insignificantly different are in black.

PPI Network Integration

We used the STRING database to find the gene sets significantly correlated with YAP in EMS. A total of 155 DEGs with significant differences in the interaction with YAP in EMS were found from the GSE51981 chip samples through the

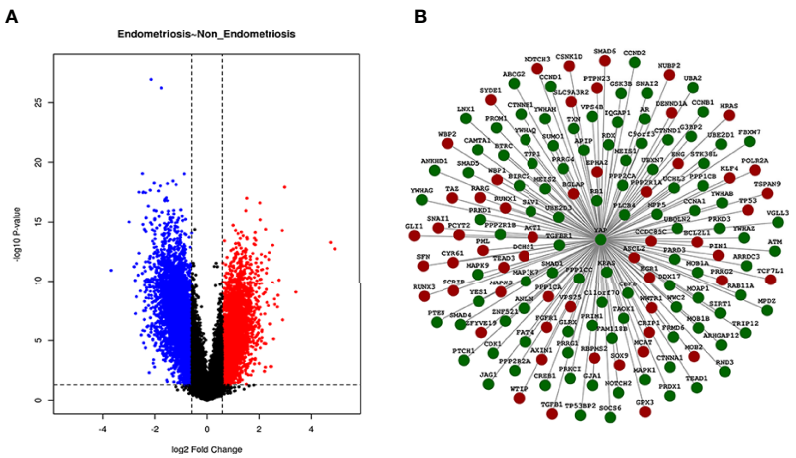


FIGURE 1 | Volcano Plot and PPI network of DEGs. In the Volcano Plot, genes that are upregulated are in red, those that are downregulated are in blue, and those that are insignificantly different are in black **(A)**. Different protein interaction graph with YAP interaction scores greater than 0.4 in GSE51981 samples were shown in the PPI network. Green indicates downregulated expression and red indicates upregulated expression **(B)**.

analysis of the protein interaction network (protein–protein interaction score > 0.4 was set as significant). The heatmap of these 155 DEGs with significant differences in the interaction with YAP is shown in **Supplementary Figure S1**, and DEGs are listed in **Supplementary Table S2**. A PPI network of DEGs was performed as shown in **Figure 1B**.

GO Biological Process Analysis and KEGG Pathway Enrichment of DEGs

GO analysis of genes includes biological processes (BP), cell composition (CC), and molecular function (MF). In our study, GO analysis was used to perform the functional process of the DEGs with significant differences in the interaction with YAP in EMS. A p-value <0.05 and enrichment score >1.0 were defined to identify regulated genes in GO functional enrichments. The results are shown in **Figure 2A**. GO biological process analysis found that DEGs were mainly enriched in the regulation of apoptotic signaling pathway, gland development, epithelial cell proliferation, urogenital system development, and positive regulation of apoptotic signaling pathway. In the cell composition part, the DEGs were involved in the transcription regulator complex, focal adhesion, cell–substrate junction, and cell–cell junction. In the molecular function section, the genes participated in cadherin binding, ubiquitin-like protein ligase binding, ubiquitin protein ligase binding, and protein C-terminus binding. These results suggested that DEGs with significant differences in the interaction with YAP were mostly involved in cell pathways, cell cycle, cell junction, and binding. All GO analysis is listed in **Supplementary Table S3**.

We also performed KEGG pathway analysis. A p-value <0.05 and enrichment fold >2.0 were defined to identify regulated genes in KEGG pathway analysis enrichments. The results are shown in **Figure 2B**. Among these enriched signaling pathways, the Hippo signaling pathway was the most significantly associated with YAP interaction DEGs, while other enriched signaling pathways included cell cycle and division-related pathways (cellular senescence, cell cycle, and oocyte meiosis), liver disease-associated pathways (hepatocellular carcinoma, hepatitis C, and hepatitis B), multiple cancer-related pathways (breast cancer, gastric cancer, colorectal cancer, prostate cancer, and endometrial cancer), and the PI3K-Akt signaling pathway, which is closely associated with tumor immunity. It is worth noting that DEGs with significant differences in the interaction with YAP in EMS are enriched in the autophagy signaling pathway (p<0.05) (**Supplementary Table S4**). It suggests that YAP may be correlated with autophagy in EMS.

The Protein Locations of YAP and mTOR in the Eutopic ESCs

Our previous study detected the mRNA and protein levels of YAP and mTOR, negative regulator of autophagy, in the eutopic ESCs. To explore the protein locations of YAP and mTOR in the eutopic ESCs, we performed immunofluorescence. The results showed that YAP was mainly expressed in the nucleus of the eutopic ESCs and a little in the cytoplasm, whereas in the normal ESCs, YAP was mainly located in the cytoplasm and slightly expressed in the nucleus (**Figure 3**). mTOR was mainly expressed in the cytoplasm of the eutopic ESCs and a little in the nucleus, whereas mTOR was expressed in small amounts in the cytoplasm of normal ESCs, but it was hardly expressed in the nucleus (**Figure 3**).

Overexpression of YAP in the Eutopic ESCs of Endometriosis Inhibited Autophagy Level

Based on our previous finding that knockdown of YAP in the eutopic ESCs of EMS increases autophagy level, we supplemented the YAP-OE experiment to further explore and clarify the regulatory mechanism of YAP on cell autophagy. The eutopic ESCs were transfected with a YAP-OE plasmid.

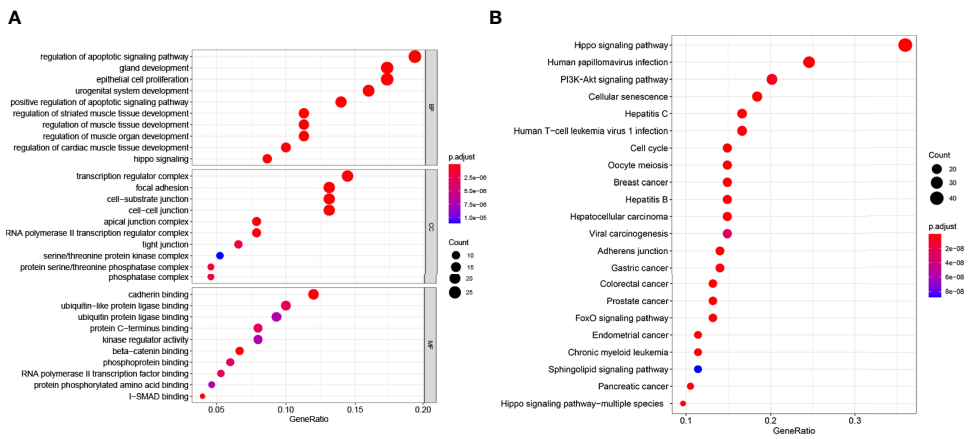


FIGURE 2 | GO and KEGG enrichment analysis of the 155 genes significantly associated with YAP. **(A)** The abscissa is the percentage of genes under the GO functional module (ratio); the ordinate is the GO functional module: BP (biological process), CC (cell composition), and MF (molecular function). **(B)** The x-coordinate in the figure is the number of DEGs annotated to KEGG pathway/the total number of differentially expressed genes (ratio); the ordinate is the KEGG pathway. The size of the point represents the number of DEGs annotated to the KEGG pathway.

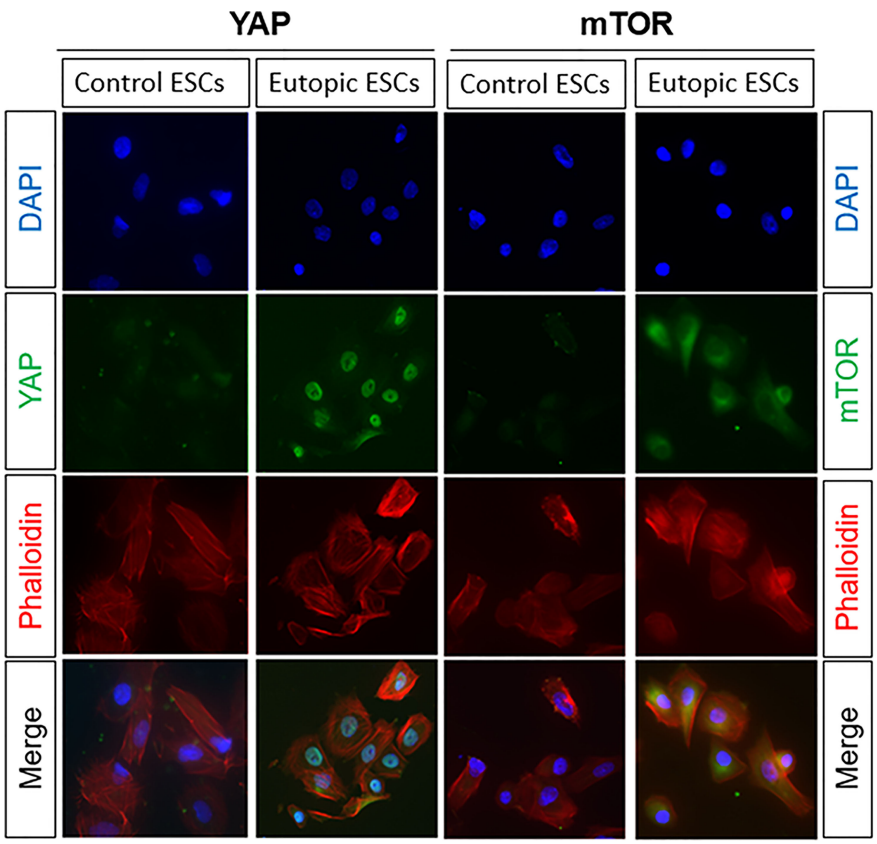


FIGURE 3 | The protein locations of YAP and mTOR in the eutopic ESCs. The results of immunofluorescence showed that YAP was mainly expressed in the nucleus of the eutopic ESCs and a little in the cytoplasm, whereas in the normal ESCs, YAP was mainly located in the cytoplasm and slightly expressed in the nucleus. mTOR was mainly expressed in the cytoplasm of the eutopic ESCs and a little in the nucleus, whereas mTOR was expressed in small amounts in the cytoplasm of normal ESCs, but it was hardly expressed in the nucleus.

The empty plasmid was used as control. qPCR showed that the expression of YAP mRNA was increased significantly (3.60 ± 0.16 vs. 1.00 ± 0.21; $p = 0.0006$) after transfection with YAP-OE plasmid in the eutopic ESCs compared with controls (**Figure 4A**). Western blotting revealed that we obtained a high OE efficiency at the YAP protein level (3.04 vs. 1.00) (**Figure 4B**). It combined to suggest that the overexpression of YAP worked. The expression of mTOR protein (2.11 vs. 1.00) was significantly increased in the eutopic ESCs compared with controls following YAP-OE. By contrast, there was a significantly decreased ratio of the autophagy marker protein LC3-II/LC3-I (0.44 vs. 1.00) (**Figure 4C**). TEM observation also showed fewer autophagosomes in the YAP-OE group compared with the control cells (**Figure 5**). These data demonstrated that overexpression of YAP in the eutopic ESCs of endometriosis inhibited the level of cell autophagy.

Rapamycin Promotes the *In Vitro* Decidualization of ESCs

To explore the effect of the YAP-autophagy signal on the *in vitro* decidualization of ESCs, we further induced decidualization by Verteporfin and Rapamycin treatments or negative controls (DMSO) exposed to decidual induction *in vitro*. The results showed that compared to the cultured control group, the morphology of most ESCs in the Verteporfin treatment group had no obvious changes after 24 h, 48 h, and 72 h of *in vitro* induction, and a large number of ESCs showed a spindle-like shape. After only 72 h of induction, a small number of ESCs in the control group were rounded and enlarged, with slight decidual-like changes, whereas the ESCs of the Verteporfin group did not change significantly (**Figure 6A**). ELISA results suggested that there was no significant difference in the dPRL levels of decidualization marker in the culture medium of ESCs in the groups of Verteporfin and the control group after 24 h, 48 h, and 72 h of decidualization ($p > 0.05$) (**Figure 6B**).

Regarding the Rapamycin group, compared with the control group, most ESCs in the Rapamycin-treated group were spindle shaped 24 h after *in vitro* decidualization. After 48 h of *in vitro* induction, some of the ESCs in the Rapamycin-treated and control groups were rounded and enlarged. Seventy-two hours after *in vitro* induction, compared with the control group, the Rapamycin-treated ESCs showed obvious decidual-like changes, with a large number of cells becoming round, enlarged, and rich in cytoplasm (**Figure 6C**). ELISA results suggested that there was

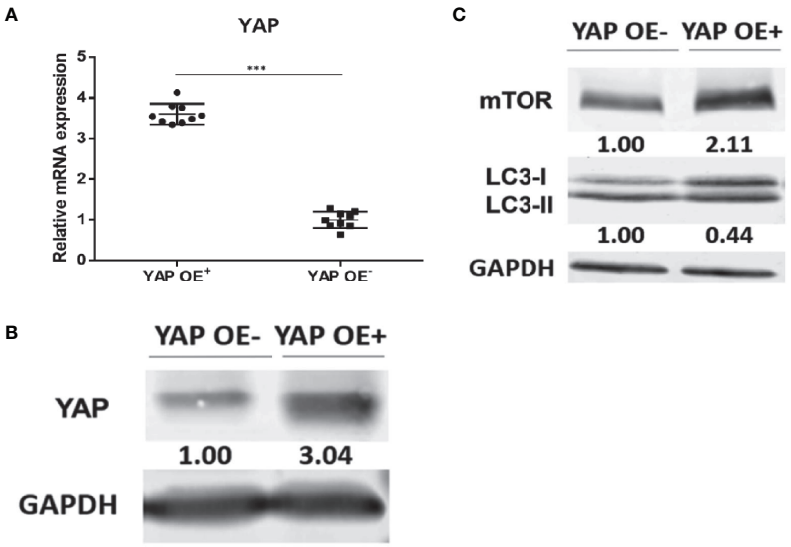


FIGURE 4 | Overexpression of YAP in the eutopic ESCs of endometriosis inhibited autophagy level. The qPCR showed that the expression of YAP mRNA was increased significantly (3.60 ± 0.16 vs. 1.00 ± 0.21; $P = 0.0006$) after transfection with YAP-overexpression plasmid in the eutopic ESCs compared with controls (the eutopic ESCs transfected with the empty plasmid) **(A)**. Western blotting revealed that we obtained a high OE efficiency at the YAP protein level (3.04 vs. 1.00) **(B)**. The expression of mTOR protein (2.11 vs. 1.00) was significantly increased in the eutopic ESCs compared with controls following YAP-OE. By contrast, there was a significantly decreased ratio of the autophagy marker protein LC3-II/LC3-I (0.44 vs. 1.00) **(C)**. ***p = 0.0006.

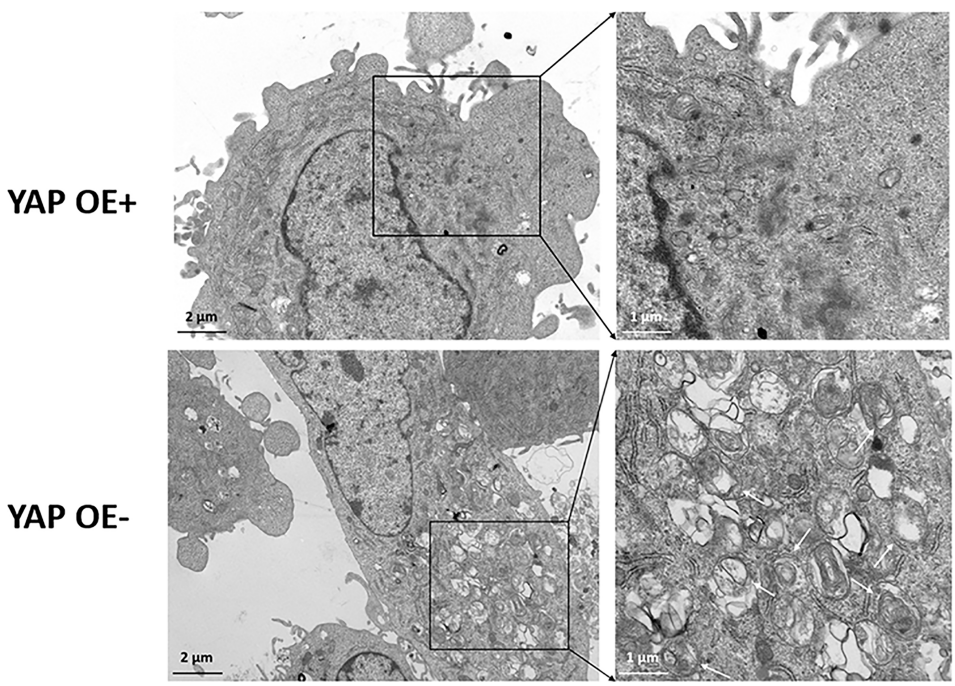


FIGURE 5 | Transmission electron microscope (TEM) observation of autophagosomes after overexpression of YAP in the eutopic ESCs of endometriosis. TEM observation showed the fewer autophagosomes in the YAP-OE group compared with the control cells.

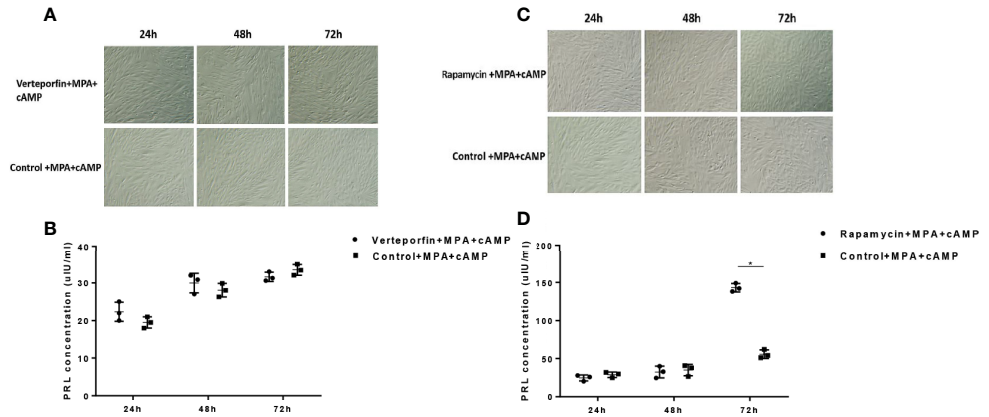


FIGURE 6 | Effects of Verteporfin and Rapamycin treatments on the *in vitro* decidualization of ESCs. Compared to the cultured control group, the morphology of ESCs in the Verteporfin treatment group had no obvious decidual changes after 24 h, 48 h, and 72 h of *in vitro* induction **(A)**. ELISA results suggested that there was no significant difference in the dPRL levels in the culture medium of ESCs in the groups of Verteporfin and the control group after 24 h, 48 h, and 72 h of decidualization ($p > 0.05$) **(B)**. After 24 h and 48 h of *in vitro* induction, some of the ESCs in the Rapamycin-treated and control groups were rounded and enlarged. Seventy-two hours after *in vitro* induction, compared with the control group, the Rapamycin-treated ESCs showed obvious decidual-like changes **(C)**. After 72 h of decidualization, the dPRL level was significantly increased in the cell culture medium of the Rapamycin group compared with that of the control group ($p < 0.05$) **(D)**. *$p < 0.05$.

no significant difference in the dPRL level of decidualized marker between the Rapamycin group and control group after 24 h and 48 h of decidualization ($p > 0.05$). After 72 h of decidualization, the dPRL level was significantly increased in the cell culture medium of the Rapamycin group compared with that of the control group ($p < 0.05$) (**Figure 6D**).

DISCUSSION

In this study, we first found a significant correlation in the YAP interaction set of 155 genes through bioinformatics analysis, from the GSE51981 chip samples of endometriosis-associated research (Endometriosis vs. Non-Endometriosis), analyzed the GO enrichment analysis and KEGG pathway enrichment, and found that these genes interact with YAP and the enrichment of autophagy function significantly; YAP may be correlated with cell autophagy in EMS.

On this basis, *in vitro* cell experiments showed that the protein level of the negative regulator of autophagy, mTOR, was significantly increased in the eutopic endometrial stromal cells in EMS than in the normal endometrial stromal cells, while the ratio of autophagy marker protein LC3-II/LC3-I was significantly decreased in the eutopic ESCs than in the normal endometrial stromal cells. When autophagy is forming, cytoplasmic LC3 (LC3-I) will enzymatically degrade a small polypeptide and transform into membrane-type autophagosome (LC3-II), which means that LC3-II will increase significantly in autophagy cells (31). Therefore, the high expression of negative autophagy regulatory factor mTOR and the reduced LC3-II/LC3-I ratio in the eutopic endometrial stromal cells indicated that the autophagy level of the endometrial stromal cells in EMS was significantly inhibited. To the extent of protein locations, our immunofluorescence showed the protein locations of YAP and mTOR in the eutopic ESCs from the nucleus to cytoplasm and *vice versa*. It suggests that YAP is activated after nuclear localization and is combined with related transcription factors. At this time, the Hippo-YAP signaling pathway is activated to exert its regulatory role on downstream genes. Similarly, the increased expression of mTOR in the cytoplasm of eutopic ESCs indicates that the mTOR pathway was active, and the autophagy level was impaired in the eutopic ESCs. This is consistent with the results of studies on the decreased level of autophagy in the eutopic and ectopic endometrium tissues in many cases of EMS (20, 32). Based on these findings, we speculated that there is a deeper connection between the Hippo-YAP signaling pathway and autophagy in EMS, and mTOR may be a key mediator molecule.

Many oncology studies have shown that YAP is involved in the regulation of autophagy signals (33–35). For example, YAP reduces cisplatin-induced apoptosis by activating autophagy in ovarian cancer cells (33). In undifferentiated pleomorphic sarcoma cells, YAP inhibits autophagy independently of NF-κB signaling (35). Studies on gastric cancer have found that the downregulation of YAP truncates weak mitochondrial autophagy, leading to the apoptosis of gastric cancer cells (36). In addition, YAP also promotes multi-drug resistance of liver cancer cells through the RAC1-Ros-MTOR pathway, thereby inhibiting autophagy-related cell death (26). So, does the increased expression of YAP in endometrial stromal cells in EMS participate in the regulation of the weakened autophagy signal in endometrial cells? In the following studies, we intend to conduct in-depth studies on the regulation mechanism of YAP on autophagy in the eutopic endometrial stromal cells in EMS and the biological functions involved in both.

To further clarify the regulatory relationship between YAP signal and autophagy in EMS, a YAP overexpression experiment was conducted in endometrial stromal cells in EMS. mTOR and autophagy levels were detected by direct interference with YAP expression. The results showed that the mTOR protein level and lc3-II/LC3-I ratio in the eutopic ESCs increased after the overexpression of YAP. Since autophagosomes are subcellular structures, the formation of autophagosomes cannot be observed under an ordinary light microscope, so direct observation of autophagosomes requires TEM. As the gold standard for the detection of autophagy, TEM results showed that the number of autophagosomes in the ESCs overexpressing YAP was significantly reduced compared with the control group, suggesting that the level of autophagy was inhibited. In recent years, several studies have found that YAP and autophagy are closely related. For example, the YAP/TAZ-autophagy axis can control the survival and proliferation of cells (37). As an upstream transcriptional regulator, YAP activates the mTOR pathway and inhibits autophagy (38). Our study revealed the correlation between YAP and autophagy in EMS and opened new ideas and directions for future basic research on signaling pathways in EMS and autophagy function. Combined with our previous finding that the mTOR protein level decreased and the LC3-II/LC3-I ratio significantly increased after silencing YAP in the eutopic ESCs, the level of autophagy was enhanced. In conclusion, this part of the study shows that YAP may be involved in the regulation process of the decreased autophagy level of the eutopic ESCs in EMS through the upregulation of the mTOR pathway, thus participating in the occurrence and development of EMS.

YAP is the main effector molecule of the Hippo signaling pathway. The Hippo signaling pathway has extensive and complex cross-linking with many other signaling pathways, and its different effects depend on different cells, different external environments, different co-activators, and different feedback of various upstream and downstream factors. Since YAP has no DNA binding region, it needs to be combined with TEADs or other transcription factors to participate in the regulation of downstream gene expression (39). Studies have found (40) that TEAD binding sites on prolactin (PRL) promoters play an important role in maintaining the basal level of PRL promoter activity, and the overexpression of TEAD1 inhibits the expression of PRL in human decidual cells, which may be realized through the interaction with other transcription factors. In 2017, our group found that YAP was highly expressed in decidual cells and promoted decidualization of ESCs cultured *in vitro*, indicating that YAP is involved in the regulation of normal endometrial stromal cell decidualization (41). A study published in *Hum Reprod* (42) found that decidual induction was performed in immortalized human endometrial stromal cells, and the level of autophagy marker protein LC3-II was significantly increased in decidual cells compared with the control group, indicating that the decidual process of endometrial stromal cells may be related to cell autophagy. Therefore, we speculated that the YAP-autophagy signal may be involved in the regulation of decidualization of the eutopic ESCs in endometriosis and play an important role in embryo implantation.

Previous studies have confirmed that EMS has decreased receptivity in the endometrium, and numerous molecules and signal pathways are involved in the regulation of decidualization of ESCs (11, 43). Local inflammation, stromal differentiation, and improper endometrial reconstruction of endometrium in endometriosis all lead to the endometrial condition that embryo implantation is not acceptable (8, 12). In this study, it has been found that the decidualization of the eutopic ESCs after treatment by Verteporfin, a YAP signal inhibitor, has not affected the decidualization of eutopic ESCs. The decidualization of the endometrium is a very complex and delicate process, which is regulated by many factors, including cytokines, immune cells, and hormones, and accompanied by various epigenetic changes during decidualization. In addition, the molecular and biological characteristics of the endometrium in EMS are fundamentally different from those in non-endometriosis patients (44–46). Therefore, it is understandable that Verteporfin does not change the decidual process of ESCs in endometriosis. As a non-photosensitizer, it inhibits the transcription and translation of YAP and destroys the complex formed by YAP and other downstream transcription factors. However, this blocking effect does not affect or participate in the decidual regulation process of endometrial stromal cells. Based on the former part of the negative regulation effect of YAP-mTOR signals on cell autophagy in the eutopic ESCs, we treated the eutopic ESCs with mTOR inhibitor Rapamycin (autophagy inducer), and induced decidual cells *in vitro*. It is found that Rapamycin had no significant effect on decidualization of ESCs after 24-h and 48-h induction, but Rapamycin treatment promoted the decidualization of the eutopic ESCs after 72 h, and this difference may be due to the induction of decidual function caused by delay and cell gradually. At present, there is no unanimous conclusion on the effect of mTOR autophagy on endometrial decidualization. Studies have reported (47–49) that the mTOR pathway plays an important role in the early embryo implantation process, and the activation of the mTOR pathway promotes NM23 to regulate the decidualization process of mouse and human endometrium. It was also found (42) that the autophagy level was upregulated during decidualization of the endometrium in mice, which was consistent with our findings. In other words, after the administration of the autophagy-inducing agent Rapamycin, decidual changes in the eutopic ESCs were significant, which may be related to the regulation of Rapamycin on the autophagy of the eutopic ESCs.

This study preliminarily explored the effect of the YAP-autophagy signal on the decidualization of the eutopic ESCs and found that the decidual process of the eutopic ESCs was promoted after inhibiting mTOR activity and activating autophagy, suggesting that autophagy may be involved in the regulation of decidualization of the eutopic ESCs in endometriosis. However, whether the increased YAP in the eutopic ESCs of patients with EMS-associated infertility is directly involved in the regulation of decidualization of the endometrium, how enhanced autophagy level regulates the

process of decidualization of the endometrium, and whether there are other pathways involved are still unknown and need to be further studied.

There are still some limitations in this study. For example, the mechanisms of YAP regulation of autophagy in the eutopic ESCs was explored *in vitro* only in patients with EMS-associated infertility, which would be more convincing if validated in an *in vivo* model. In addition, any signaling pathway is not a single one, and there are extensive crossover and network-like interactions among them, and a certain pathway cannot explain all the research problems. In the future, more transcriptomics and proteomics studies are needed to study the histological and cellular structure of the endometrium, as well as the interactions between genes, proteins and molecules from an overall perspective, so as to provide new ideas for exploring the pathogenesis of EMS.

Therefore, subsequent studies will continue to explore the regulatory effects of the YAP-autophagy signal on the decidual and endometrial receptivity of ESCs, to provide a theoretical basis for exploring the pathogenesis of EMS and improving endometrial receptivity and provide new options for the treatment of endometriosis-associated infertility.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

This study was approved by the Ethics Committee of West China Second University Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TP performed bioinformatic analysis and immunofluorescence and wrote the manuscript, which was commented on by all authors. BL performed the cell transfection and *in vitro* decidualization experiments. WH evaluated all data and contributed to discussion. DL, YL, and LX analyzed the qRT-PCR and WB data. XH and YO contributed to human sample collection. HZ supervised the study and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Key R&D Program of China (grant number 2017YCF1001200); The National Natural Science Foundation of China (grant number 82101719); The National Natural Science Foundation of China (grant number 82071625); The Key Project of Department of Science and Technology of Sichuan (grant number 2018SZ0124); and the International Cooperation of Chengdu Science and Technology Bureau (grant number 2017GH0200060HZ).

ACKNOWLEDGMENTS

The authors thank Dr. Jiayu Liang (Department of Urology, West China Hospital of Sichuan University) for helpful discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.813165/full#supplementary-material

REFERENCES

1. Shafrir AL, Farland LV, Shah DK, Harris HR, Kvaskoff M, Zondervan K, et al. Risk for and Consequences of Endometriosis: A Critical Epidemiologic Review. *Best Pract Res Clin Obstet Gynaecol* (2018) 51:1–15. doi: 10.1016/j.bpobgyn.2018.06.001
2. *The World Bank. Population Ages 15-64 (% of Population)* (2017). Available at: https://data.worldbank.org/indicator/SP. POP.1564.TO.ZS.
3. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *N Engl J Med* (2020) 382: (13):1244–56. doi: 10.1056/NEJMra1810764
4. Eskenazi B, Warner ML. Epidemiology of Endometriosis. *Obstet Gynecol Clin North Am* (1997) 24(2):235–58. doi: 10.1016/S0889-8545(05)70302-8
5. Giudice LC. Clinical Practice Endometriosis. *N Engl J Med* (2010) 362 (25):2389–98. doi: 10.1056/NEJMcp1000274
6. Vercellini P, Viganò P, Somigliana E, Fedele L. Endometriosis: Pathogenesis and Treatment. *Nat Rev Endocrinol* (2014) 10(5):261–75. doi: 10.1038/nrendo.2013.255
7. D'Hooghe TM, Debrock S, Hill JA, Meuleman C. Endometriosis and Subfertility: Is the Relationship Resolved? *Semin Reprod Med* (2003) 21 (2):243–54. doi: 10.1055/s-2003-41330
8. Lessey BA, Kim JJ. Endometrial Receptivity in the Eutopic Endometrium of Women With Endometriosis: It Is Affected, and Let Me Show You Why. *Fertil Steril* (2017) 108(1):19–27. doi: 10.1016/j.fertnstert.2017.05.031
9. Marquardt RM, Kim TH, Shin JH, Jeong JW. Progesterone and Estrogen Signaling in the Endometrium: What Goes Wrong in Endometriosis? *Int J Mol Sci* (2019) 20(15):3822. doi: 10.3390/ijms20153822
10. Pei T, Liu C, Liu T, Xiao L, Luo B, Tan J, et al. MiR-194-3p Represses the Progesterone Receptor and Decidualization in Eutopic Endometrium From Women With Endometriosis. *Endocrinology* (2018) 159(7):2554–62. doi: 10.1210/en.2018-00374
11. Uimari O, Rahmioglu N, Nyholt DR, Vincent K, Missmer SA, Becker C, et al. Genome-Wide Genetic Highlight Mitogen-Activated Protein Kinase (MAPK) Signaling in the Pathogenesis of Endometriosis. *Hum Reprod* (2017) 32 (4):780–93. doi: 10.1093/humrep/dex024
12. Altmae S, Aghajanova L. What do We Know About Endometrial Receptivity in Women With Endometriosis? *Reprod BioMed Online* (2015) 31(5):581–3. doi: 10.1016/j.rbmo.2015.09.008
13. Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, et al. Elucidation of a Universal Size-Control Mechanism in Drosophila and Mammals. *Cell* (2007) 130(6):1120–33. doi: 10.1016/j.cell.2007.07.019
14. Fallahi E, O'Driscoll NA, Matallanas D. The MST/Hippo Pathway and Cell Death: A Non-Canonical Affair. *Genes (Basel)* (2016) 7(6):28. doi: 10.3390/genes7060028

15. Song Y, Fu J, Zhou M, Xiao L, Feng X, Chen H, et al. Activated Hippo/YAP Pathway Promotes Cell Proliferation and Anti-Apoptosis in Endometrial Stromal Cells of Endometriosis. *J Clin Endocrinol Metab* (2016) 101 (4):1552–61. doi: 10.1210/jc.2016-1120
16. Choi J, Jo M, Lee E, Oh YK, Choi D. The Role of Autophagy in Human Endometrium. *Bio Reprod* (2012) 86(3):70. doi: 10.1095/biolreprod.111.096206
17. McKinnon BD, Kocbek V, Nirgianakis K, Bersinger NA, Mueller MD. Kinase Signaling Pathways in Endometriosis Potential Targets for Non-Hormonal Therapeutics. *Hum Reprod Update* (2016) 22(3):382–403. doi: 10.1093/humupd/dmv060
18. Liang N, Zhang C, Dill P, Panasyuk G, Pion D, Koka V, et al. Regulation of YAP by mTOR and Autophagy Reveals a Therapeutic Target of Tuberous Sclerosis Complex. *J Exp Med* (2014) 211(11):2249–63. doi: 10.1084/jem.20140341
19. Wilkinson DS, Jariwala JS, Anderson E, Mitra K, Meisenhelder J, Chang JT, et al. Phosphorylation of LC3 by Hippo Kinases STK3/STK4 Is Essential for Autophagy. *Mol Cell* (2015) 57(1):55–68. doi: 10.1016/j.molcel.2014.11.019
20. Zhang L, Liu Y, Xu Y, Wu H, Wei Z, Cao Y. The Expression of the Autophagy Gene Becilin-1 mRNA and Protein in Ectopic and Eutopic Endometrioum of Patients With Endometriosis. *Int J Fertil Steril* (2015) 8(4):429–36. doi: 10.22074/ijfs.2015.4183
21. Ruiz A, Rockfield S, Taran N, Haller E, Engelman RW, Flores I, et al. Effect of Hydroxychloroquine and Characterization of Autophagy in a Mouse Model of Endometriosis. *Cell Death Dis* (2016) 7(1):e2059. doi: 10.1038/cddis.2015.361
22. Mei J, Zhu XY, Jin LP, Duan ZL, Li DJ, Li MQ, et al. Estrogen Promotes the Survival of Human Secretory Phase Endometrial Stromal Cells *via* CHCL12/CXCR4 Up-Regulation Mediated Autophagy Inhibition. *Hum Reprod* (2015) 30(7):1677–89. doi: 10.1093/humrep/dev100
23. Choi JY, Jo MW, Lee EY, Kim HJ, Choi D. Differential Induction of Autophagy by mTOR Is Associated With Abnormal Apoptosis in Ovarian Endometriotic Cysts. *Mol Hum Reprod* (2014) 20(4):309–17. doi: 10.1093/molehr/gat091
24. Pei T, Huang X, Long Y, Duan C, Liu T, Li Y, et al. Increased Expression of YAP Is Associated With Decreased Cell Autophagy in the Eutopic Endometrial Stromal Cells of Endometriosis. *Mol Cell Endocrinol* (2019) 491:110432. doi: 10.1016/j.mce.2019.04.012
25. Wei X, Jia YJ, Lou H, Ma J, Huang Q, Meng Y, et al. Targeting YAP Suppresses Ovarian Cancer Progression Through Regulation of the PI3K/Akt/mTOR Pathway. *Oncol Rep* (2019) 42(6):2768–76. doi: 10.3892/or.2019.7370
26. Zhou Y, Wang Y, Zhou WH, Chen T, Wu Q, Chutturghoon VK, et al. YAP Promotes Multi-Drug Resistance and Inhibits Autophagy-Related Cell Death in Hepatocellular Carcinoma *via* the RAC1-ROS-mTOR Pathway. *Cancer Cell Int* (2019) 19:179. doi: 10.1186/s12935-019-0898-7
27. Tamaresis JS, Irwin JC, Goldfien GA, Chen T, Wu Q, Chutturghoon VK, et al. Molecular Classification of Endometriosis and Disease Stage Using High-Dimensional Genomic Data. *Endocrinology* (2014) 155(12):4986–99. doi: 10.1210/en.2014-1490
28. Wu J, Vallenius T, Ovaska K, Westermarck J, Mäkelä TP, Hautaniemi S, et al. Integrated Network Analysis Platform for Protein-Protein Interactions. *Nat Methods* (2009) 6(1):75–7. doi: 10.1038/nmeth.1282
29. Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A, et al. STRING V9.1: Protein-Protein Interaction Networks, With Increased Coverage and Integration. *Nucleic Acids Res* (2013) 41:D808–15. doi: 10.1093/nar/gks1094
30. Yue Z, Li H, Yang Y, Hussain S, Zheng CH, Xia J, et al. Identification of Breast Cancer Candidate Genes Using Gene Co-Expression and Protein-Protein Interaction Information. *Oncotarget* (2016) 7(24):36092–100. doi: 10.18632/oncotarget.9132
31. Kimmelman AC, White E. Autophagy and Tumor Metabolism. *Cell Metab* (2017) 25(5):1037–43. doi: 10.1016/j.cmet.2017.04.004
32. Ren Y, Mu L, Ding X, Zheng W. Decreased Expression of Beclin 1 in Eutopic Endometrium of Women With Adenomyosis. *Arch Gynecol Obstet* (2010) 282 (4):401–6. doi: 10.1007/s00404-009-1280-0
33. Xiao L, Shi XY, Zhang Y, Zheng W. YAP Induces Cisplatin Resistance Through Activation of Autophagy in Human Ovarian Carcinoma Cells. *Onco Targets Ther* (2016) 9:1105–14. doi: 10.2147/OTT.S102837
34. Hou L, Chen L, Fang L. Scutellarin Inhibits Proliferation, Invasion, and Tumorigenicity in Human Breast Cancer Cells by Regulating HIPPO-YAP Signaling Pathway. *Med Sci Monit* (2017) 23:5130–8. doi: 10.12659/MSM.904492
35. Rivera-Reyes A, Ye S, Marino GE, Egolf S, Ciotti GE, Chor S, et al. YAP1 Enhances NF-κb-Dependent and Independent Effects on Clock-Mediated Unfolded Protein Responses and Autophagy in Sarcoma. *Cell Death Dis* (2018) 9(11):1108. doi: 10.1038/s41419-018-1142-4
36. Yan H, Qiu C, Sun W, Gu M, Xiao F, Zou J, et al. Yap Regulates Gastric Cancer Survival and Migration *via* SIRT1/Mfn2/mitophagy. *Oncol Rep* (2018) 39(4):1671–81. doi: 10.3892/or.2018.6252
37. Pavel M, Renna M, Park S, Menzies FM, Ricketts T, Füllgrabe J, et al. Contact Inhibition Controls Cell Survival and Proliferation *via* YAP/TAZ-Autophagy Axis. *Nat Commun* (2018) 9(1):2961. doi: 10.1038/s41467-018-05388-x
38. Tumaneng K, Schlegelmilch K, Russell R, et al. YAP Mediates Crosstalk Between the Hippo and PI3K-TOR Pathways by Suppressing PTEN *via* miR-29. *Nat Cell Biol* (2012) 14(12):1322–9. doi: 10.1038/ncb2615
39. Pobbati AV, Hong W. Emerging Roles of TEAD Transcription Factors and Its Coactivators in Cancers. *Cancer Biol Ther* (2013) 14(5):390–8. doi: 10.4161/cbt.23788
40. Kessler CA, Bachurski CJ, Schroeder J, Stanek J, Handwerger S. TEAD1 Inhibits Prolactin Gene Expression in Cultured Human Uterine Decidual Cells. *Mol Cell Endocrinol* (2008) 295(1-2):32–8. doi: 10.1016/j.mce.2008.08.007
41. Yang H, Mei J, Chang K, Zhou W, Huang L, Li M. Autophagy in Endometriosis. *Am J Transl Res* (2017) 9(11):4707–25.
42. Rhee JS, Saben JL, Mayer AL, Schulte MB, Asghar Z, Stephens C, et al. Diet-Induced Obesity Impairs Endometrial Stromal Cell Decidualization: A Potential Role for Impaired Autophagy. *Hum Reprod* (2016) 31(6):1315–26. doi: 10.1093/humrep/dew048
43. Su RW, Strug MR, Joshi NR, Jeong JW, Miele L, Lessey BA, et al. Decreased Notch Pathway Signaling in the Endometrium of Women With Endometriosis Impairs Decidualization. *J Clin Endocrinol Metab* (2015) 100 (3):E433–42. doi: 10.1210/jc.2014-3720
44. Guo SW, W u Y, Strawn E, Basir Z, Wang Y, Halverson G, et al. Genomic Alterations in Endometrium May Be a Proximate Cause for Endometriosis. *Eur J Obstet Gynecol Reprod Biol* (2004) 116(1):89–99. doi: 10.1016/j.ejogrb.2004.02.004
45. Meola J, Rosa E, Silva JC, da Silva WA Jr, Veiga-Castelli LC, Bernardes LA, et al. Differentially Expressed Genes in Eutopic and Ectopic Endometrium of Wonlen With Endometriosis. *Fertil Steril* (2010) 93(6):1750–73. doi: 10.1016/j.fertnstert.2008.12.058
46. Sha G, W u D, Zhang L, Chen X, Lei M, Sun H, et al. Differentially Expressed Genes in Human Endometrial Endothelial Cells Derived From Eutopic Endometrium of Patients With Endometriosis Compared With Those From Patients Without Endometriosis. *Hum Reprod* (2007) 22(12):3159–69. doi: 10.1093/humrep/dem266
47. Am JU, Gong WJ, Su Y, Mou ZB. Imperatorin Shows Selective Antitumor Effects in SGC - 7901 Human Gastric Adenocarcinoma Cells by Inducing Apoptosis, Cell Cycle Arrest and Targeting PI3K/Akt/mTOR Signalling Pathway. *J BUON* (2017) 22(6):1471–6.
48. Yarahmadi A, Khademi F, Mostafavi-Pour Z. *In-Vitro* Analysis of Glucose and Quercetin Effects on mTOR and Nrf-2 Expression in HepG2 Cell Line (Diabetes and Cancer Connection). *Nutr Cancer* (2018) 70(5):770–5. doi: 10.1080/01635581.2018.1470654
49. Ozturk E, Aksoy SA, Ugras N, Tunca B, Ceylan S, Tezcan G, et al. Coexistence of MACC1 and NM23-H1 Dysregulation and Tumor Budding Promise Early Prognostic Evidence for Recurrence Risk of Early-Stage Colon Cancer. *APMIS* (2018) 126(2):99–108. doi: 10.1111/apm.12801

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 04 May 2022
doi: 10.3389/fendo.2022.853563

Downregulation of LHCGR Attenuates COX-2 Expression and Induces Luteinized Unruptured Follicle Syndrome in Endometriosis

Ting Geng[1,2,3†], *Yifan Sun*[1,2,3†], *Lin Cheng*[1,2,3], *Yuming Cao*[1,2,3], *Ming Zhang*[1,2], *Zhidan Hong*[1,2], *Ling Ma*[1,2] *and Yuanzhen Zhang*[1,2,3*]

[1] *Reproductive Medicine Center, Zhongnan Hospital of Wuhan University, Wuhan, China,* [2] *Hubei Clinical Research Center for Prenatal Diagnosis and Birth Health, Wuhan, China,* [3] *Department of Obstetrics and Gynecology, Zhongnan Hospital of Wuhan University, Wuhan, China*

OPEN ACCESS

Edited by:
Ye Chun Ruan,
Hong Kong Polytechnic University,
Hong Kong SAR, China

Reviewed by:
Holly A. LaVoie,
University of South Carolina,
United States
Pascale Crepieux,
Centre National de la Recherche Scientifique (CNRS), France

***Correspondence:**
Yuanzhen Zhang
zhangyuanzhen@whu.edu.cn

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *12 January 2022*
Accepted: *21 March 2022*
Published: *04 May 2022*

Citation:
Geng T, Sun Y, Cheng L, Cao Y, Zhang M, Hong Z, Ma L and Zhang Y (2022) Downregulation of LHCGR Attenuates COX-2 Expression and Induces Luteinized Unruptured Follicle Syndrome in Endometriosis. Front. Endocrinol. 13:853563. doi: 10.3389/fendo.2022.853563

An association between endometriosis and luteinized unruptured follicle syndrome (LUFs) has long been identified. Although inactivating mutation of luteinizing hormone/choriogonadotropin receptor (LHGCR) results in LUFs, whether LHCGR contributes to promoting LUFs in endometriosis remains elusive. To investigate the effect of LHCGR signaling in the development of endometriosis-associated LUFs and dissect the underlying mechanism *in vivo* mouse endometriosis model was established to measure the effect on ovarian folliculogenesis. *In vitro* cultures of primary human GCs collected from patients undergoing *in vitro* fertilization were performed and treated with human chorionic gonadotropin (hCG), dibutyryl cyclic-AMP (db-cAMP), LHCGR or CCAAT/enhancer binding protein-α (C/EBPα) small interfering RNA to identify the potential mechanisms. KGN cell line was used to investigate the mechanistic features of transcriptional regulation. Results showed an increased incidence of LUFs was observed in mice with endometriosis. The expression of LHCGR was decreased in the GCs of endometriosis mice. In *in vitro* cell models, LHCGR signaling increased the expression of C/EBPα and cyclooxygenase-2(COX-2), while inhibiting C/EBPα mitigated the induced COX-2 expression. Mechanically, C/EBPα bounded to the promoter region of COX-2 and increased the transcriptional activity under the stimulation of hCG or db-cAMP. Taken together, this study demonstrated that the LHCGR signaling was reduced in GCs of endometriosis and resulted in a decrease in gonadotropin-induced COX-2 expression. Our study might provide new insights into the dysfunction of GCs in endometriosis.

Keywords: endometriosis, luteinized unruptured follicle syndrome, LHCGR, ovulation, COX-2

Abbreviations: C/EBPα, CCAAT/enhancer binding protein-α; cAMP, cyclic adenosine monophosphate; ChIP, chromatin immunoprecipitation; CL, corpus luteum; COCs, cumulus–oocyte complexes; COX-2, cyclooxygenase-2; db-cAMP, dibutyryl cyclic-AMP; EGF, epidermal growth factor; GCs, granulosa cells; hCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; JIA, juvenile idiopathic arthritis; LH, luteinizing hormone; LHGCR, luteinizing hormone/choriogonadotropin receptor; LUFs, unruptured follicle syndrome; NSAIDs, non-steroidal anti-inflammatory drugs; PGE2, prostaglandin E2; PMSG, pregnant mare serum gonadotropin; siRNA, Small interfering RNA.

INTRODUCTION

Endometriosis (EMs) is an estrogen-dependent chronic inflammatory condition that affects women in their reproductive period and causes infertility and pelvic pain. EMs has long been identified to have an association with luteinized unruptured follicle syndrome (LUFs), one of the ovulatory dysfunction subtypes, due to the intrafollicular endocrine milieu (1, 2). LUFs has been considered a subtle cause of endometriosis-associated infertility (3). The incidence of LUFs accessed by laparoscopic examination is 35% in endometriosis patients, while it is 11% in others (4). Increased incidence of LUFs is also observed in the animal model with endometriosis (5, 6). Although the mechanisms of ovulatory dynamics are similar to inflammatory responses (7), the precise underlying reasons for LUFs associated with endometriosis remain uncovered.

It has been observed that the dysregulation of follicle maturation and ovulation in endometriosis are tightly associated with endocrine and paracrine factors produced by granulosa cells (GCs) (8). The cyclooxygenase-2(COX-2)/prostaglandin E2 (PGE2), one of the major GCs derived factors, plays an essential role in the maintenance of normal oocyte maturation, follicle rupture and ovulation (9, 10). Moreover, COX-2 is aberrantly decreased in endometriosis, which may result in insufficient cumulus expansion and subsequently lead to impairment of the oocyte quality (11). However, the regulation mechanism of COX2 in endometriosis is largely unclear.

COX-2 is considered inducible by gonadotropin and participates in the regulation of reproduction, in addition, the luteinizing hormone (LH) surge regulates the expression of COX-2 and promotes biosynthesis of PGE2 within the ovulatory follicle (12). The biological activity of LH is mainly mediated by receptor-mediated signal transduction cascades and activated LH subsequently provokes the expression of numerous endocrine factors, either in ovarian granulosa or thecal cells. Therefore, changes in the LHCGR, which plays a vital role during ovarian development and corpus luteum function (13, 14) in women, might impact the correct course of these processes. Recent observations have demonstrated that abnormal LH signaling may be involved in the coexistence of anovulation and endometriosis (15). The clinical observation that patients with endometriosis have dysfunctional LHCGR expression (16), further suggesting a failure in the mechanism associated with LH action in the ovulation process. These observations led us to investigate whether LHCGR involved in COX-2 induced ovulation disorder.

Despite accumulating evidence exploring the mechanisms involving normal folliculogenesis and ovulation, the specific mechanism of LUF syndrome in endometriosis currently has not been elucidated. In this study, we found that LHCGR expression decreased in endometriosis granulosa cells. Functional studies in mice model and primary cultured granulosa cells revealed that attenuated LH signaling induced ovulatory disorder, mechanically, the inactivation of LHCGR induced decreased C/EBPα, which upregulated COX2 expression by binding to its promoter. Collectively, these results indicated that the decline of LHCGR may result in LUFs, and this may be associated with endometriosis-associated infertility.

MATERIALS AND METHODS

Mice Model of Surgical-Induced Endometriosis

To improve our understanding of the pathophysiology underlying this enigmatic disease, animal models have been employed due to the ethical limitations of performing controlled studies of infertile women with endometriosis. The procedures on animals were carried out following institutional guidelines and the Institutional Animal Care and Use Committee of Wuhan University approved the experimental protocol (Approval No. WP2020-08005).

Five-week-old female C57BL/6 mice (Vital River Laboratory, China) were housed under well-controlled conditions (12 h light/12 h dark cycle maintained at a temperature of 22–25°C). After a week of acclimation, mice were injected with 17 β-estradiol (Sigma-Aldrich, E2758) (3 μg/mouse, s.c.) for 1 week, then the endometriosis model was conducted by autologous transplantation of uterine tissue (17). Briefly, after euthanized, the left uterine horns were isolated. Obtained uterine tissue was cut into three equal-sized parts as implants, auto-transplanted was performed around three arteries of the intestinal mesentery. Sham-operated control mice (sham) were subjected to the same steps, but no implant was sutured to the intestinal mesentery. To allow the recuperation and development of endometriotic implants, the subsequent experiment began after 3 weeks.

Superovulation, Oocyte Collection and Sample Harvest

After 3 weeks, mice were superovulated with 5 IU pregnant mare serum gonadotropin (PMSG) (Solarbio, P9970) followed 48 h later by 5 IU human chorionic gonadotropin (hCG) (LIVZON, China) to induce follicle development and ovulation. When mimicking the poor response to LH surge of ovary *in vivo*, the mice were treated with full-dose PMSG (5IU) followed by half-dose hCG (2.5 IU) to trigger ovulation. Ovarian tissues for follicular morphology were collected before superovulation or 48 h after PMSG administration. Granulosa cells for gene expression analysis were isolated at different times after hCG administration (0, 2, 4, and 8 h). To exam the number of ovulated oocytes, ampullae were collected at 14–16 h after hCG injection and then secured to release the clutch of cumulus–oocyte complexes (COCs). For morphology analysis of the post-ovulatory ovary, the samples were collected at 24 h after hCG administration.

H&E Staining and Immunohistochemistry

For follicle counting, ovaries from each group were collected at 14–16 h after hCG injection and hematoxylin and eosin (H&E) staining was performed as described previously (18). Briefly, the right ovaries were fixed in 4% paraformaldehyde, routinely paraffin-embedded, then cut thoroughly into sections of 5-μm thickness followed by staining. In every fifth section, follicles containing oocytes with a visible nucleus were counted and properly classified into different follicle stages (19). The number of luteinized unruptured follicle and corpus luteum (CL) were also recorded.

Mice ovaries were collected as mentioned above and Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections using immunoperoxidase staining kit (PV-9001, ZSGB-BIO, Beijing, China) according to the procedure of the manufacturer. After deparaffinized and rehydrated, antigen retrieval was carried out with sodium citrate. Approximately 3% hydrogen peroxide was used to eliminate the activity of endogenous peroxidase. The sections were then treated with bovine serum albumin (BSA) blocking, followed by incubation with primary antibody for LHCGR (19968-1-AP; 1:200 dilution; Proteintech), C/EBPα (18311-1-AP; 1:200 dilution; Proteintech), COX-2 (ab15191; 1:200 dilution; Abcam) and corresponding secondary antibody. The omission of the primary antibody served as a negative control. The H-score was processed and calculated as described previously (20). We used the following equation: H-score = Σ Pi (i), where i was the intensity of staining with a value of 1, 2, or 3 (weak, moderate, or strong, respectively) and Pi was the percentage of stained cells for each intensity, varying from 0 to 100%.

Human Granulosa Cell Collection and KGN Cell Line Culture

The study protocol was approved by the Institutional Review Board (No. 2018047). Human GCs were obtained from patients undergoing *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment due to tubal factor or male subfertility at the Reproductive Medical Center of Zhongnan Hospital. After controlled ovarian hyperstimulation, 10,000 IU hCG was administered to trigger ovulation. The follicular fluid was immediately collected and centrifuged for 10 min at 2,000 rpm after oocyte pick-up. Then the pellet was resuspended in an enzymatic solution to digest clusters of cells. GCs were highly isolated through Percoll density gradient and red blood cells were removed using lysis buffer. The cell pellet was resuspended in DMEM/F12 medium (Gibco) supplemented with100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal bovine. The cells were then seeded at a density of 2×10^5 cells/ml in a 6-well plate and incubated for 3 days at 37°C in humidified atmosphere with 5% CO_2. The media was replenished every 24 h. To mimic the effect of LH and cAMP *in vivo*, we used hCG (10 IU/ml; LIVZON, China) and dibutyryl-cAMP (db-cAMP, 1 mM; HY-B0764A, MedChemExpress, USA) respectively to stimulate cells and further cultured for stated hours *in vitro* according to previous studies (21). KGN cell line was cultured in DMEM/F12 medium as mentioned above.

Small Interfering RNA (siRNA) and Transfection

For gene silencing experiments, human GCs were transfected with 50 uM small interfering (siRNA) oligonucleotides against LGCGR, C/EBPα or negative control (NC) siRNA (Huzhou Hippo Biotechnology Co., Ltd.) using lipofectamine 3000 transfection reagent (Invitrogen, USA) according to the instructions provided by the manufacturer. The specific sequences of target genes were as follows: si-LHCGR, 5′-UGC CUU CAA AGU ACC UCU UAU TT-3′ (sense) and 5′-AUA AGA GGU ACU UUG AAG GCA TT-3′ (antisense); si-C/EBPα, 5′-GGA GCU GAC CAG UGA CAA UTT-3′ (sense) and 5′-AUU GUC ACU GGU CAG CUC CAG-3′ (antisense); si-LHCGR scrambled NC, 5′-GUC AUU AUC CUU UCG CAC UAA dTdT-3′(sense) and 5′-UUA GUG CGA AAG GAU AAU GAC dTdT-3′ (antisense); si-C/EBPα scrambled NC, 5′-GGU AAC GGG ACC GAC UUA AdTdT-3′ (sense) and 5′-UUA AGU CGG UCC CGU UAC CdTdT-3′ (antisense). For further experiments which were focused on the mechanisms of signal pathways, the cells were incubated with or without db-cAMP or hCG for further 24 h after 24 h of transfection (22).

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted with an RNA extraction kit (RN0302, Aidlab, China). RNA (1 ug) was reverse transcribed with a cDNA Synthesis Kit (R212-01, Vazyme, China). Quantitative real-time PCR (qRT-PCR) was performed using ChamQ SYBR qPCR Master Mix (Q311-02, Vazyme, China) and a CFX96 PCR system machine (Bio-Rad Laboratories, USA). Each reaction was performed with a total volume of 20 μl, consisting of 2× ChamQ SYBR qPCR Master Mix (10 μl), 5'- and 3'-primer (0.4 μl, respectively), cDNA (1 μl), and ddH2O (8.2 μl). With the following primers: LHCGR: 5′-TCC TTT CCA GGG AAT CAA TC-3′ (sense) and 5′-GGC CGG TCT CAC TCG AC-3′ (antisense); C/EBPα: 5′-CAC GAA GCA CGA TCA GTC CAT-3′ (sense) and 5′-CGG AGA GTC TCA TTT TGG CAA G-3′ (antisense); COX-2: 5′-TAA GTG CGA TTG TAC CCG GAC-3′ (sense) and 5′-TTT GTA GCC ATA GTC AGC ATT GT-3′ (antisense); GAPDH: 5′-CTG TTC GAC AGT CAG CCG CATC-3′ (sense) and 5′-GCG CCC AAT ACG ACC AAA TCC G-3′ (antisense). Data analysis was performed using Bio-Rad CFX manager system, using GAPDH as a reference transcript.

Western Blot Analysis

Whole-cell protein extract was lysed and isolated from cultured cells or mouse ovaries. After measuring protein concentrations using a BCA Protein Assay Kit (P0010, Beyotime, China), equal amounts of denatured protein were separated by electrophoresis in 10% SDS polyacrylamide gels and transferred to polyvinyl difluoride membranes (Millipore, Billerica, USA), which were then saturated with blocking buffer for 1 h. After that, the membranes were incubated with rabbit polyclonal anti-LHCGR (19968-1-AP; 1:1,000 dilution; Proteintech), rabbit polyclonal anti-CEBPα (8178; 1:1,000 dilution; Cell Signaling), or rabbit polyclonal anti-COX2 antibodies (ab 15191; 1:1,000 dilution; Abcam) O/N at 4°C. The blots were incubated with HRP–conjugated anti-rabbit IgG for 1 h. Peroxidase activity was detected using the ECL system (Touch Imager, e-Blot, China).

Immunofluorescence

After treatment for stated hours *in vitro*, cells were fixed with paraformaldehyde. Then, the cells were treated with 0.5% Triton solution to encourage intracellular labeling. Cells were next

blocked with 5% BSA for 1–2 h at RT and incubated with primary antibody against CEBPα (8178; 1:200 dilution; Cell Signaling) O/N at 4°C. Secondary antibody incubation was performed at RT for 60 min. Cells were washed and treated with antifade mounting medium, which contains DAPI (P0131, Beyotime, China).

Luciferase Reporter Assays

The effect of C/EBPα on the transcriptional activity of COX-2 promoter was determined by analyzing the dual-luciferase activities using a commercial assay kit (E2920, Promega, USA). The expression construct used for luciferase-based assays was pcDNA3.1 C/EBPα (NM_004364) while reporter construct used was pGL3-PTGS2 promoter (−1.2 kb/+137). KGN cells were co-transfected with the indicated plasmids with the help of lipofectamine 3000 transfection reagent (Invitrogen, USA) as previously described (23). The samples were lysed at 48 h after transfection, detection of luciferase activity was conducted. Firefly luciferase measurements were normalized to Renilla luciferase.

Chromatin Immunoprecipitation Assay

After treatment with hCG or db-cAMP, KGN cells (2×10^7) were collected and processed as described previously (24). Briefly, cells were washed and fixed in 1% formaldehyde for 15 min then cross-linking was terminated using 0.125 M glycine. Then the chromatin immunoprecipitation (ChIP) assay was performed to determine whether CEBPα interacts with the putative binding site in COX-2 promoter using a Simple ChIP Kit (56383, Cell Signaling) according to the protocols of the manufacturer. Approximately 1% of the chromatin fragments were stored at −20°C to be used later for input for normalization. For each immunoprecipitation (IP) reaction, every 5 ug chromatin sample was incubated with 4 ug CEBPα antibody (18311-1-AP, Proteintech) O/N at 4°C or with 1 ul IgG antibody (2729, CST) as a negative control for nonspecific IP. The primers for the COX2 promoter used in ChIP-PCR analyses were as follows: 5′-TCTAGGAAGCCTTTCTCCTCCT-3′ (sense) and 5′-TGATCCACGCTCTTAGTTGAAAT-3′ (antisense). The resulting signals were normalized to input values, with the IgG-negative control values subtracted as background.

Statistical Analysis

Data were calculated as percentages or ratios relative to the corresponding negative controls, presented as means ± SEM, and were appropriately analyzed by ANOVA, or unpaired *t*-test with GraphPad Prism (Version 8.1.1, California). Values of $P < 0.05$ were considered statistically significant.

RESULTS

The Presence of LUFs in Mice With Surgical-Induced Endometriosis

After confirming the induction of surgical-induced endometriosis three weeks after the operation, we initially collected the ovaries and evaluated the impact of endometriosis on the general morphology and ovarian reserve (**Figures 1A, B**). The anatomical observation indicated both morphology and ovarian weight were similar between EMs and sham mice. Counts of primary, secondary and antral follicles in the endometriosis model (78.00 ± 9.72, 46.67 ± 9.14, and 16.00 ± 2.00, respectively) were comparable to those of sham-operated mice (84.00 ± 11.24, 44.33 ± 10.33, and 16.67 ± 2.94, respectively) (**Figure 1C**). These results suggested that endometriosis mice presented similar healthy follicles to sham mice.

Ovarian responsiveness to gonadotropins was further evaluated to examine if folliculogenesis and ovulation are affected by endometriosis. Firstly, ovaries were collected 48 h after PMSG treatment when follicles developed to preovulatory stage (**Figure 1D**). Quantification of ovarian follicles indicated that the number of preovulatory follicles showed no significant differences in animals of both groups (7.33 ± 1.50 in sham vs. 7.17 ± 1.94 in EMs). Subsequently, the number of ovulated oocytes was assessed after a superovulation protocol to further investigate whether ovulation was affected by endometriosis (**Figure 1E**). After 16 h of hCG administration, fewer cumulus–oocyte complexes (COCs) were released in EMs mice compared to that in sham-operated mice (9.12 ± 2.31 in EMs vs. 13.67 ± 1.37 in sham), indicating the ovulatory capacity was compromised in endometriosis mice.

During ovulation, the follicle ruptures and oocyte is released, the remaining GCs and theca cells under the influence of LH are luteinized to form a corpus luteum (CL). EMs ovaries showed increased luteinized unruptured follicles (**Figures 1F, G**), characterized by the oocytes destined for ovulation becoming entrapped in preovulatory follicles or corpora lutea within a full investment of luteinized granulosa cells. These observations established that, in endometriosis, LUFs leads to reduced ovulation and abnormal CL formation, and it may be a cause of endometriosis-associated infertility.

Attenuated Responsiveness of LHCGR to its Ligand Leads to LUFs in Endometriosis

To uncover the mechanism responsible for LUFs, we mimicked the poor response to LH surge of ovary *in vivo* by treating the mice with full-dose PMSG (5I U) followed by half-dose hCG (2.5 IU) to trigger ovulation. Although similar trends had been observed in full-dose hCG mice occurred in sham mice, the EMs mice displayed a significant reduction of ovulatory oocytes and more frequent incidence of LUFs after administration of half dose of hCG *in vivo* (**Figures 2A, B**). Moreover, the expression pattern of LHCGR protein in the GCs collected at different time points after hCG administration was determined by Western blotting (**Figure 2C**). The results showed that LHCGR protein levels were lower in the GCs of EMs mice in the early ovulatory phase compared with controls, the reduced trends even lasted to late ovulatory phases. IHC staining confirmed that the intensity of LHCGR staining appeared weaker and sporadic in GCs of hCG-primed (0 and 8 h) EM mice than that in sham mice (**Figures 2D, E**). These results suggested that decreased LHCGR induces a poor response to LH surge and therefore contribute to

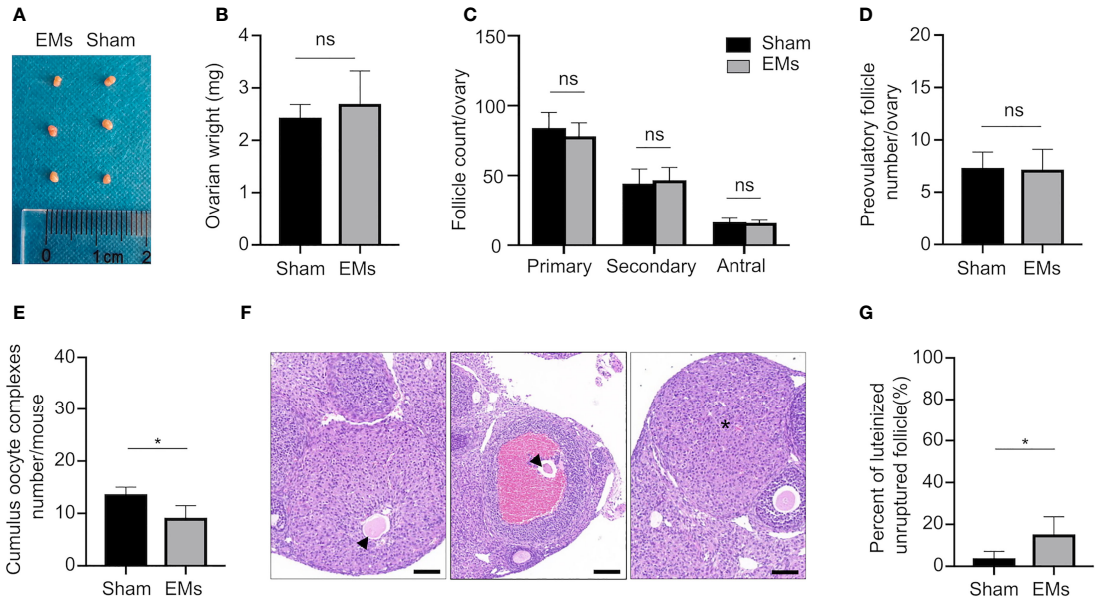


FIGURE 1 | EMs mice with ovulatory dysfunction and LUFs. **(A, B)** Ovarian morphology and weights of EMs and sham mice. ns, no significance. **(C)** Average number of each follicle classes per ovary (every fifth section of serially sectioned ovaries was counted; n = 6, each group). ns, no significance. **(D)** Number of preovulatory follicles per ovary (n = 6, each group). **(E)** Number of ovulated oocytes per mouse (n = 6, each group). *$P < 0.05$ (Student's *t*-test). ns, no significance. **(F)** Representative H&E-stained ovarian tissue sections depicting unruptured follicle and normal CL. Asterisk denoted normal CL after ovulation. Arrows pointed to the trapped oocytes within CLs. Scale bars, 100 μm. **(G)** The percentage of luteinized unruptured follicle for all CLs. The values were the mean ± SEM. *$P < 0.05$ (Student's *t*-test).

the pathogenesis of LUFs. More importantly, the dysfunction presents before endogenous LH surge.

LHCGR Modulates COX-2 Expression in Human Granulosa Cells

LH signaling primes many key ovulatory genes in granulosa cells. To further look into the mechanism responsible for LUFs, we first investigate whether LHCGR is involved in the expression of ovulation-related genes (25–27). The qRT-PCR analysis indeed showed that the expression levels of known genes, such as VEGFA, COX-2, AREG, and EREG were significantly diminished in the human GCs of LHCGR knockdown than those of the negative control (NC) (**Figure 3A**). We further analyzed the gene expression between EMs and sham mice. Both VEGFA and COX-2 were significantly decreased in the GCs of EMs mice (**Figure 3B**). Since abnormal COX-2 function is also associated with ovulation failure (28), these observations led us to investigate the correlation between LH signaling and COX-2.

To determine the effect of LH signaling on COX-2, human GCs were stimulated with 10 IU/ml hCG to mimic the *in vivo* induction. The expression pattern of COX-2 in the human GCs collected at different time points after hCG administration was determined (**Figures 3C, D**). As expected, hCG treatment induced the expression of COX-2 in levels of mRNA and proteins at 24 h, and the levels remained appreciable even at 36 h after hCG treatment (**Figures 3C, D**). To reveal the functional role of LHCGR involved in the expression of COX-2 during the periovulatory period, RNA interference (RNAi) approach was employed to knock down LHCGR transcripts in the presence or absence of hCG. The knockdown of LHCGR *per se*, rather than negative control, recapitulated the hCG induced COX-2 upregulation (**Figures 3E, F**).These results revealed that LHCGR is involved in the hCG-induced upregulation of COX-2 expression in human GCs.

We further assess the expression of COX-2 during ovulation, granulosa cells were collected at different time points after hCG (0, 2, 4, and 8 h) treatment for analysis (**Figure 3G**). COX-2 protein of the granulosa cells from EMs mice were significantly decreased compared to sham mice. IHC staining for COX-2 showed that COX-2 was mainly localized to granulosa and theca cells of dominant follicles during both early and late ovulatory phases, and abundant COX-2 was found at 8 h after hCG priming, whereas little staining was observed in EMs mice (**Figure 3H**). Therefore, we conjecture that the endometriosis-related abnormal actions of LHCGR modulate downregulation of COX-2 in GCs, then results in reduced ovulation with impaired follicle rupture.

LHCGR Regulates COX-2 Expression Through C/EBPα Protein

We further sought to identify underlying mechanisms of the LHCGR-induced COX2 upregulation. There is evidence indicating the expression and functional activation of C/EBP family members is essential for events associated with reproduction (29, 30). We reasoned that C/EBPα may participate in the LHCGR-induced COX2 expression. Accordingly, Western blot was conducted to investigate the expression pattern of C/EBPα

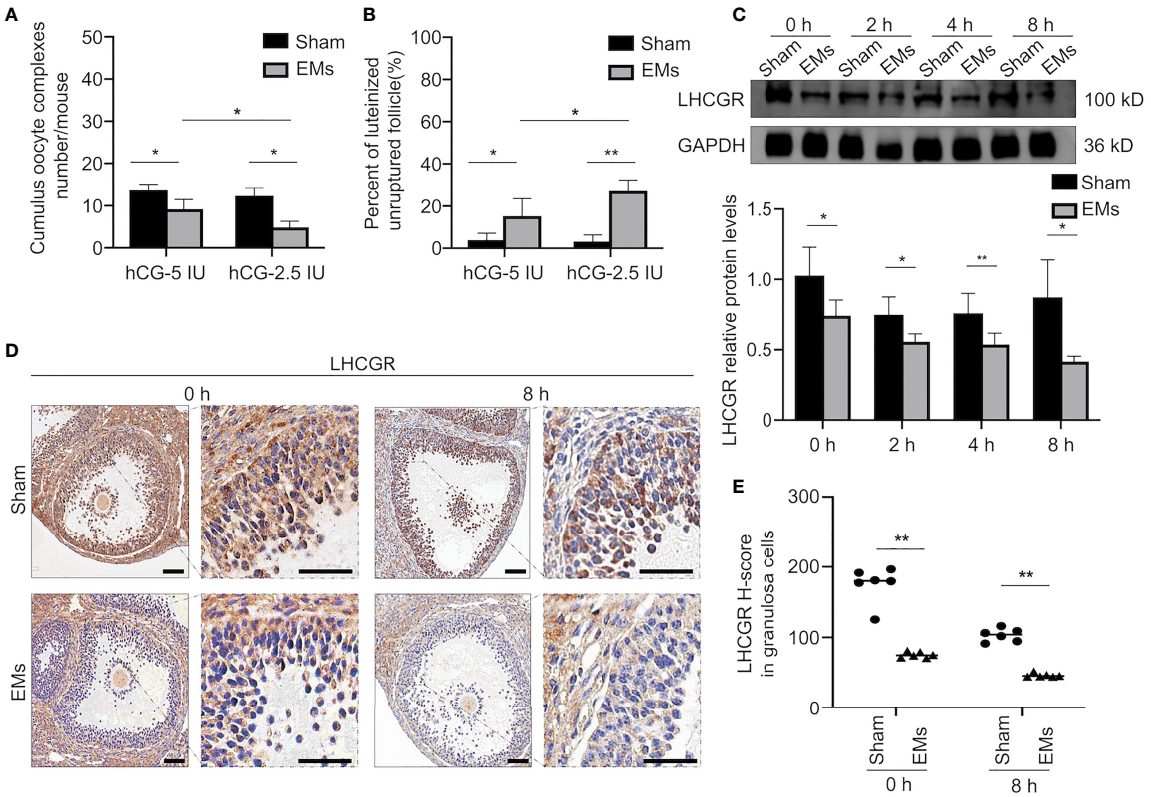


FIGURE 2 | Decreased responsiveness of LHCGR to its ligand in endometriosis. **(A)** Average number of ovulated oocytes following superovulation protocols with a different dose of hCG (n = 6, each group). *$P < 0.05$ (two-way ANOVA). **(B)** The percentage of luteinized unruptured follicle for all CLs tracked per ovary. *$P < 0.05$, **$P < 0.001$ (two-way ANOVA). **(C)** Western blotting of LHCGR protein expression in GCs of EMs and sham mice at different time points after hCG administration (0, 2, 4, and 8 h). GAPDH was used as a sample loading control. * $P < 0.05$, **$P < 0.001$ (Student's *t*-test). **(D, E)** Immunohistochemical H-score and representative images of immunohistochemical staining for LHCGR in the GCs from EMs and sham mice after PMSG-priming (48 h). Scale bar, 100 μm. **$P < 0.001$ (Student's *t*-test).

in granulosa cells collected at different time points after hCG administration and the results showed that C/EBPα was diminished in EMs mice (**Figure 4A**). IHC staining showed that, after PMSG administration (48 h), follicles were either mature or in the process of ovulation, C/EBPα was expressed in the granulosa and theca cells of superovulated mouse ovaries. After treatment with hCG, the protein level was significantly increased. Interestingly, C/EBPα staining in the EMs mice appeared to be attenuated in the granulosa cells (**Figure 4B**).

The results demonstrated that hCG escalated the expression of C/EBPα in granulosa cells after 24 h in both mRNA and protein levels (**Figures 4C, D**). We also confirmed that hCG significantly induced C/EBPα expression in the nuclei of human GCs (**Figure 4E**).

Consistently, knockdown of LHCGR affected the basal levels of C/EBPα expression; it also further significantly diminished the hCG-induced C/EBPα expression (**Figures 4F, G**). These results provided evidence that C/EBPα may involve in regulation by LHCGR signaling in human GCs. To further substantiate our observation, siRNA-mediated down-regulation of endogenous C/EBPα was employed, and we found that the expression of COX-2 was down-regulated after knockdown endogenous C/EBPα in GCs (**Figures 4H–J**).

To interrogate the bona fide regulation of C/EBPα on COX-2, dual-luciferase reporter assay using KGN cells was conducted and the results showed that C/EBPα was sufficient to operate as a transactivator of COX-2 transcription since the luciferase activity of cells transfected with COX-2 wild-type reporter plasmid (COX-2-wt) was strongly improved after co-transfected with C/EBPα-overexpression (C/EBPα-oe) plasmid in a dose-dependent manner (**Figure 4L**). These results indicated that C/EBPα activated the transcription of COX-2. To address the potential binding region of C/EBPα in the COX-2 promoter region, a bioinformatics analysis was conducted by JASPAR database (http://jaspar.genereg.net/), a putative C/EBPα-binding site located at position −416/−403 of the COX-2 promoter was identified (**Figure 4K**). The results of dual-luciferase reporter assay verified that the luciferase activity in cells co-transfected with COX-2 mutant reporter plasmid (COX-2-mut) and C/EBPα-oe plasmid was not altered (**Figure 4L**). Furthermore, ChIP-PCR assays were conducted to validate the molecular interaction between C/EBPα and the identified biding site in

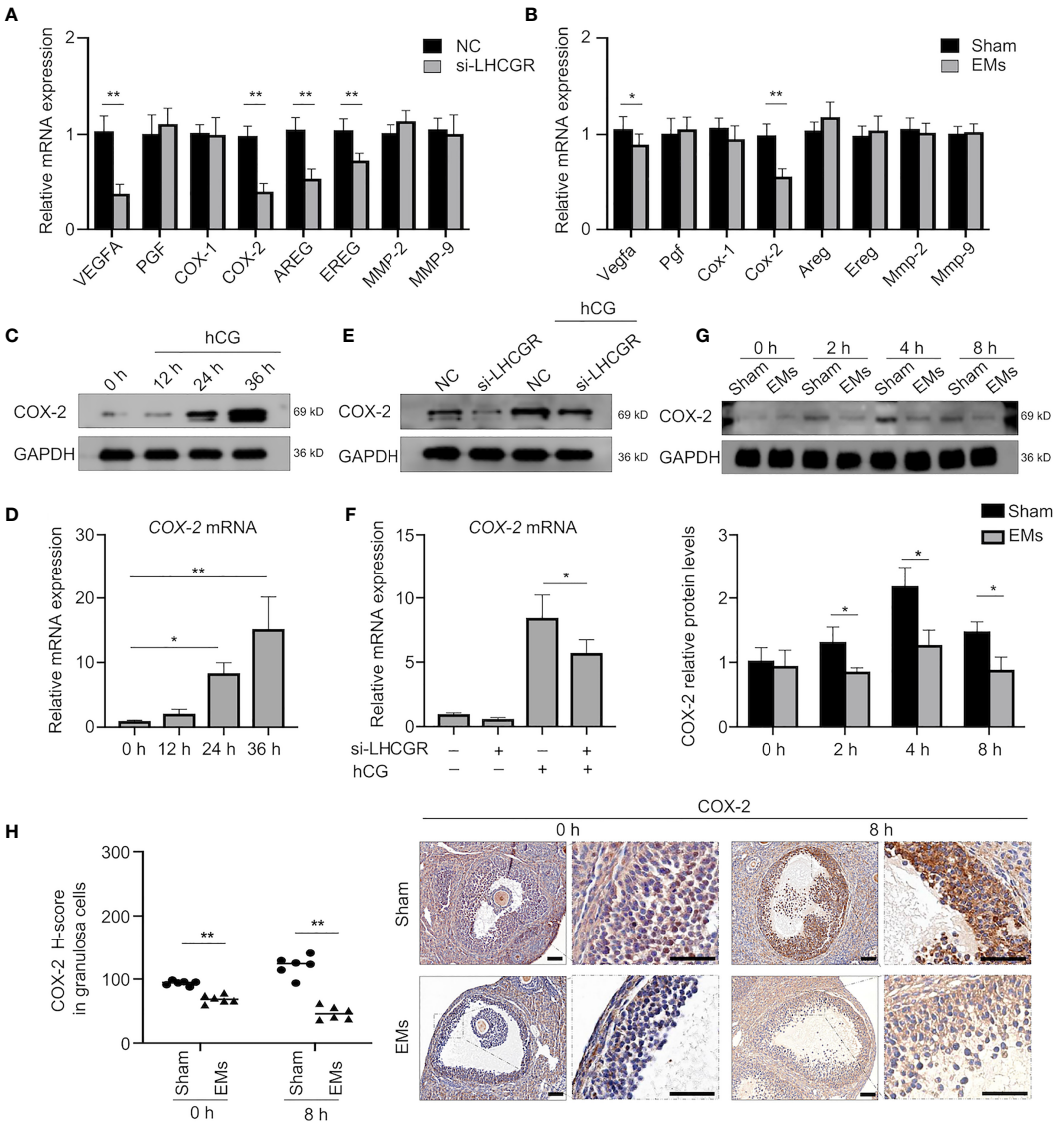


FIGURE 3 | COX-2 involved in the hCG-induced ovulation is downregulated in EMs granulosa cells. **(A)** qRT-qPCR analysis of expression of ovulation-related genes VEGFA, PGF, COX-1, COX-2, AREG, EREG, MMP-2, and MMP-9 in human GCs treated with nontargeting negative control siRNA (NC) or LHCGR siRNA.***P* < 0.001 (Student's *t* -test). **(B)** qRT-PCR analysis of ovulation-related gene expression in GCs of EMs and sham mice after PMSG-priming (48 h). **P* < 0.05, ***P* < 0.001 (Student's *t* -test). **(C)** Human GCs were treated with10 IU/ml hCG for 0, 12, 24, and 36 h, the protein levels of COX-2 were examined by Western blot. **(D)** The mRNA levels of COX-2 in hCG-treated human GCs at different time points were analyzed by qRT-PCR. **P* < 0.05, ***P* < 0.001 (ANOVA). **(E, F)** Human GCs were transfected with 50 nM siRNA against LHCGR for 24 h and then treated with 10 IU/ml hCG for another 24 h. The mRNA and protein levels of COX-2 were analyzed. **P* < 0.05 (ANOVA). **(G)** Western blotting of COX-2 during ovulation in GCs from EMs and sham mice.**P* < 0.05 (Student's *t* -test). **(H)** Immunohistochemical H-score and representative images of immunohistochemical staining for COX-2 in the GCs from EMs and sham mice after PMSG-priming (48 h). Scale bar, 100 μm. ***P* < 0.001 (Student's *t* -test).

COX-2 promoter in KGN cell line collected 24 h after treatment with hCG (**Figure 4M**). The result unveiled that the immunoprecipitation of the C/EBPα antibody-enriched DNA fragments containing identified binding sites, demonstrating CEBPα was strongly bound to the promoter region upstream from the transcriptional start site of COX-2 gene.

Further experiments showed that hCG-increased expression of COX-2 was attenuated by knock down of C/EBPα in human granulosa cells (**Figures 4N–P**). Characterization of C/EBPα showed the same pattern of expression. These results suggest that C/EBPα plays an important role in hCG-induced COX-2 expression in human GCs.

cAMP Modulates the Activity of C/EBPα to Stimulate COX-2 Transcription *In Vitro*

Cognate receptor of LH is G-protein coupled receptor that is predominantly mediated by activation of adenylate cyclase and cAMP-dependent mechanisms in ovarian follicle growth and

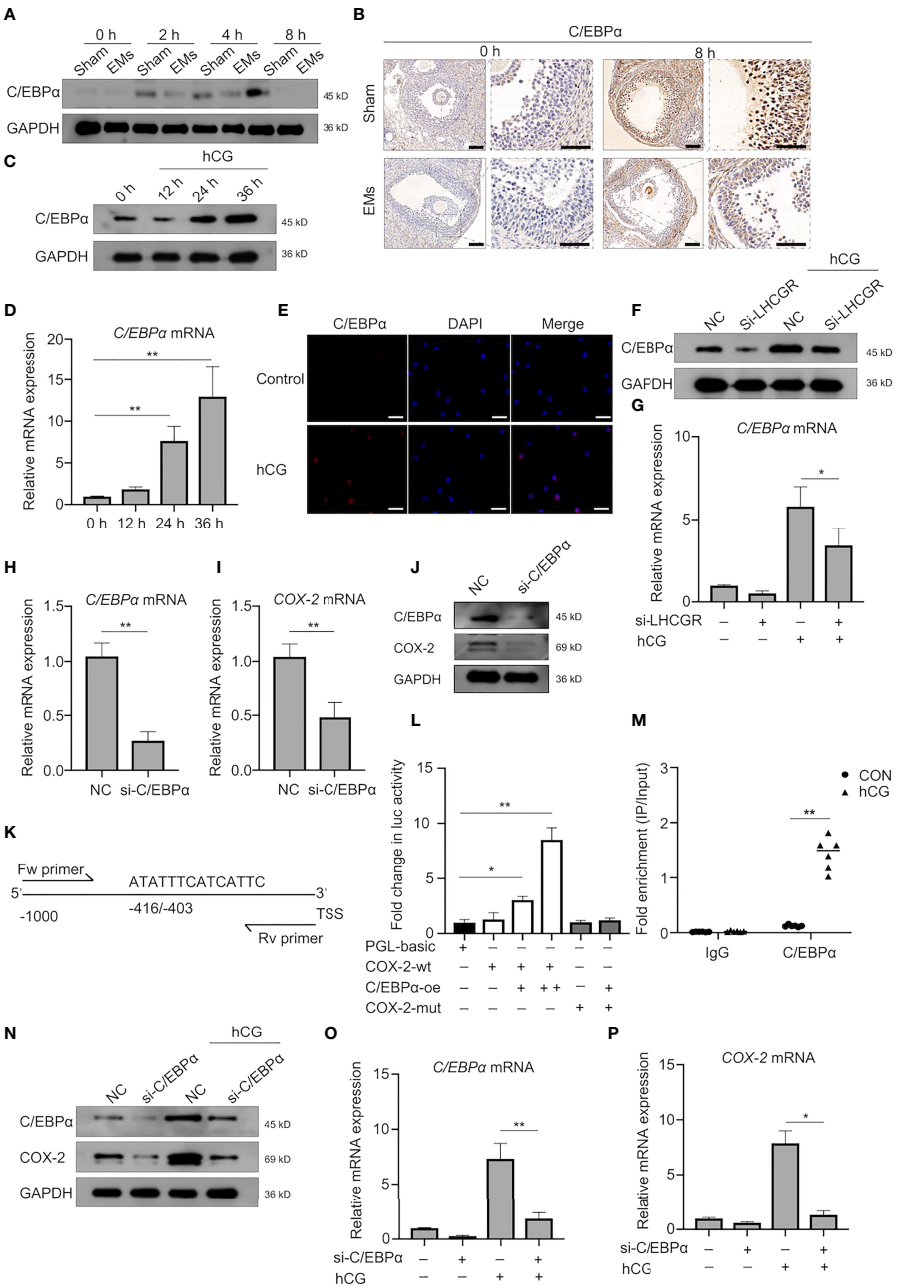


FIGURE 4 | C/EBPα is necessary for hCG-induced COX-2 expression in granulosa cells. **(A)** Western blotting of C/EBPα during ovulation in GCs from EMs and sham mice. **(B)** Representative images of immunohistochemical staining for C/EBPα in the GCs from EMs and sham mice after PMSG-priming (48 h). Scale bar, 100 μm. **(C, D)** The protein and mRNA levels of C/EBPα in hCG-treated human GCs at different time points were analyzed by Western blot and qRT-PCR, respectively. $^{**}P < 0.001$ (ANOVA). **(E)** hCG-treated (24 h) human GCs were analyzed by immunofluorescence to identify the subcellular localization and protein expression levels of C/EBPα (red). Nuclei were stained with DAPI (blue). Magnification: ×100. Scale bar, 50 μm. **(F, G)** Human GCs were transfected with negative control siRNA or LHCGR siRNA and then treated with hCG. The mRNA and protein levels of C/EBPα were analyzed. NC, negative control. $^{*}P < 0.05$ (ANOVA). **(H–J)** Human GCs were transfected with negative control siRNA or C/EBPα siRNA. The expression of indicated genes and protein was analyzed by qRT-PCR and Western blot. $^{**}P < 0.001$ (Student's *t* -test). **(K)** Predicted C/EBPα-binding site in the promoter region of human *COX-2* . TSS, transcriptional start site; Fw primer, forward primer; Rev primer, reverse primer. **(L)** KGN cells were cotransfected with C/EBPα-overexpressing plasmid vectors, and luciferase reporter constructs harboring the COX-2 promoters, along with a Renilla luciferase construct for internal control. Firefly luciferase (Luc) activity was normalized to Renilla activity. Data are shown as mean ± SEM and expressed as fold increase in firefly luciferase activity compared with empty vector (PGL-basic). $^{*}P < 0.05$, $^{**}P < 0.001$ (Student's t-test). **(M)** KGN cells were left untreated or stimulated with hCG for 24 h. ChIP assays were performed using anti-C/EBPα antibody or isotype control antibody (IgG). qRT-PCR was used to determine C/EBPα occupancy at the potential biding site under the conditions tested. $^{**}P < 0.001$ (Student's t-test). **(N–P)** Human GCs were transfected with negative control siRNA or C/EBPα siRNA and then treated with hCG. The expression of indicated genes and protein was analyzed by qRT-PCR and Western blot. $^{**}P < 0.001$ (ANOVA).

maturation (31). As C/EBPα is identified with both constitutive and cAMP inducible activities (32), we next examined whether COX-2 expression was mediated through a cAMP-regulated pathway.

First, ChIP-PCR assay was conducted to clarify the enrichment of C/EBPα bound to the COX-2 promoter (**Figure 5A**). The interaction was also confirmed by results obtained in KGN cells treated with db-cAMP (24 h), indicating that C/EBPα directly binds to the promoter of COX-2 gene to regulate its expression in a cAMP-dependent manner.

To further determine the role of cAMP, human GCs were treated with db-cAMP *in vitro*. The observations showed that db-cAMP improved C/EBPα expression in the nuclei of human GCs as well (**Figure 5B**). Treatment of human GCs with db-cAMP for 24 h significantly induced the expression of C/EBPα and COX-2 (**Figures 5C–E**). Indeed, knockdown of LHCGR not only decreased the expression of C/EBPα and COX-2 in basal treatment, but it also exerted a significant inhibitory effect of cAMP-induced expression of C/EBPα and COX-2 (**Figures 5F–H**). Additionally, knockdown of C/EBPα counteracted the cAMP-induced COX2 upregulation (**Figures 5I–K**). Here, we documented that C/EBPα and COX-2 may be induced by LHCGR signaling in a cAMP-dependent manner in granulosa cells. Furthermore, cAMP can promote the transcriptional activity of C/EBPα.

DISCUSSION

To date, the underlying molecular mechanisms involved in endometriosis-related LUFs remain largely elusive. COX-2 and its major derivative product, PGE2, are recognized to be indispensable factors in the formation of LUFs (9, 33). In this study, we found deceased LHCGR expression in GCs of mice model. The dysfunction may further result in inactivation of cAMP-dependent C/EBPα, which severed as a key transcription factor to regulate COX-2 activation (**Figures 6**). We demonstrated that endometriosis was associated with LUFs because of impaired ovulation function and partially unveiled the underlying mechanism.

LUFs has long been associated with endometriosis in primates (34), rodents (35), and humans (4). In surgical-induced EMs mice, we clearly observed evidence of ovulatory dysfunction due to unruptured follicle which has already developed to preovulatory stage. Although previous studies have claimed that inhibitors (36) and environmental endocrine disruptors (37) may play an important role in impaired ovulation, dysfunctional gene expression in patients with endometriosis draws focus on the expression of LHCGR (16). It is indispensable for granulosa cells to acquire the ability to respond to gonadotropin in follicle differentiation and maturation. As folliculogenesis proceeds, the dominant follicle acquires much higher expression of LHCGR, a gonadotropin-induced G protein–coupled receptor, to allow it to promote ovulation in response to LH (38). In this study, superovulation was initially induced by a standard dose of gonadotropin (5 IU), however, when a lower dose of hCG (2.5 IU) was administered more failed ovulation was observed in EMs mice. It was a matter of interest that the increased unruptured follicles were not found in the sham-operated mice. These results demonstrate that endometriosis reduces GC response to LH, which normally peaks before ovulation. Undoubtedly, the induction of the LHCGR in granulosa cells is a key step in reproductive physiology. Endometriosis is an estrogen-dependent chronic inflammatory condition that affects women in their reproductive period. The local intrafollicular environment and local environment of peritoneal fluid are immunologically dynamic and links the reproductive and immune systems. Alterations in ovarian follicle morphology and function have been documented in affected women. Nevertheless, we documented that the expression of LHCGR was decreased in EMs granulosa cells 48 h after PMSG in this study and it may be a key mediator of endometriosis-associated LUFs. These observations concurred with previous study indicating reduced expression of LHCGR is a key observation in cases of LUFs (39). It is further confirmed by the results obtained in *Lhcgr* knockout zebrafish showing increased unruptured follicles after LH surge (40). Furthermore, the administration of hCG during gonadotrophin ovulation prevents or treats LUFs (41), while a lower dose of hCG may induce LUFs (42). It seems that not only an adequate decrease in intrafollicular prostaglandin but decreased LH or LHCGR responsiveness contributes to the occurrence of LUFs as well. Taken together, it is possible that endometriosis induces attenuation of LHCGR during folliculogenesis. Although the follicle can develop to the preovulatory stage in a follicle-stimulating hormone-dependent manner, the endometriosis-associated pathological states result in decreased responsiveness of granulosa cells to LH peak and subsequently lead to the occurrence of unruptured follicle.

Following activation by LH, LHCGR interacts with a heterotrimeric G-protein (αγβ), generally Gs, that leads to increased intracellular biosynthesis of cAMP (43). Persistent cAMP from internalized LHCGR contributes to transmitting LH signals inside follicles and ultimately to the oocyte (44).Moreover, inactivating mutation of LHCGR has been identified in some women, although follicles of ovulatory size develop fail to ovulate due to decreased cAMP levels (45). In a word, the LHCGR-provoked cAMP, which spreads throughout the follicle is critical to identify the mechanisms involved in the pathogenesis of unruptured follicles, especially after LH surge. Previous studies have originally confirmed that cAMP signaling can increase the transcriptional activity of cAMP-response element-binding protein (CREB) (46), but recent researches provide compelling evidence that C/EBPs also serve as cAMP-responsive transcription factors due to their functionally cAMP-inducible activities (47). Occupying specific cis-elements in the cAMP response unit (CRU), C/EBPα has proved to play a critical role in this process (48). In this study, we found that the attenuation of C/EBPα in endometriosis GCs and a previous study had clarified C/EBPα loss may cause infertility due to LUFs (49). Our results further demonstrated that both hCG and db-cAMP can strongly induce the expression and transcriptional activity of C/EBPα, and the hCG-induced expression can be eliminated by LHCGR knock-down. Thus, it is possible that C/EBPα is hormonally regulated in the ovary and plays an important role during ovarian follicular development and ovulation.

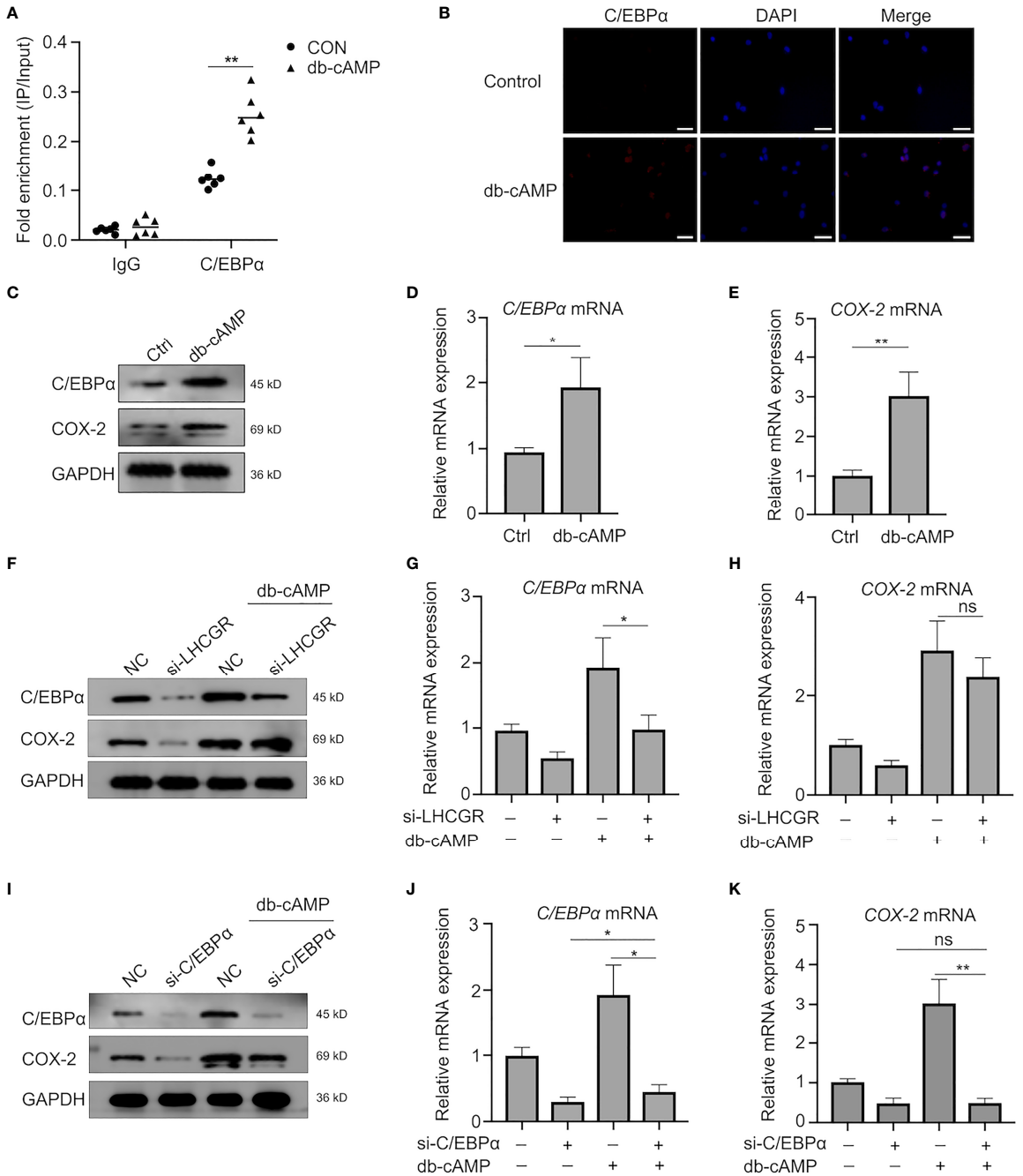


FIGURE 5 | Transcriptional activity of C/EBPα is regulated in a Cyclic AMP-Independent manner. **(A)** KGN cells were left untreated or stimulated with db-cAMP. ChIP assays were performed using anti-C/EBPα antibody or isotype control antibody (IgG). qRT-PCR was used to determine C/EBPα occupancy at the potential biding site under the conditions tested. **P <0.001 (Student's t-test). **(B)** db-cAMP-treated (24 h) human GCs were analyzed by immunofluorescence to identify the subcellular localization and protein expression levels of C/EBPα (red). Nuclei were stained using DAPI (blue). Magnification: ×100. Scale bar, 50 μm. **(C–E)** Western blotting and qRT-PCR analysis of indicated genes and protein in human GCs after treatment with db-cAMP. *$P < 0.05$, **$P < 0.001$ (Student's *t* -test). **(F–H)** Human GCs were transfected with negative control siRNA or LHCGR siRNA and then treated with 1 mM db-cAMP for 24 h. The expression of indicated genes and protein was analyzed by qRT-PCR and Western blot. ns, no significance, *$P < 0.05$ (ANOVA). **(I–K)** The protein and mRNA levels of C/EBPα and COX-2 in human GCs, which were treated with 1 mM db-cAMP for 24 h following exposure to siRNAs against C/EBPα. ns, no significance, *$P < 0.05$, **$P < 0.001$ (ANOVA).

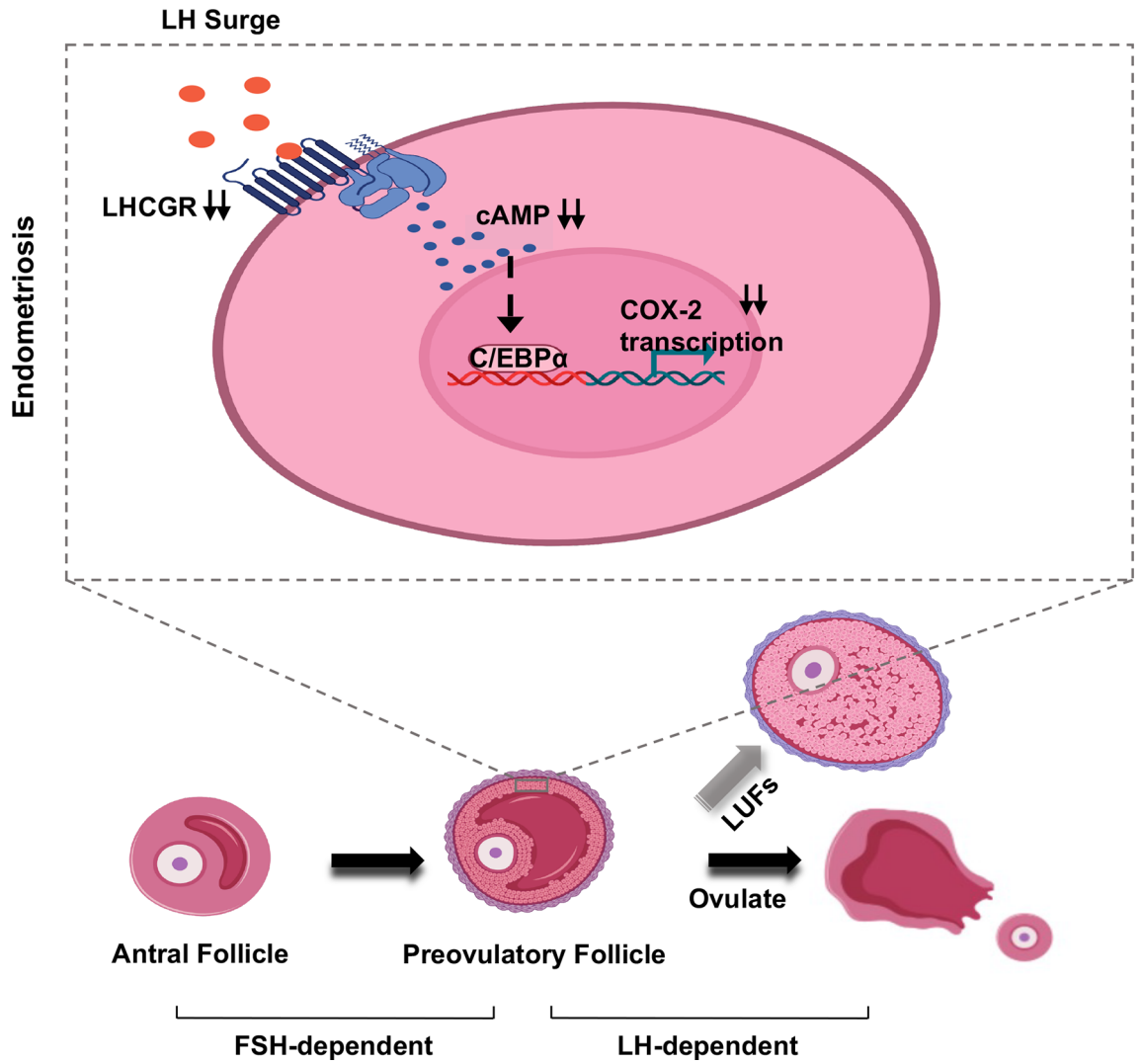


FIGURE 6 | Model of LUFs in endometriosis. Schematic depiction of the effect of LH signaling in preovulatory granulosa cells in endometriosis. During midcycle LH surge, attenuated LHCGR deactivating C/EBPα in a cAMP-dependent manner, then the transcription of COX-2 was repressed in granulosa cells. Ultimately, inducing ovulation failure and oocyte trapped in CLs.

Ovulation is a complex process initiated by the preovulatory LH surge that activates the signal transduction cascades and provokes the expression of numerous endocrine factors. More particularly, many studies have highlighted the important role played by the gonadotrophin-dependent induction of COX-2, which is a key enzyme required for prostaglandin synthesis in the periovulatory follicles (50). In fact, using non-steroidal anti-inflammatory drugs (NSAIDs) would lead to an increase in LUFs in juvenile idiopathic arthritis (JIA) patients due to the effect of inhibition of cyclooxygenase (28, 51). Moreover, animal study has revealed that selective COX-2 inhibitor is a more potent inducers of LUFs (28). Apart from eutopic and ectopic endometrium (52), abnormal expression of COX-2 is also found in cumulus cells of infertile women with endometriosis (53, 54). Our experiments showed that COX-2 expression was decreased in granulosa cells of endometriosis mice. Both C/EBPα and C/EBPβ are expressed in granulosa cells, and are dynamically initiated by LH and hCG to regulate genes that control luteinization and ovulation (55, 56). Although C/EBPβ is the known major regulator of the COX-2 gene, C/EBPβ-deficient ovaries lack corpora lutea and fail to down-regulate expression of COX-2 (32). Therefore, we can speculate that C/EBPα may be involved in this critical progress in ovary. It is well established that the expression of C/EBPα is under the positive control of hCG (57), furthermore, C/EBP-α could serve as a factor mediating COX-2 expression and PGE2 production (58). As C/EBPα gene deletion has resulted in moderately reduced ovulation in mice (49), we further investigated whether C/EBPα is involved in the effects of hCG and cAMP on COX-2 expression in human GCs. Our results indicated that both cAMP

and hCG stimulation of COX-2 was eliminated by knock-down of C/EBPα. Using immortalized human granulosa cells, KGN, we further presented molecular and functional evidence that C/EBPα is responsible for regulating COX-2 expression by directly modulating transcriptional activation.

Here, we provide evidence that attenuation of LHCGR in granulosa cells is involved in the increased incidence of LUFs in surgical-induced endometriosis mice. In an *in vitro* cell model system of human granulosa cells, we identify a previously unappreciated role for LHCGR activating transcription factor C/EBPα in a cAMP-dependent manner to sustain COX-2 expression that is necessary for mature follicle rupture and ovulation. We showed molecular and functional evidence that reveals GC dysfunction for the LHCGR as a central mediator of COX-2 expression and may result in LUFs in EMs. Clinical studies and samples acquired from patients are needed in further study to dissect the pathophysiology of this enigmatic syndrome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Zhongnan Hospital of Wuhan University (No. 2018047). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Wuhan University (Approval No. WP2020-08005).

AUTHOR CONTRIBUTIONS

TG and YS conceived the study, performed the experiments, processed the data and wrote the manuscript. YC and LC processed the data and revised the manuscript. ZH, MZ, and LM collected the clinical samples. YZ conceived the study, contributed to the study design and final approval of the version to be submitted. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work was supported by the National Key Research and Development Program of China (No. 2020YFA0803900) and the National Natural Science Foundation (No. 81771543).

ACKNOWLEDGMENTS

The authors are grateful for the technical assistance of Dr. Rongzhang Dou. Additionally, we would like to thank Dr. Juling Qin, Dr. Lei Shi, Dr. Yanhong Mao and Dr. Rong Zhou for their assistance with the collection of human granulosa cells from the Zhongnan Hospital.

REFERENCES

1. Cuervo-Arango J, Beg MA, Ginther OJ. Follicle and Systemic Hormone Interrelationships During Induction of Luteinized Unruptured Follicles With a Prostaglandin Inhibitor in Mares. *Theriogenology* (2011) 76(2):361–73. doi: 10.1016/j.theriogenology.2011.02.015
2. Bourne TH, Reynolds K, Waterstone J, Okokon E, Jurkovic D, Campbell S, et al. Paracetamol-Associated Luteinized Unruptured Follicle Syndrome: Effect on Intrafollicular Blood Flow. *Ultrasound Obstet Gynecol: Off J Int Soc Ultrasound Obstet Gynecol* (1991) 1(6):420–5. doi: 10.1046/j.1469-0705.1991.01060420.x
3. Holtz G, Williamson HO, Mathur RS, Landgrebe SC, Moore EE. Luteinized Unruptured Follicle Syndrome in Mild Endometriosis. Assessment With Biochemical Parameters. *J Reprod Med* (1985) 30(9):643–5. doi: 10.1515/jpme.1985.13.6.305
4. Mio Y, Toda T, Harada T, Terakawa N. Luteinized Unruptured Follicle in the Early Stages of Endometriosis as a Cause of Unexplained Infertility. *Am J Obstet Gynecol* (1992) 167(1):271–3. doi: 10.1016/S0002-9378(11)91673-1
5. Moon CE, Bertero MC, Curry TE, London SN, Muse KN, Sharpe KL, et al. The Presence of Luteinized Unruptured Follicle Syndrome and Altered Folliculogenesis in Rats With Surgically Induced Endometriosis. *Am J Obstet Gynecol* (1993) 169(3):676–82. doi: 10.1016/0002-9378(93)90642-v
6. Schenken RS, Asch RH, Williams RF, Hodgen GD. Etiology of Infertility in Monkeys With Endometriosis: Luteinized Unruptured Follicles, Luteal Phase Defects, Pelvic Adhesions, and Spontaneous Abortions. *Fertil Steril* (1984) 41(1):122–30. doi: 10.1016/s0015-0282(16)47552-7
7. Duffy DM, Ko C, Jo M, Brannstrom M, Curry TE. Ovulation: Parallels With Inflammatory Processes. *Endocr Rev* (2019) 40(2):369–416. doi: 10.1210/er.2018-00075
8. Prins JR, Marissen LM, Scherjon SA, Hoek A, Cantineau AEP. Is There an Immune Modulating Role for Follicular Fluid in Endometriosis? A Narrative Review. *Reproduction* (2020) 159(1):R45–54. doi: 10.1530/Rep-19-0050
9. Duffy DM. Novel Contraceptive Targets to Inhibit Ovulation: The Prostaglandin E2 Pathway. *Hum Reprod Update* (2015) 21(5):652–70. doi: 10.1093/humupd/dmv026
10. Peluffo MC, Murphy M, Zelinski MB, Lindenthal B, Stouffer RL, Hennebold JD. Cumulus-Oocyte Expansion in Rhesus Macaques: A Critical Role for Prostaglandin E2 (PGE2) and the PGE2 Receptor Subtype-2 (PTGER2). *Biol Reprod* (2011) Suppl_1:146. doi: 10.1093/biolreprod/85.s1.146
11. Donabela F, Barcelos I, Padovan C, Meola J, Ferriani RA, Navarro P. PTGS2 Gene Expression (COX2) in Cummulus Oophorus Cells of Endometriosis and Control Infertile Patients Submitted to ICSI. *Fertil Steril* (2011) 96(3-supp-S):S236–7. doi: 10.1016/j.fertnstert.2011.07.909
12. Berisha B, Rodler D, Schams D, Sinowatz F, Pfaffl MW. Prostaglandins in Superovulation Induced Bovine Follicles During the Preovulatory Period and Early Corpus Luteum. *Front Endocrinol* (2019) 10:467. doi: 10.3389/fendo.2019.00467
13. Filicori M, Cognigni GE, Samara A, Melappioni S, Perri T, Cantelli B, et al. The Use of LH Activity to Drive Folliculogenesis: Exploring Uncharted Territories in Ovulation Induction. *Hum Reprod Update* (2002) 8(6):543–57. doi: 10.1093/humupd/8.6.543

14. Convissar S, Winston NJ, Fierro MA, Scoccia H, Zamah AM, Stocco C. Sp1 Regulates Steroidogenic Genes and LHCGR Expression in Primary Human Luteinized Granulosa Cells. *J Steroid Biochem* (2019) 190:183–92. doi: 10.1016/j.jsbmb.2019.04.003
15. Cahill DJ, Harlow CR, Wardle PG. Pre-Ovulatory Granulosa Cells of Infertile Women With Endometriosis are Less Sensitive to Luteinizing Hormone. *Am J Reprod Immunol* (2003) 49(2):66–9. doi: 10.1034/j.1600-0897.2003.01156.x
16. Kauppila A, Rajaniemi H, Ronnberg L, Low LH. (hCG) Receptor Concentration in Ovarian Follicles in Endometriosis. *Acta Obstet Gyn Scan* (1982) 61(1):81–3. doi: 10.3109/00016348209156957
17. Pelch KE, Sharpe-Timms KL, Nagel SC. Mouse Model of Surgically-Induced Endometriosis by Auto-Transplantation of Uterine Tissue. *J Vis Exp* (2012) 59):e3396. doi: 10.3791/3396
18. Wang H, Zhong C, Yang R, Yin Y, Tan R, Gao L, et al. Hfm1 Participates in Golgi-Associated Spindle Assembly and Division in Mouse Oocyte Meiosis. *Cell Death Dis* (2020) 11(6):490. doi: 10.1038/s41419-020-2697-4
19. Stubbs SA, Jaroslav S, Dilworth SM, Stephen F, Kate H. Abnormal Preantral Folliculogenesis in Polycystic Ovaries Is Associated With Increased Granulosa Cell Division. *J Clin Endocr Metab* (2007) 11):4418–26. doi: 10.1210/jc.2007-0729
20. Kim TH, Yoo JY, Choi KC, Shin JH, Leach RE, Fazleabas AT, et al. Loss of HDAC3 Results in Nonreceptive Endometrium and Female Infertility. *Sci Transl Med* (2019) 11(474):eaaf7553. doi: 10.1126/scitranslmed.aaf7533
21. Hobeika E, Armouti M, Fierro MA, Winston N, Scoccia H, Zamah AM, et al. Regulation of Insulin-Like Growth Factor 2 by Oocyte-Secreted Factors in Primary Human Granulosa Cells. *J Clin Endocrinol Metab* (2020) 105(1):327–35. doi: 10.1210/clinem/dgz057
22. Shinagawa M, Tamura I, Maekawa R, Sato S, Shirafuta Y, Mihara Y, et al. C/EBPbeta Regulates Vegf Gene Expression in Granulosa Cells Undergoing Luteinization During Ovulation in Female Rats. *Sci Rep* (2019) 9(1):714. doi: 10.1038/s41598-018-36566-y
23. Chopra S, Giovanelli P, Alvarado-Vazquez PA, Alonso S, Song M, Sandoval TA, et al. IRE1 Alpha-XBP1 Signaling in Leukocytes Controls Prostaglandin Biosynthesis and Pain. *Science* (2019) 365(6450):248–+. doi: 10.1126/science.aau6499
24. Vitellius G, Delemer B, Caron P, Chabre O, Bouligand J, Pussard E, et al. Impaired 11 Beta-Hydroxysteroid Dehydrogenase Type 2 in Glucocorticoid-Resistant Patients. *J Clin Endocr Metab* (2019) 104(11):5202–13. doi: 10.1210/jc.2019-00800
25. Fang LL, Cheng JC, Chang HM, Sun YP, Leung PCK. EGF-Like Growth Factors Induce COX-2-Derived PGE2 Production Through ERK1/2 in Human Granulosa Cells. *J Clin Endocr Metab* (2013) 98(12):4932–41. doi: 10.1210/jc.2013-2662
26. Shimada M, Umehara T, Hoshino Y. Roles of Epidermal Growth Factor (EGF)-Like Factor in the Ovulation Process. *Reprod Med Biol* (2016) 15 (4):201–16. doi: 10.1007/s12522-016-0236-x
27. Rico C, Dodelet-Devillers A, Paquet M, Tsoi M, Lapointe E, Carmeliet P, et al. HIF1 Activity in Granulosa Cells Is Required for FSH-Regulated Vegfa Expression and Follicle Survival in Mice. *Biol Reprod* (2014) 90(6):135. doi: 10.1095/biolreprod.113.115634
28. Micu MC, Micu R, Ostensen M. Luteinized Unruptured Follicle Syndrome Increased by Inactive Disease and Selective Cyclooxygenase 2 Inhibitors in Women With Inflammatory Arthropathies. *Arthritis Care Res (Hoboken)* (2011) 63(9):1334–8. doi: 10.1002/acr.20510
29. Piontkewitz Y, Enerb?Ck S, Hedin L. Expression of CCAAT Enhancer Binding Protein-α (C/Ebpα) in the Rat Ovary: Implications for Follicular Development and Ovulation. *Dev Biol* (1996) 179(1):288–96. doi: 10.1006/dbio.1996.0258
30. Ren YA, Liu Z, Mullany LK, Fan CM, Richards JS. Growth Arrest Specific-1 (GAS1) Is a C/EBP Target Gene That Functions in Ovulation and Corpus Luteum Formation in Mice. *Biol Reprod* (2016) 94(2):44–4. doi: 10.1095/biolreprod.115.133058
31. Gupta A, Pandey AN, Sharma A, Tiwari M, Yadav PK, Yadav AK, et al. Cyclic Nucleotide Phosphodiesterase Inhibitors: Possible Therapeutic Drugs for Female Fertility Regulation. *Eur J Pharmacol* (2020) 883:173293. doi: 10.1016/j.ejphar.2020.173293
32. Gillio-Meina C, Hui YY, LaVoie HA. Expression of CCAAT/enhancer Binding Proteins Alpha and Beta in the Porcine Ovary and Regulation in Primary Cultures of Granulosa Cells. *Biol Reprod* (2005) 72(5):1194–204. doi: 10.1095/biolreprod.104.035246
33. Niringiyumukiza JD, Cai HC, Xiang WP. Prostaglandin E2 Involvement in Mammalian Female Fertility: Ovulation, Fertilization, Embryo Development and Early Implantation. *Reprod Biol Endocrin* (2018) 16. doi: 10.1186/s12958-018-0359-5
34. D'Hooghe TM. Clinical Relevance of the Baboon as a Model for the Study of Endometriosis. *Fertil Steril* (1997) 68(4):613–25. doi: 10.1016/S0015-0282(97)00277-X
35. Stilley J, Woods-Marshall R, Sutovsky M, Sutovsky P, Sharpe-Timms KL. Reduced Fecundity in Female Rats With Surgically Induced Endometriosis and in Their Daughters: A Potential Role for Tissue Inhibitors of Metalloproteinase 1. *Biol Reprod* (2009) 80(4):649–56. doi: 10.1095/biolreprod.108.073411
36. Li CY, Liu ZJ, Li WJ, Zhang LL, Zhou JL, Sun MH, et al. The FSH-HIF-1 Alpha-VEGF Pathway Is Critical for Ovulation and Oocyte Health But Not Necessary for Follicular Growth in Mice. *Endocrinology* (2020) 161(4): bqaa038. doi: 10.1210/endocr/bqaa038
37. Lopez-Rodriguez D, Franssen D, Bakker J, Lomniczi A, Parent AS. Cellular and Molecular Features of EDC Exposure: Consequences for the GnRH Network. *Nat Rev Endocrinol* (2021) 17(2):83–96. doi: 10.1038/s41574-020-00436-3
38. Ereno RL, Loureiro B, Castilho AC, Machado MF, Pegorer MF, Satrapa RA, et al. Expression of mRNA Encoding the LH Receptor (LHR) and LHR Binding Protein in Granulosa Cells From Nelore (Bos Indicus) Heifers Rround Follicle Deviation. *Reprod Domest Anim.* (2015) 50(6):952–7. doi: 10.1111/rda.12614
39. Koskimies AI, Liukkonen S, Tenhunen A, Huhtaniemi I. Low LH Receptor Content in Corpora Lutea in Luteinized Unruptured Follicle (LUF) Syndrome. *Hum Reprod* (1987) 2(5):367–9. doi: 10.1093/oxfordjournals.humrep.a136552
40. Liu DT, Hong WS, Chen SX, Zhu Y. Upregulation of Adamts9 by Gonadotropin in Preovulatory Follicles of Zebrafish. *Mol Cell Endocrinol* (2020) 499:110608. doi: 10.1016/j.mce.2019.110608
41. Azmoodeh A, Pejman Manesh M, Akbari Asbagh F, Ghaseminejad A, Hamzehgardeshi Z. Effects of Letrozole-HMG and Clomiphene-HMG on Incidence of Luteinized Unruptured Follicle Syndrome in Infertile Women Undergoing Induction Ovulation and Intrauterine Insemination: A Randomised Trial. *Global J Health Sci* (2015) 8(4):244–52. doi: 10.5539/gjhs.v8n4p244
42. Bashir ST, Gastal MO, Tazawa SP, Tarso SGS, Hales DB, Cuervo-Arango J, et al. The Mare as a Model for Luteinized Unruptured Follicle Syndrome: Intrafollicular Endocrine Milieu. *Reproduction* (2016) 151(3):271–83. doi: 10.1530/Rep-15-0457
43. Puett D, Li Y, Angelova K, Demars G, Meehan TP, Fanelli F, et al. Structure-Function Relationships of the Luteinizing Hormone Receptor. *Ann N Y Acad Sci* (2005) 1061:41–54. doi: 10.1196/annals.1336.006
44. Lyga S, Volpe S, Werthmann RC, Gotz K, Sungkaworn T, Lohse MJ, et al. Persistent cAMP Signaling by Internalized LH Receptors in Ovarian Follicles. *Endocrinology* (2016) 157(4):1613–21. doi: 10.1210/en.2015-1945
45. Arnhold IJ, Lofrano-Porto A, Latronico AC. Inactivating Mutations of Luteinizing Hormone Beta-Subunit or Luteinizing Hormone Receptor Cause Oligo-Amenorrhea and Infertility in Women. *Horm Res* (2009) 71 (2):75–82. doi: 10.1159/000183895
46. Paradiso E, Lazzaretti C, Sperduti S, Antoniani F, Fornari G, Brigante G, et al. Sphingosine-1 Phosphate Induces cAMP/PKA-Independent Phosphorylation of the cAMP Response Element-Binding Protein (CREB) in Granulosa Cells. *Mol Cell Endocrinol* (2021) 520:111082. doi: 10.1016/j.mce.2020.111082
47. Wilson HL, Roesler WJ. CCAAT/enhancer Binding Proteins: Do They Possess Intrinsic cAMP-Inducible Activity? *Mol Cell Endocrinol* (2002) 188(1-2):15–20. doi: 10.1016/s0303-7207(01)00754-7
48. Roesler WJ. What is a cAMP Response Unit? *Mol Cell Endocrinol* (2000) 162 (1-2):1–7. doi: 10.1016/S0303-7207(00)00198-2
49. Fan HY, Liu Z, Johnson PF, Richards JAS. CCAAT/Enhancer-Binding Proteins (C/EBP)-α and -β Are Essential for Ovulation, Luteinization, and the Expression of Key Target Genes. *Mol Endocrinol* (2011) 2):253–68. doi: 10.1210/me.2010-0318
50. Sirois J, Sayasith K, Brown KA, Stock AE, Bouchard N, Dore M. Cyclooxygenase-2 and its Role in Ovulation: A 2004 Account. *Hum Reprod Update* (2004) 10(5):373–85. doi: 10.1093/humupd/dmh032

51. Tomioka RB, Ferreira GRV, Aikawa NE, Maciel GAR, Serafini PC, Sallum AM, et al. Non-Steroidal Anti-Inflammatory Drug Induces Luteinized Unruptured Follicle Syndrome in Young Female Juvenile Idiopathic Arthritis Patients. *Clin Rheumatol* (2018) 37(10):2869–73. doi: 10.1007/s10067-018-4208-x
52. Ota H, Igarashi S, Sasaki M, Tanaka T. Distribution of Cyclooxygenase-2 in Eutopic and Ectopic Endometrium in Endometriosis and Adenomyosis. *Hum Reprod* (2001) 16(3):561–6. doi: 10.1093/humrep/16.3.561
53. Yin YX, Mao YD, Liu A, Shu L, Yuan C, Cui YG, et al. Insufficient Cumulus Expansion and Poor Oocyte Retrieval in Endometriosis-Related Infertile Women. *Reprod Sci* (2021) 28(5):1412–20. doi: 10.1007/s43032-020-00410-4
54. da Luz CM, da Broi MG, Donabela FC, de Paz CCP, Meola J, Navarro PA. PTGS2 Down-Regulation in Cumulus Cells of Infertile Women With Endometriosis. *Reprod BioMed Online* (2017) 35(4):379–86. doi: 10.1016/j.rbmo.2017.06.021
55. Zhen YH, Wang L, Riaz H, Wu JB, Yuan YF, Han L, et al. Knockdown of CEBPbeta by RNAi in Porcine Granulosa Cells Resulted in S Phase Cell Cycle Arrest and Decreased Progesterone and Estradiol Synthesis. *J Steroid Biochem Mol Biol* (2014) 143:90–8. doi: 10.1016/j.jsbmb.2014.02.013
56. Piontkewitz Y, Enerback S, Hedin L. Expression of CCAAT Enhancer Binding Protein-Alpha (C/EBP Alpha) in the Rat Ovary: Implications for Follicular Development and Ovulation. *Dev Biol* (1996) 179(1):288–96. doi: 10.1006/dbio.1996.0258
57. Dos Santos E, Dieudonne MN, Leneveu MC, Pecquery R, Serazin V, Giudicelli Y. *In Vitro* Effects of Chorionic Gonadotropin Hormone on Human Adipose Development. *J Endocrinol* (2007) 194(2):313–25. doi: 10.1677/JOE-06-0101
58. Pan HC, Yang CN, Hung YW, Lee WJ, Tien HR, Shen CC, et al. Reciprocal Modulation of C/EBP-Alpha and C/EBP-Beta by IL-13 in Activated Microglia Prevents Neuronal Death. *Eur J Immunol* (2013) 43(11):2854–65. doi: 10.1002/eji.201343301

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 29 August 2022
DOI 10.3389/fendo.2022.932339

OPEN ACCESS

EDITED BY
Sijia Lu,
Yikon Genomics, China

REVIEWED BY
Kai-Fai Lee,
The University of Hong Kong, Hong Kong SAR, China
Min Jin,
Zhejiang University, China
Chi Chiu Wang,
The Chinese University of Hong Kong, China

*CORRESPONDENCE
Jintao Peng
pengjt3@mail.sysu.edu.cn
Haitao Zeng
zenghtao@mail.sysu.edu.cn

†These authors have contributed equally to this work and share first authorship

SPECIALTY SECTION
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

RECEIVED 29 April 2022
ACCEPTED 04 July 2022
PUBLISHED 29 August 2022

CITATION
Xiang R, Chen P, Zeng Z, Liu H, Zhou J, Zhou C, Peng J and Zeng H (2022) Transcriptomic analysis shows that surgical treatment is likely to influence the endometrial receptivity of patients with stage III/IV endometriosis.
Front. Endocrinol. 13:932339.
doi: 10.3389/fendo.2022.932339

Transcriptomic analysis shows that surgical treatment is likely to influence the endometrial receptivity of patients with stage III/IV endometriosis

Rui Xiang[1†], Peigen Chen[1†], Zhi Zeng[1†], Huijun Liu[1], Juan Zhou[2], Chuanchuan Zhou[1], Jintao Peng[1*] and Haitao Zeng[1*]

[1]Reproductive Medicine Center, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China, [2]Department of Gynecology and Obstetrics, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

Background: Endometriosis negatively affects fertility, and it is a common disease in assisted reproductive practice. Surgical removal of endometriotic lesions is widely carried out to relieve symptoms and promote fertility. But it is not intensively investigated what changes in the secretory eutopic endometrium of patients with endometriosis after surgery.

Methods: Eighteen patients with stage III/IV endometriosis were included in the study, and they were divided into the untreated group and the treated group (6 vs. 12). Basic clinical data were compared, and transcriptomic data of the secretory eutopic endometrium were analyzed with DESeq2, Cytoscape, ClueGO, CluePedia, and Gene Set Enrichment Analysis (GSEA). CIBERSORT was used to calculate the relative abundance of 22 immune cells in the samples.

Results: We determined 346 differentially expressed genes (DEGs) using DESeq2. These DEGs were used to enrich seven Gene Ontology terms including three associated with immune processes and one correlated to prostaglandin using ClueGO and CluePedia. GSEA enriched 28 Gene Ontology terms in the treated group mainly associated with immune and blood pressure regulation process. Compared to the untreated group, the relative abundance of resting CD4+ memory T cells [0.218 (0.069, 0.334) vs. 0.332 (0.181, 0.429), P = 0.022] and the even less abundant memory B cells [0.001 (0.000, 0.083) vs. 0.033 (0.007, 0.057), P = 0.049] are significantly decreased in the treated group.

Conclusion: Surgical treatment of stage III/IV endometriosis influences some genes and biological processes related to endometrial receptivity, but more evidence is needed.

KEYWORDS

endometriosis (EM), surgical treatment, endometrial receptivity, RNA sequencing (RNA-seq), secretory endometrium (SE), immune cell profile

Introduction

Endometriosis is recognized as a chronic estrogen-dependent complex syndrome and inflammatory disease that affects around 5%~10% of women, characterized by the abnormally existing endometrium-like tissues outside the uterus, commonly located on pelvic organs and tissues (1). Endometriosis significantly disturbs female fertility partly due to abnormal endometrial receptivity since studies in human and animal models show defective implantation (2).

Endometrial receptivity, coined as the ability of the endometrium to allow normal implantation, is fundamental for a successful pregnancy and is affected in patients with endometriosis. Multi-omics were applied to identify potential biomarkers for assessing endometrial receptivity. Clinical treatment of endometriosis improves pregnancy rates, and basic research showed abnormal endometrial milieu. For instance, prokineticin-1 (*PROK1*), which is important in the vascular function of peri-implantation endometrium and early pregnancy, is significantly less expressed in the eutopic endometrium of patients with endometriosis (3). Other factors that are associated with decidualization and implantation such as L-selectin ligand, $\alpha v\beta 3$ integrin, and leukemia inhibitory factor (*LIF*) are also decreased in the eutopic endometrium (4–6).

Surgery that removes ectopic endometrial tissue is a common measure to relieve symptoms and promote fertility. Some evidence shows that fertility is improved after surgery (7, 8), but the changes in the eutopic endometrium after surgery are not well-illustrated. We hereby conducted this research to preliminarily discover the effect of surgery on the secretory eutopic endometrium.

Materials and methods

Criteria of patients included in the research groups

Six patients in the untreated group were diagnosed with endometriosis by ultrasound or magnetic resonance imaging (MRI), as endometrioma exists and did not receive surgical treatment for endometriosis. Twelve patients in the treated group were confirmed to have endometriosis during laparoscopic or transabdominal surgery in less than 24 months before endometrial sampling, and the endometriotic lesions were removed during surgery. All 18 patients were of stage III/IV according to the revised American Fertility Society (r-AFS) classification. The exclusion criteria are as follows: 1) follicle stimulation hormone (FSH) >12 mIU/ml and anti-Müllerian hormone (AMH) <1.1 ng/ml; 2) abnormal uterine cavity morphology by ultrasound, MRI, or hysteroscopy; 3) signs of malignancies.

Endometrial preparation and sample collection protocol

Patients received 2 mg of estradiol valerate (Progynova, Bayer Pharma AG, Berlin, Germany) twice daily for 28 days starting on the second day of menstrual bleeding. From the 15th day onward, 10 mg of dydrogesterone (Duphaston, Abbott Biologicals B.V., Netherlands) twice daily was applied.

Endometrial sampling was conducted on P+5 (the 5th day of Duphaston administration) using a sterile endometrium sampler kit with a separate package (Type I, Run Ting). The patient was kept in the lithotomy position and received vulvar and vaginal disinfection with the introduction of the disinfected speculum, and a sterile swab was inserted into the cervical canal to avoid contamination. Then, a disposable sterile endometrial sampler (Yikon Inc.) was introduced into the uterine cavity to suck the endometrium. After that, the external surface of the catheter was cleaned with a sterile gauze and half of its content was transferred to cryotubes containing 1.5 ml of tissue preservation solution (XK-039-3, Yikon Inc.) at -20°C for future RNA extraction and PCR test, and the other half was stored in formalin for hematoxylin and eosin staining and immunohistochemistry staining for CD138. Chronic endometritis (CE) is determined as at least one plasma cell is found in the endometrial biopsy using immunohistochemistry staining for CD138 (9, 10).

RNA extraction and RNA sequencing procession

Total RNA was extracted using RNeasy Micro Kit (74004, Qiagen) according to the manufacturer's instructions. Then, RNA quantitative detection was conducted with Qubit RNA HS Kit (Q32855; Thermo Fisher Scientific). Agilent Bioanalyzer

2100 (Agilent) was used to check the integrity of the extracted total RNA. Only samples whose RNA integrity number (RIN) is greater than 7 were considered qualified samples and used for subsequent testing.

Next, RNA reverse transcription and amplification were conducted using MALBAC® Platinum Single Cell RNA Amplification Kit (KT110700796; Xukang Co., Ltd.). The positive and negative controls were 500 ng of high-quality host total RNA and ultrapure water, respectively. After that, 1 μl cDNA was 10-fold diluted and detected with Agilent Bioanalyzer 2100 (Agilent).

Finally, the library was constructed using gene sequencing and library preparation kit (XK-038, Xukang Co., Ltd.) according to the manufacturer's instructions. Following purification, the library was quantified with Qubit dsDNA HS kit (Q32584, Invitrogen). According to the quantitative results of Qubit, each sample was taken from 10 ng library and mixed in equal proportions, followed by Qubit quantitative detection again. Pair-end sequencing of the products was performed on the NextSeq CN500 platform (Illumina).

RNA extraction, quantitative real-time PCR, and public database search

Total RNA was extracted from five samples from the untreated group and 12 samples from the treated group using RNeasy Micro Kit (Qiagen, MD, USA; Cat# 74004) according to manufacturer's instructions. Reverse transcription of first-strand complementary DNAs (cDNAs) was carried out using HiScript III RT SuperMix for qPCR (Vazyme, Nanjing, China, Cat# R323-01). Quantitative PCR (qPCR) was conducted using 2× RealStar Green Power Mixture (Genstar; Cat# A311-101) on a Roche LightCycler 480 II (Roche Diagnostics, Mannheim, Germany). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as an endogenous control. The primers used were as follows:

HOXB2: forward: GATGAAAGAGAAGAAATCCGCC, reverse: AAGTGGAATTCCTTCTCCAGTT;

GAPDH: forward: GGTCGGAGTCAACGGATTT, reverse: CCAGCATCGCCCCACTTG.

The Human Protein Atlas (11) is used for the *HOXB2* gene expression feature identification.

RNA sequencing analysis procession

Firstly, FastQC v.0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to evaluate fastq files, and then the files were processed with trim_galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to remove reads containing adapters, more than 10% unknown nucleotides (N), and more than 50% of low-quality (Q-value ≤20) bases. Next, HISAT2 (12) was used to map the processed paired-end reads to the human genome GRCh38 from Gencode v26. FeatureCount (13) was used to generate the count value of gene expression in the form of a matrix. After that, principal component analysis was done with TPM (Transcripts Per Kilobase of exon model per Million mapped reads) value normalized from the matrix. The differentially expressed genes (DEGs) between the two groups were selected by using DESeq2 R package (14) with the *P* value <0.05 and log fold change (logFC) >1 as the cutoff. Then, functional enrichment analysis of DEGs was performed with ClueGO v.2.5.8 (15) and CluePedia 1.5.8 (16) plugins of Cytoscape v.3.9.1 (17). GSEA 4.2.1 (http://www.gsea-msigdb.org/gsea/index.jsp) was used to conduct functional enrichment analysis of whole gene expression features.

Calculation of endometrial immune cell abundance

CIBERSORT (18) was used to estimate the abundance of 22 types of immune cells in the endometrium.

Statistical analysis

Clinical characteristics of participants, including age, body mass index (BMI), FSH, and AMH, are presented as the mean ± standard deviation (SD) and were compared between groups using the *t*-test for equality of means. The relative abundance of immune cells is presented as the median (range) and was

TABLE 1 Basic information of the untreated and treated groups.

Items	The untreated	The treated	*P* value
No. of patients	6	12	–
Age (years)	34.17 ± 3.76	33.08 ± 2.23	0.451
BMI	21.07 ± 1.40	20.99 ± 2.43	0.945
FSH (IU/L)	6.17 ± 2.77	6.62 ± 2.06	0.698
AMH (ng/ml)	4.06 ± 1.70	2.58 ± 1.23	0.050
CE	16.67% (1/6)	8.33% (1/12)	>0.999

BMI, body mass index; FSH, follicle stimulating hormone; AMH, anti-Müllerian hormone; CE, chronic endometritis.

compared between groups using the nonparametric Mann–Whitney U-test. CE rate is presented as n% (positive/all) and was compared between groups using the Fisher's exact test. Above statistical analysis is conducted with SPSS 22.0 software (IBM Corporation, Armonk, NY, USA). The relative mRNA expression ratio was compared between groups using the nonparametric Mann–Whitney U-test by GraphPad Prism 9.0.0 software (Bethesda, MD, USA). Statistical significance was set at P value <0.05.

Results

Clinical features of enrolled patients

The age, BMI, FSH, AMH, and CE rates are demonstrated in Table 1.

Transcriptomic features of the secretory eutopic endometrium in between the untreated and treated groups

We first conducted PCA that shows relatively obvious discrimination between the two groups (Figure 1).

Then, we used DESeq2 to determine the DEGs between the groups. A total of 346 DEGs were filtered with logFC >|1| and P value <0.05. Among these DEGs, 140 and 206 genes are upregulated and downregulated in the treated group, respectively. The top 10 upregulated and downregulated genes with high expression levels are exhibited in Table 2. All 346 DEGs are presented in Supplementary Material 1. Of the 20 top expressed DEGs, we found *HOXB2* to be of importance, since it is a downstream gene of type I interferon (IFN) response and we validated its expression by qPCR (Figure 2A). We then searched for the expression feature of *HOXB2* in the human protein atlas database, and it turns out that *HOXB2* expresses highly in endometrial ciliated cells, natural killer (NK) cells, and T cells (Figure 2B).

Functional analysis of the transcriptomic features

Functional analysis derived from ClueGO suggests that "regulation of αβT cell proliferation," "regulation of natural killer cell mediated immunity," "regulation of defense response to bacterium," "regulation of response to wounding," "epithelial fluid transport," "nucleoside biphosphate biosynthetic process," and "prostaglandin transport" are different between the untreated and treated groups (Figure 3A). Of the seven Gene Ontology (GO) terms, three are associated with immune processes. Gene counts, gene ratio, and Bonferroni-corrected P value of each term are shown in Figure 3B.

To have a broader view of the potential changes based on the whole transcriptomic features, we ran GSEA and enriched 28 GO terms in the treated group, including 17 terms of biological process, 8 terms of molecular function, and 3 terms of cellular component (Table 3). Of these GO terms, "defense response to Gram negative bacterium," "antibacterial humoral response," "humoral immune response mediated by circulating

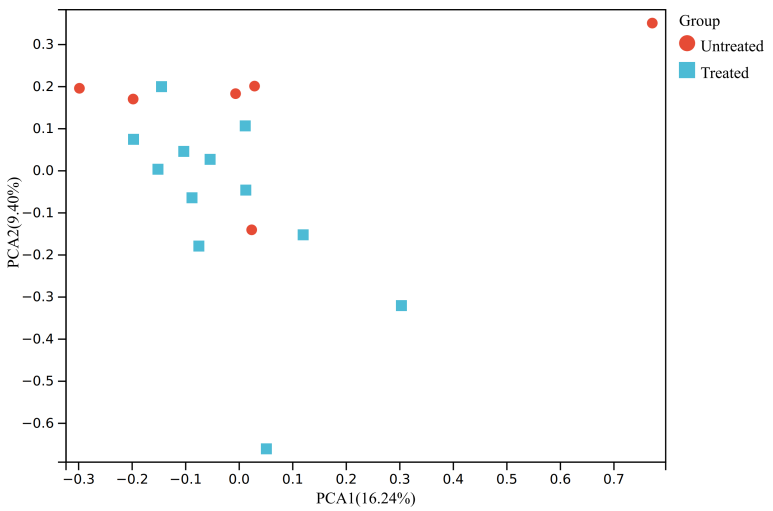


FIGURE 1
PCA of the transcriptomic data. The raw expression matrix was normalized to TPM before PCA. PCA, principal component analysis; TPM, Transcripts Per Kilobase of exon model per Million mapped reads; Untreated, the untreated group; Treated, the treated group.

TABLE 2 Top 10 upregulated and downregulated genes with a high expression level.

ENSEMBL ID	Gene Symbol	Gene Name	Biotype	Base Mean	logFC	*P* value
Up-regulated genes						
ENSG00000157613	CREB3L1	cAMP-responsive element-binding protein 3 like 1	Protein coding	915.372	1.109	0.004
ENSG00000100075	SLC25A1	Solute carrier family 25 member 1	Protein coding	259.557	1.165	0.001
ENSG00000179271	GADD45GIP1	GADD45G interacting protein 1	Protein coding	120.709	1.119	<0.001
ENSG00000078808	SDF4	Stromal cell derived factor 4	Protein coding	108.906	1.289	<0.001
ENSG00000102760	RGCC	Regulator of cell cycle	Protein coding	65.688	1.583	0.020
ENSG00000173917	HOXB2	Homeobox B2	Protein coding	63.869	1.042	0.006
ENSG00000157933	SKI	SKI proto-oncogene	Protein coding	56.638	1.085	<0.001
ENSG00000170624	SGCD	Sarcoglycan delta	Protein coding	50.021	1.057	0.029
ENSG00000051523	CYBA	Cytochrome b-245 alpha chain	Protein coding	43.135	1.053	0.001
ENSG00000176463	SLCO3A1	Solute carrier organic anion transporter family member 3A1	Protein coding	42.782	1.325	<0.001
Down-regulated genes						
ENSG00000134333	LDHA	Lactate dehydrogenase A	Protein coding	4,270.837	-1.017	<0.001
ENSG00000165507	DEPP1	DEPP1 autophagy regulator	Protein coding	2,169.889	-1.850	0.002
ENSG00000100342	APOL1	Apolipoprotein L1	Protein coding	2,075.956	-1.111	<0.001
ENSG00000189143	CLDN4	Claudin 4	Protein coding	1,989.718	-1.147	<0.001
ENSG00000159167	STC1	Stanniocalcin 1	Protein coding	1,761.674	-1.441	0.028
ENSG00000150347	ARID5B	AT-rich interaction domain 5B	Protein coding	1,452.366	-1.049	0.008
ENSG00000067082	KLF6	Kruppel-like factor 6	Protein coding	1,253.342	-1.033	0.001
ENSG00000122884	P4HA1	Prolyl 4-hydroxylase subunit alpha 1	Protein coding	1,146.017	-1.028	<0.001
ENSG00000196352	CD55	CD55 molecule	Protein coding	773.112	-1.035	0.004
ENSG00000162896	PIGR	Polymeric immunoglobulin receptor	Protein coding	681.500	-1.157	0.006

immunoglobulin", "immunological memory process", "immunoglobulin receptor binding", "antigen binding", "peptide antigen binding", "immunoglobulin complex", "T cell receptor complex," and "MHC protein complex" correlate with immune process.

Several terms associated with blood pressure regulation such as "regulation of systemic arterial blood pressure by renin angiotensin", "serotonin receptor signaling pathway", "regulation of systemic arterial blood pressure by hormone", "regulation of systemic arterial blood pressure mediated by a chemical signal", "regulation of systemic arterial blood pressure by circulatory renin angiotensin", "vasodilation", "regulation of systemic arterial blood pressure", and "serotonin receptor activity" are also enriched.

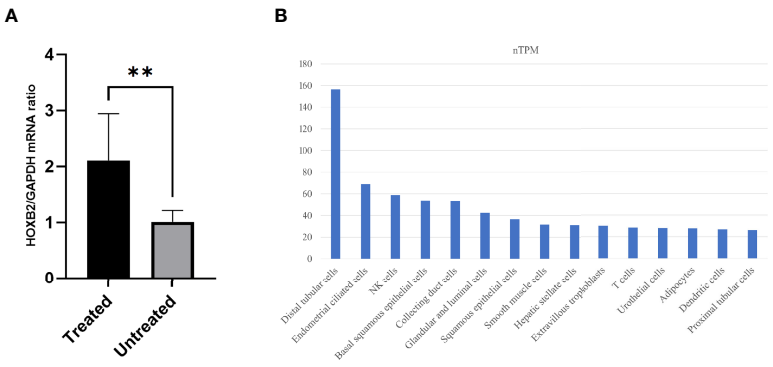


FIGURE 2
The expression of HOXB2. **(A)** Relative mRNA expression of HOXB2 in the treated (T) and untreated (UT) groups by quantitative real-time PCR. ***P* value = 0.006. **(B)** Top 15 cell types with high expression levels of HOXB2. Raw data are from the human protein atlas (www.proteinatlas.org). Untreated, the untreated group; Treated, the treated group.

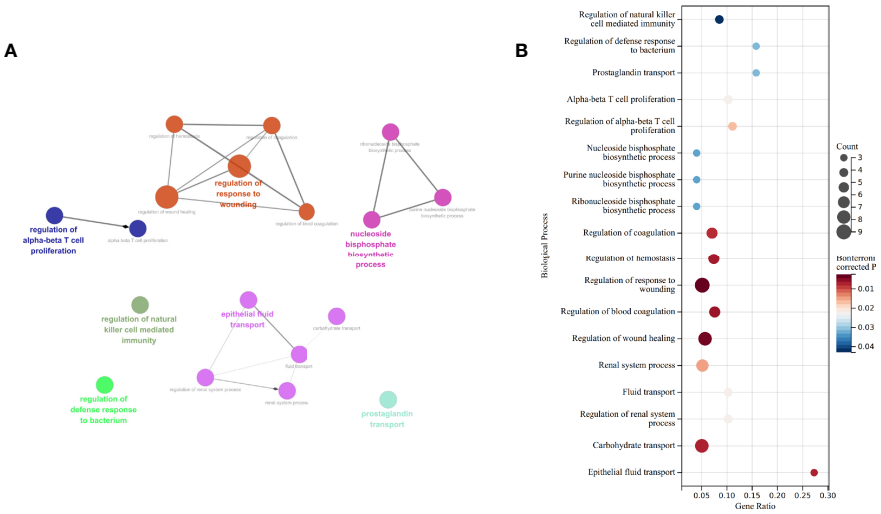


FIGURE 3
GO enrichment by ClueGO and CluePedia. **(A)** Network of biological process enriched. **(B)** Gene counts, gene ratio, and *P* value after Bonferroni correction of each GO: biological process terms. GO, gene ontology.

Calculated immune cell abundance in the secretory eutopic endometrium in between the untreated and treated groups

To further investigate the immunological changes in the endometrium, we used CIBERSORT to estimate the relative percent of 22 types of immune cells. Activated NK cells and resting CD4+ memory T cells comprise the majority of the immune cell population in the secretory eutopic endometrium (Figure 4A).

Then, the immune cell populations were compared between the groups, three of them are shown in Figure 4B. Activated NK cells are not significantly different [0.424 (0.248, 0.526) in the treated group vs. 0.365 (0.198, 0.568) in the untreated group, $P = 0.574$]. While the less abundant resting CD4+ memory T cells and the even less abundant memory B cells are significantly decreased in the treated group [0.218 (0.069, 0.334) vs. 0.332 (0.181, 0.429), $P = 0.022$, and 0.001 (0.000, 0.083) vs. 0.033 (0.007, 0.057), $P = 0.049$, respectively]. The comparison of all of the immune cells is concluded in Supplementary Material 2.

Discussion

In this study, we applied RNA-seq analysis and CIBERSORT to profile the endometrial environment during the secretory phase after surgical treatment of endometriosis. We found several DEGs and biological processes that could be associated with receptivity. Also, altered function and abundance of certain immune cells are speculated to be related to the possibly differed endometrial microbiota after surgery.

Changes identified at the gene expression level

The expression of certain genes associated with endometriosis and receptivity is altered after surgery.

HOXB2, a relatively less studied member of the homeobox genes in the endometrium is upregulated in type I IFN response (19), and one study pointed out that the response to IFN signaling is dysregulated in the eutopic endometrium of patients with adenomyosis during the secretory phase (20). IFNs bind to specific membrane receptors to exert biological activities. Upregulated transcription of IFN-α in the human endometrium during the window of implantation (21) and the effect of type I IFN-τ on receptivity and implantation in the endometrium of the ruminant animal (22) indicate the importance of IFNs in fertility. IFNs also play an important role in immune modulation, including the adaptive immune response (23). As a downstream target gene of type I IFN, the upregulated *HOXB2* expression is a putative implication of the altered IFN signaling in the endometrium of patients with endometriosis after surgery. *CREB3L1* that is identified as a target gene of the progesterone receptor is decreased in the eutopic endometrium of patients with endometriosis, and it regulates the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 in the decidualization process (24). The

TABLE 3 Enriched GO terms of 12 samples in the treated group by GSEA.

GO terms	NES	*P* value	FDR
Biological Process			
Regulation of systemic arterial blood pressure by renin angiotensin	-2.036	<0.001	0.080
Serotonin receptor signaling pathway	-2.003	<0.001	0.087
Hepatocyte differentiation	-1.936	<0.001	0.105
Regulation of systemic arterial blood pressure by hormone	-1.934	0.008	0.095
Regulation of systemic arterial blood pressure mediated by a chemical signal	-1.862	0.010	0.130
Regulation of systemic arterial blood pressure by circulatory renin angiotensin	-1.849	0.026	0.120
Defense response to Gram-negative bacterium	-1.786	<0.001	0.136
Sensory perception of smell	-1.777	<0.001	0.136
Positive regulation of organic acid transport	-1.736	0.006	0.154
Detection of chemical stimulus	-1.710	<0.001	0.174
Vasodilation	-1.670	0.015	0.214
Antibacterial humoral response	-1.670	0.011	0.206
Phagocytosis recognition	-1.659	0.015	0.203
Humoral immune response mediated by circulating immunoglobulin	-1.648	<0.001	0.209
Regulation of systemic arterial blood pressure	-1.646	<0.001	0.204
Immunological memory process	-1.641	0.015	0.203
Icosanoid secretion	-1.640	0.008	0.198
Molecular Function			
Serotonin receptor activity	-2.191	<0.001	0.030
Olfactory receptor activity	-1.978	<0.001	0.090
NAD^+ nucleosidase activity	-1.898	<0.001	0.111
Immunoglobulin receptor binding	-1.853	0.016	0.126
Carbohydrate derivative transmembrane transporter activity	-1.844	<0.001	0.116
Antigen binding	-1.804	<0.001	0.137
Peptide antigen binding	-1.769	0.023	0.136
G protein-coupled amine receptor activity	-1.666	0.013	0.203
Cellular Component			
Immunoglobulin complex	-2.313	<0.001	0.014
T-cell receptor complex	-1.804	<0.001	0.129
MHC protein complex	-1.745	0.014	0.153

NES, normalized enrichment score;
FDR, false discovery rate.

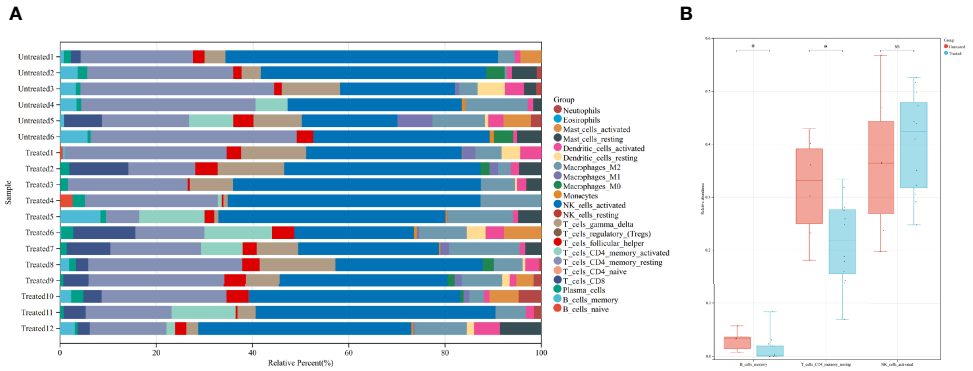


FIGURE 4
Immune cell abundance and comparison of certain immune cells between the groups. **(A)** CIBERSORT-calculated relative abundance of 22 different immune cells in the 18 samples. **(B)** Comparison of memory B cells, resting CD4+ memory T cells, and activated NK cells in the treated and untreated groups. **P* value <0.05. ns, not significant. Untreated, the untreated group; Treated, the treated group.

transcription level of *CLDN4* is significantly higher in the endometrium of idiopathic infertility and minimal endometriosis, indicating that *CLDN4* overexpression might be negatively linked to endometrial receptivity (25). *STC1* serves in many processes (26), and it correlates with receptivity markers during the window of implantation (27). In patients with endometriosis, *STC1* is upregulated in the mid-secretary eutopic endometrium (28).

In our results, *CREB3L1* and *HOXB2* are upregulated after surgery, while *CLDN4* and *STC1* are downregulated. These alterations might be important in remodeling receptivity.

Changes identified at the biological process level

Several biological processes are interconnected and found to be altered after surgery.

Prostaglandins (PGs) are bioactive in tissues and organs. PGE2 is observed to increase during the luteal phase in the animal uterus, and this hints that it might be associated with luteal function and implantation. PGE2 induces LH receptor expression on the corpus luteum through receptor EP2 (29, 30), and it increases the blood supply of the uterus and ovary by increasing a vasodilator called nitric oxide *via* receptor EP4 (31). In a recent review, the authors hypothesized that PGE2 prevents luteolysis by playing a role in the P4 secretion stimulated by estradiol (E2) and IFN-τ, and the latter is a member of type I IFNs (32). In goat endometrial stromal cells, it has been validated that IFN-τ could increase the ratio of PGE2/PGF2α *via* JAB1 and the unfolded protein response (UPR) mainly by regulating the amount of PGE2 rather than PGF2α (33). As the increased expression of *HOXB2* hints, this process is likely to be the underlying mechanism that alters luteal function.

PGE2 is also found to regulate the *CXCR4* expression through the epidermal growth factor receptor (EGFR)-phosphatidyl inositol-3 kinase (PI3K) and ERK1/2 pathway *via* prostaglandin E receptor 2 (PTGER2) in the endometrium (34–37). One study confirmed that *CXCR4* expression is especially increased at the apposition site (38). *CXCR4* is vital in embryonic vasculogenesis (39), which might be crucial in placenta attachment. Furthermore, the PI3K/ERK1/2 pathways enhance the growth, proliferation, differentiation, and survival of endometrial cells and embryos (40). PGE2 could also help increase the expression of *αvβ3* integrin, thus helping embryo adhesion (41). Fetal–maternal crosstalk between the embryo and the endometrium is essential in successful pregnancy, and PGE2 is widely involved in this process. Our analysis suggests that "prostaglandin transport" is altered after surgery, which might contribute to the altered response of the endometrium to the embryo and luteal function, as PGE2 might function in implantation by means that are mentioned above.

Changes identified at the immune environment level

The endometrium contains different kinds of immune cells and relative molecules, which are important during implantation and pregnancy.

A PGE2–myocyte enhancer factor 2A (MEF2A) axis in type I IFN induction is introduced recently. Research shows that PGE2 interferes with lipopolysaccharide (LPS)-mediated activation of ERK5 that is a known transcriptional partner of MEF2 and thus affects inflammatory gene expression (42). Given the previously mentioned regulation of IFN-τ on PGE2, it can be inferred that the PG and IFN response in the endometrium interact mutually and they maintain balance in an accurate manner, and surgery might influence that regulation process.

Implantation correlates with innate immune cells in the endometrium. Although our analysis did not show significant alteration of several innate immune cells in relative quantity after surgery, "regulation of αβT cell proliferation" and "regulation of natural killer cell mediated immunity" are enriched in the functional enrichment. In patients with endometriosis who obtained successful implantation, uterine NK progenitor cell populations are markedly higher than those in patients who have failed implantation (43). Uterine NK cells secrete angiogenic growth factor, contributing to decidualization and formation of spiral arteries, which makes them important in early pregnancy (44). However, there is evidence that NK cells are not essential for pregnancy (45). The ratio of T helper type 1 (Th1) cytokines over T helper type 2 (Th2) cytokines is related to fetal acceptance. Inflammatory Th1 immunity is dominant at the peri-implantation stage, it could benefit the invading of trophoblasts (46). Interestingly, there is evidence that type I IFN response works in the regulation of NK cells and T cells. In direct and indirect activation of NK cells, type I IFN could activate NK cells through the IFN-activating receptor on NK cells or dendritic cells are first activated by type I IFN, then they activate NK cells by trans-presentation of interleukin (IL)-15 to IL-15 receptors on NK cells, respectively (47). As for T cells, type I IFN could also alter the αβT-cell population by inducing an immune response of dendritic cells *via* both IL-15 and the IL-15R α-chain (48). Exposure to IFN-α/β results in increased expression of genes associated with cell proliferation and cell survival (49), which could be the mechanism of αβT-cell proliferation. Type I IFN leads to Th1 induction and Th2 restriction, which might contribute to better implantation (50). Due to the lack of adequate subjects, the function of T cells and NK cells after surgery needs to be further elucidated.

Implantation might correlate with adaptive immune cells in the endometrium as well. Memory B cells and resting CD4+ memory T cells are less in the treated group. We also find "antibacterial humoral response," "humoral immune response mediated by circulating immunoglobulin," and "immunological memory

process" are enriched in the treated group. Higher IL-2 seems to be adverse to pregnancy, since it induces increased NK activity of decidual NK cells (51) that leads to a less tolerant endometrial environment, and application of IL-2 causes fetal development inhibition in mice (52). Surgical treatment could reduce serum IL-2 (53), which might facilitate receptivity. CD4+ memory T cells are typical IL-2-producing cells (54), thus their reduction in the relative population might facilitate a healthier milieu for implantation. Both CD4+ memory T cells and memory B cells are generated in the adaptive immune process, so their abundance could be closely related to the microbes in the uterine cavity. Endometrial microbiota seems to be more diverse in patients with endometriosis (55). One study found a complete absence of *Atopobium* in the vaginal and cervical microbiota and an increase of potentially pathogenic *Gardnerella*, *Streptococcus*, *Escherichia*, *Shigella*, and *Ureoplasma* in the cervical microbiota in III/IV endometriosis (56). Another study categorized the endometrial microbial composition as either *Lactobacillus*-dominant (LD, >90% *Lactobacillus* spp.) or non-*Lactobacillus*-dominant (NLD, <90% *Lactobacillus* spp. with >10% of other bacteria), and NLD microbiotas are associated with adverse reproductive outcomes (57). Since the female genital tract is consecutively unobstructed and relatively short, the endometrial microbiota possibly has a crosstalk with that in the lower tract. Clinical administration of a broad-spectrum antibiotic significantly decreased several kinds of microbiomes (58). The endometrial microbiota might alter after surgery, since antibiotics were applied during the surgery and thus caused altered adaptive immune cell proportions. Based on these clues, it could be inferred that adaptive immune cells might be a potential marker of endometrial microbiotic milieu due to their interaction with the microbiota, and the microbiota could interfere with endometrial receptivity indirectly by affecting the adaptive immune cells.

Upon these findings and speculation, we hypothesized that surgical treatment might modify immune components and the endometrial microbiota, leading to the alteration of innate and adaptive immune cells. Further investigation of immune factors and endometrial microbiota after surgery is needed to prove this.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Genomics Data Center, accession number HRA002724 (https://ngdc.cncb.ac.cn/gsa-human/browse/HRA002724).

Ethics statement

This study was reviewed and approved by The Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-Sen University. All methods were carried out in accordance with the Declaration of Helsinki and the ethical guidelines and regulations of the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-Sen University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RX, JP, and HZ proposed the idea and designed the research. ZZ, HL, JZ, JP, and HZ collected clinical data and tissue samples of the patients. RX, PC, ZZ, and CZ analyzed and interpreted the transcriptomic data. RX drafted the manuscript. JP and HZ did the revision. All authors read and approved the final manuscript.

Funding

This work was supported by the National Key Research and Development Program of China (grant no. 2021YFC2700503).

Acknowledgments

We appreciate Xukang Medical·Yikon Genomics for helping with the sequencing process.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.932339/full#supplementary-material

References

1. Taylor HS, Kotlyar AM, Flores VA. Endometriosis is a chronic systemic disease: clinical challenges and novel innovations. *Lancet* (2021) 397:839–52. doi: 10.1016/S0140-6736(21)00389-5

2. Lessey BA, Kim JJ. Endometrial receptivity in the eutopic endometrium of women with endometriosis: it is affected, and let me show you why. *Fertil Steril* (2017) 108:19–27. doi: 10.1016/j.fertnstert.2017.05.031

3. Tiberi F, Tropea A, Apa R, Romani F, Lanzone A, Marana R. Prokineticin 1 mRNA expression in the endometrium of healthy women and in the eutopic endometrium of women with endometriosis. *Fertil Steril* (2010) 93:2145–9. doi: 10.1016/j.fertnstert.2009.01.105

4. Lessey BA, Castelbaum AJ, Sawin SW, Buck CA, Schinnar R, Bilker W, et al. Aberrant integrin expression in the endometrium of women with endometriosis. *J Clin Endocrinol Metab* (1994) 79:643–9. doi: 10.1210/jcem.79.2.7519194

5. Margarit L, Gonzalez D, Lewis PD, Hopkins L, Davies C, Conlan RS, et al. L-selectin ligands in human endometrium: comparison of fertile and infertile subjects. *Hum Reprod* (2009) 24:2767–77. doi: 10.1093/humrep/dep247

6. Franasiak JM, Holoch KJ, Yuan L, Schammel DP, Young SL, Lessey BA. Prospective assessment of midsecretory endometrial leukemia inhibitor factor expression versus αvβ3 testing in women with unexplained infertility. *Fertil Steril* (2014) 101:1724–31. doi: 10.1016/j.fertnstert.2014.02.027

7. Zhong C, Gao L, Shu L, Hou Z, Cai L, Huang J, et al. Analysis of IVF/ICSI outcomes in endometriosis patients with recurrent implantation failure: Influence on cumulative live birth rate. *Front Endocrinol (Lausanne)* (2021) 12:640288. doi: 10.3389/fendo.2021.640288

8. Bafort C, Beebeejaun Y, Tomassetti C, Bosteels J, Duffy JM. Laparoscopic surgery for endometriosis. *Cochrane Database Syst Rev* (2020) 10:CD011031. doi: 10.1002/14651858.CD011031.pub3

9. Bayer-Garner IB, Nickell JA, Korourian S. Routine syndecan-1 immunohistochemistry aids in the diagnosis of chronic endometritis. *Arch Pathol Lab Med* (2004) 128:1000–3. doi: 10.5858/2004-128-1000-RSIAIT

10. Kitaya K, Takeuchi T, Mizuta S, Matsubayashi H, Ishikawa T. Endometritis: new time, new concepts. *Fertil Steril* (2018) 110:344–50. doi: 10.1016/j.fertnstert.2018.04.012

11. Pontén F, Jirström K, Uhlen M. The human protein atlas–a tool for pathology. *J Pathol* (2008) 216:387–93. doi: 10.1002/path.2440

12. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* (2015) 12:357–60. doi: 10.1038/nmeth.3317

13. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* (2014) 30:923–30. doi: 10.1093/bioinformatics/btt656

14. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* (2014) 15:550. doi: 10.1186/s13059-014-0550-8

15. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. ClueGO: a cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* (2009) 25:1091–3. doi: 10.1093/bioinformatics/btp101

16. Bindea G, Galon J, Mlecnik B. CluePedia cytoscape plugin: pathway insights using integrated experimental and in silico data. *Bioinformatics* (2013) 29:661–3. doi: 10.1093/bioinformatics/btt019

17. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* (2003) 13:2498–504. doi: 10.1101/gr.1239303

18. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods* (2015) 12:453–7. doi: 10.1038/nmeth.3337

19. Vestergaard AL, Knudsen UB, Munk T, Rosbach H, Martensen PM. Transcriptional expression of type-I interferon response genes and stability of housekeeping genes in the human endometrium and endometriosis. *Mol Hum Reprod* (2011) 17:243–54. doi: 10.1093/molehr/gaq100

20. Prašnikar E, Kunej T, Gorenjak M, Potočnik U, Kovačič B, Knez J. Transcriptomics of receptive endometrium in women with sonographic features of adenomyosis. *Reprod Biol Endocrinol* (2022) 20:2. doi: 10.1186/s12958-021-00871-5

21. Li Q, Zhang M, Kumar S, Zhu LJ, Chen D, Bagchi MK, et al. Identification and implantation stage-specific expression of an interferon-alpha-regulated gene in human and rat endometrium. *Endocrinology* (2001) 142:2390–400. doi: 10.1210/endo.142.6.8101

22. Roberts RM. Interferon-tau, a type 1 interferon involved in maternal recognition of pregnancy. *Cytokine Growth Factor Rev* (2007) 18:403–8. doi: 10.1016/j.cytogfr.2007.06.010

23. Zivcec M, Spiropoulou CF, Spengler JR. The use of mice lacking type I or both type I and type II interferon responses in research on hemorrhagic fever viruses. part 2: Vaccine efficacy studies. *Antiviral Res* (2020) 174:104702. doi: 10.1016/j.antiviral.2019.104702

24. Ahn JI, Yoo J-Y, Kim TH, Kim YI, Ferguson SD, Fazleabas AT, et al. cAMP-response element-binding 3-like protein 1 (CREB3L1) is required for decidualization and its expression is decreased in women with endometriosis. *Curr Mol Med* (2016) 16:276–87. doi: 10.2174/1566524016666160225153659

25. Mikołajczyk M, Wirstlein P, Skrzypczak J. Aberrant claudin-4 transcript levels in eutopic endometrium of women with idiopathic infertility and minimal endometriosis. *Ginekol Pol* (2013) 84:90–4. doi: 10.17772/gp/1546

26. Bishop A, Cartwright JE, Whitley GS. Stanniocalcin-1 in the female reproductive system and pregnancy. *Hum Reprod Update* (2021) 27:1098–114. doi: 10.1093/humupd/dmab028

27. Allegra A, Marino A, Coffaro F, Lama A, Rizza G, Scaglione P, et al. Is there a uniform basal endometrial gene expression profile during the implantation window in women who became pregnant in a subsequent ICSI cycle? *Hum Reprod* (2009) 24:2549–57. doi: 10.1093/humrep/dep222

28. Aghajanova L, Altmäe S, Kasvandik S, Salumets A, Stavreus-Evers A, Giudice LC. Stanniocalcin-1 expression in normal human endometrium and dysregulation in endometriosis. *Fertil Steril* (2016) 106:681–91.e1. doi: 10.1016/j.fertnstert.2016.05.023

29. Ashley RL, Arreguin-Arevalo JA, Nett TM. Binding characteristics of the ovine membrane progesterone receptor alpha and expression of the receptor during the estrous cycle. *Reprod Biol Endocrinol* (2009) 7:42. doi: 10.1186/1477-7827-7-42

30. Weems YS, Nett TM, Rispoli LA, Davis TL, Johnson DL, Uchima T, et al. Effects of prostaglandin e and f receptor agonists *in vivo* on luteal function in ewes. *Prostaglandins Other Lipid Mediat* (2010) 92:67–72. doi: 10.1016/j.prostaglandins.2010.03.004

31. Hristovska A-M, Rasmussen LE, Hansen PBL, Nielsen SS, Nüsing RM, Narumiya S, et al. Prostaglandin E2 induces vascular relaxation by e-prostanoid 4 receptor-mediated activation of endothelial nitric oxide synthase. *Hypertension* (2007) 50:525–30. doi: 10.1161/HYPERTENSIONAHA.107.088948

32. Niringiyumukiza JD, Cai H, Xiang W. Prostaglandin E2 involvement in mammalian female fertility: ovulation, fertilization, embryo development and early implantation. *Reprod Biol Endocrinol* (2018) 16:43. doi: 10.1186/s12958-018-0359-5

33. Yang D, Jiang T, Liu J, Hong J, Lin P, Chen H, et al. Interferon-τ regulates prostaglandin release in goat endometrial stromal cells *via* JAB1 - unfolded protein response pathway. *Theriogenology* (2018) 113:237–46. doi: 10.1016/j.theriogenology.2018.03.007

34. Banerjee P, Sapru K, Strakova Z, Fazleabas AT. Chorionic gonadotropin regulates prostaglandin e synthase *via* a phosphatidylinositol 3-kinase-extracellular regulatory kinase pathway in a human endometrial epithelial cell line: implications for endometrial responses for embryo implantation. *Endocrinology* (2009) 150:4326–37. doi: 10.1210/en.2009-0394

35. Jabbour HN, Boddy SC. Prostaglandin E2 induces proliferation of glandular epithelial cells of the human endometrium *via* extracellular regulated kinase 1/2-mediated pathway. *J Clin Endocrinol Metab* (2003) 88:4481–7. doi: 10.1210/jc.2003-030297

36. Sales KJ, Katz AA, Millar RP, Jabbour HN. Seminal plasma activates cyclooxygenase-2 and prostaglandin E2 receptor expression and signalling in cervical adenocarcinoma cells. *Mol Hum Reprod* (2002) 8:1065–70. doi: 10.1093/molehr/8.12.1065

37. Sales KJ, Grant V, Catalano RD, Jabbour HN. Chorionic gonadotrophin regulates CXCR4 expression in human endometrium *via* e-series prostanoid receptor 2 signalling to PI3K-ERK1/2: implications for fetal-maternal crosstalk for embryo implantation. *Mol Hum Reprod* (2011) 17:22–32. doi: 10.1093/molehr/gaq069

38. Dominguez F, Galan A, Martin JJL, Remohi J, Pellicer A, Simón C. Hormonal and embryonic regulation of chemokine receptors CXCR1, CXCR4, CCR5 and CCR2B in the human endometrium and the human blastocyst. *Mol Hum Reprod* (2003) 9:189–98. doi: 10.1093/molehr/gag024

39. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, et al. Defects of b-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* (1996) 382:635–8. doi: 10.1038/382635a0

40. Gentilini D, Busacca M, Di Francesco S, Vignali M, Viganò P, Di Blasio AM. PI3K/Akt and ERK1/2 signalling pathways are involved in endometrial cell migration induced by 17beta-estradiol and growth factors. *Mol Hum Reprod* (2007) 13:317–22. doi: 10.1093/molehr/gam001

41. Huang X, Liu H, Li R. Prostaglandin E2 promotes BeWo spheroids implantation in RL95-2 cell monolayers. *Gynecol Endocrinol* (2017) 33:548–52. doi: 10.1080/09513590.2017.1296125

42. Cilenti F, Barbiera G, Caronni N, Iodice D, Montaldo E, Barresi S, et al. A PGE2-MEF2A axis enables context-dependent control of inflammatory gene expression. *Immunity* (2021) 54:1665–82.e14. doi: 10.1016/j.immuni.2021.05.016

43. Glover LE, Crosby D, Thiruchelvam U, Harmon C, Chorcora CN, Wingfield MB, et al. Uterine natural killer cell progenitor populations predict successful implantation in women with endometriosis-associated infertility. *Am J Reprod Immunol* (2018) 79:e12817. doi: 10.1111/aji.12817

44. Fu B, Zhou Y, Ni X, Tong X, Xu X, Dong Z, et al. Natural killer cells promote fetal development through the secretion of growth-promoting factors. *Immunity* (2017) 47:1100–13.e6. doi: 10.1016/j.immuni.2017.11.018

45. Barber EM, Pollard JW. The uterine NK cell population requires IL-15 but these cells are not required for pregnancy nor the resolution of a listeria monocytogenes infection. *J Immunol* (2003) 171:37–46. doi: 10.4049/jimmunol.171.1.37

46. Wang W, Sung N, Gilman-Sachs A, Kwak-Kim J. T Helper (Th) cell profiles in pregnancy and recurrent pregnancy losses: Th1/Th2/Th9/Th17/Th22/Tfh cells. *Front Immunol* (2020) 11:2025. doi: 10.3389/fimmu.2020.02025

47. Ml S, Aj L, Aa A. Type I interferon regulation of natural killer cell function in primary and secondary infections. *Expert Rev Vaccines* (2013) 12:875-84. doi: 10.1586/14760584.2013.814871

48. Mattei F, Schiavoni G, Belardelli F, Tough DF. IL-15 is expressed by dendritic cells in response to type I IFN, double-stranded RNA, or lipopolysaccharide and promotes dendritic cell activation. *J Immunol* (2001) 167:1179–87. doi: 10.4049/jimmunol.167.3.1179

49. Matikainen S, Sareneva T, Ronni T, Lehtonen A, Koskinen PJ, Julkunen I. Interferon-alpha activates multiple STAT proteins and upregulates proliferation-associated IL-2Ralpha, c-myc, and pim-1 genes in human T cells. *Blood* (1999) 93:1980–91.

50. Huber JP, Farrar JD. Regulation of effector and memory T-cell functions by type I interferon. *Immunology* (2011) 132:466–74. doi: 10.1111/j.1365-2567.2011.03412.x

51. Saito S, Umekage H, Nishikawa K, Morii T, Narita N, Enomoto M, et al. Interleukin 4 (IL-4) blocks the IL-2-induced increased in natural killer activity and DNA synthesis of decidual CD16-CD56bright NK cells by inhibiting expression of the IL-2 receptor alpha, beta, and gamma. *Cell Immunol* (1996) 170:71–7. doi: 10.1006/cimm.1996.0135

52. Tezabwala BU, Johnson PM, Rees RC. Inhibition of pregnancy viability in mice following IL-2 administration. *Immunology* (1989) 67:115–9.

53. Monsanto SP, Edwards AK, Zhou J, Nagarkatti P, Nagarkatti M, Young SL, et al. Surgical removal of endometriotic lesions alters local and systemic proinflammatory cytokines in endometriosis patients. *Fertil Steril* (2016) 105:968–77.e5. doi: 10.1016/j.fertnstert.2015.11.047

54. Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJC, et al. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity* (2013) 38:187–97. doi: 10.1016/j.immuni.2012.09.020

55. Wessels JM, Domínguez MA, Leyland NA, Agarwal SK, Foster WG. Endometrial microbiota is more diverse in people with endometriosis than symptomatic controls. *Sci Rep* (2021) 11:18877. doi: 10.1038/s41598-021-98380-3

56. Ata B, Yildiz S, Turkgeldi E, Brocal VP, Dinleyici EC, Moya A, et al. The endobiota study: Comparison of vaginal, cervical and gut microbiota between women with stage 3/4 endometriosis and healthy controls. *Sci Rep* (2019) 9:2204. doi: 10.1038/s41598-019-39700-6

57. Moreno I, Codoñer FM, Vilella F, Valbuena D, Martinez-Blanch JF, Jimenez-Almazán J, et al. Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am J Obstet Gynecol* (2016) 215:684–703. doi: 10.1016/j.ajog.2016.09.075

58. Khan KN, Fujishita A, Muto H, Masumoto H, Ogawa K, Koshiba A, et al. Levofloxacin or gonadotropin releasing hormone agonist treatment decreases intrauterine microbial colonization in human endometriosis. *Eur J Obstet Gynecol Reprod Biol* (2021) 264:103–16. doi: 10.1016/j.ejogrb.2021.07.014

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 20 March 2023
DOI 10.3389/fendo.2023.1144323

OPEN ACCESS

EDITED BY
Achmad Kemal Harzif,
University of Indonesia, Indonesia

REVIEWED BY
Seido Takae,
St. Marianna University School of Medicine, Japan
Xiufeng Ling,
Nanjing Medical University, China

*CORRESPONDENCE
Fardin Amidi
famidi@sina.tums.ac.ir

SPECIALTY SECTION
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

RECEIVED 14 January 2023
ACCEPTED 08 March 2023
PUBLISHED 20 March 2023

CITATION
Rostami S, Alyasin A, Saedi M, Nekoonam S, Khodarahmian M, Moeini A and Amidi F (2023) Astaxanthin ameliorates inflammation, oxidative stress, and reproductive outcomes in endometriosis patients undergoing assisted reproduction: A randomized, triple-blind placebo-controlled clinical trial.
Front. Endocrinol. 14:1144323.
doi: 10.3389/fendo.2023.1144323

Astaxanthin ameliorates inflammation, oxidative stress, and reproductive outcomes in endometriosis patients undergoing assisted reproduction: A randomized, triple-blind placebo-controlled clinical trial

Sahar Rostami [1], Ashraf Alyasin [2,3], Mojtaba Saedi [1], Saeid Nekoonam [1], Mahshad Khodarahmian [4], Ashraf Moeini [5,6] and Fardin Amidi [1*]

[1]Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran, [2]Department of Infertility, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran, [3]Department of Obstetrics and Gynecology, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran, [4]Department of Infertility, Arash Women's Hospital, Tehran University of Medical Sciences, Tehran, Iran, [5]Department of Gynecology and Obstetrics, Arash Women's Hospital, Tehran University of Medical Sciences, Tehran, Iran, [6]Department of Endocrinology and Female Infertility, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, Academic Center for Education, Culture, and Research (ACECR), Tehran, Iran

Purpose: In a randomized, triple-blind, placebo-controlled clinical trial (RCT) including 50 infertile women with endometriosis candidate for assisted reproductive techniques (ART), we studied the effect of Astaxanthin (AST) on pro-inflammatory cytokines, oxidative stress (OS) markers, and early pregnancy outcomes.

Methods: Before and after 12 weeks of AST treatment (6 mg per day), blood serum and follicular fluid (FF) samples were collected from 50 infertile women with endometriosis stage III/IV undergoing ART. Pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and OS markers (malondialdehyde [MDA], superoxide dismutase [SOD], catalase [CAT], and total antioxidant capacity [TAC]) were measured in the serum and FF. ART outcomes were also compared between the groups.

Results: Increased serum levels of TAC (398.661 ± 57.686 *vs*. 364.746 ± 51.569; P = 0.004) and SOD (13.458 ± 7.276 *vs*. 9.040 ± 5.155; P = 0.010) were observed after AST therapy in the treatment group. Furthermore, serum MDA (14.619 ± 2.505 *vs*. 15.939 ± 1.512; P = 0.031) decreased significantly following antioxidant treatment. In addition, significantly lower serum levels of IL-1β (4.515 ± 0.907 *vs*. 6.8760 ± 0.8478; P = 0.000), IL-6 (5.516 ± 0.646 *vs*. 5.0543 ± 0.709; P = 0.024) and TNF-α (2.520 ± 0.525 *vs*. 2.968 ± 0.548; P = 0.038) were observed after AST

treatment. In addition, AST supplementation led to an improved number of oocytes retrieved (14.60 ± 7.79 *vs*. 9.84 ± 6.44; P = 0.043), number of mature (MII) oocytes (10.48 ± 6.665 *vs*. 6.72 ± 4.3; P = 0.041), and high-quality embryos (4.52 ± 2.41 *vs*. 2.72 ± 2.40; P = 0.024).

Conclusion: AST pretreatment can modulate inflammation and OS in endometriosis-induced infertile patients. ART outcomes also improved after 12 weeks of AST therapy. Our results suggest that AST can be a potential therapeutic target for infertile patients with endometriosis undergoing ART.

KEYWORDS

endometriosis, assisted reproductive techniques (ARTs), female infertility, astaxanthin (AST), antioxidants

Introduction

Endometriosis, the presence of endometrial-like tissue (glands and stroma) outside the uterine cavity, is a chronic estrogen-dependent inflammatory disease affecting approximately 10-15% of reproductive-age women and 35–50% of women with pelvic pain and/or infertility worldwide (1). There is an enigmatic association between endometriosis and infertility. Distorted pelvic anatomy, changes in the peritoneal fluid milieu, endocrine and ovulatory abnormalities, compromised ovarian function and impaired folliculogenesis, diminished ovarian reserve due to surgery, disrupted endometrial receptivity, and inability to have regular intercourse due to dyspareunia might all have detrimental effects on natural conception (2–4).

A complex network of molecular, cellular, and hormonal factors could help unravel the underlying pathogenic mechanism for declined reproductive outcomes of endometriosis patients (5). Endometriosis lesions are present in a unique microenvironment rich in inflammation, steroid hormones (especially estrogen), oxidative stress (OS), and iron originating from erythrocytes and menstrual debris in the peritoneal cavity (6, 7). Reduced activity of endogenous antioxidant enzymes and increased accumulation of reactive oxygen species (ROS) have been detected in endometriosis patients (8, 9). Elevated OS caused by the accumulation of iron in the peritoneal fluid and endometriotic cells contributes to the activation of the nuclear factor–κB (NF-κB) pathway in endometriotic lesions and peritoneal macrophages involved in the inflammatory reaction caused by endometriosis (10). NF-κB pathway activity leads to increased levels of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, in the peritoneal fluid, serum, and follicular fluid (FF) of women with endometriosis (11, 12). These factors contribute to poor oocyte quality, impaired fertilization, and embryo implantation failure (5, 13).

The assisted reproductive technique (ART) is the main treatment option for endometriosis-induced infertility (14). It may not, however, be absolutely and solely successful, as fertility rates are lower in patients with stage I/II endometriosis, and follicle-stimulating hormone (FSH) requirements are higher and fewer oocytes are collected in patients with stage III/IV endometriosis (13, 15). In recent decades, the use of complementary and alternative medicine in treating endometriosis has been welcomed due to its promising efficiency and few side effects (9, 16). Since redox dysregulation plays a vital role in the pathophysiology of endometriosis, a considerable number of studies have indicated the positive effects of antioxidants such as vitamin C, vitamin E, resveratrol, curcumin, melatonin, and epigallocatechin-3-gallate (EGCG) as a supplement along with common endometriosis treatments (17).

Astaxanthin (dihydroxy-4,4′-dione- β,β′-carotene: AST), called the king of antioxidants, is a red-orange, lipid-soluble xanthophyll photo-pigmented ketocarotenoid isolated from the alga *Haematococcus pluvialis* with an antioxidant activity 10 times stronger than that of other natural carotenoids (18). In addition to an outstanding singlet oxygen-quenching activity, this multi-target pharmacological agent has been shown to exert great immunomodulatory, anti-inflammatory, anti-proliferative, anti-apoptotic, anti-diabetic, and neuroprotective properties (19).

AST targets several molecules and pathways, including phosphoinositide 3-kinases (PI3K)/Akt and Janus kinase-2/signal transducer and activator of transcription-3 (JAK2/STAT3) signaling pathways, NF-κB family, mitogen-activated protein kinases (MAPKs), nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1), and peroxisome proliferator-activated receptor gamma (PPARγ) to efficiently modulate the dynamic oxidant: antioxidant equilibrium (19). Various preclinical studies have shown that AST exerts antioxidant effects mainly *via* the Nrf2/HO-1 antioxidant signaling pathway, and increases the expression of its antioxidant target genes, including the phase II biotransformation enzyme (20). Moreover, AST alleviates oxidative damage to DNA by lowering 8-hydroxy-20 deoxyguanosine (8-OHdG), inhibits lipid oxidation by decreasing malondialdehyde (MDA), a decomposition product of peroxidized polyunsaturated fatty acids in membrane lipids, and reduces 8-isoprostane (ISP), an eicosanoid product of the free radical-catalyzed oxidation of primarily arachidonic acid. It can also

increase plasma total antioxidant capability (TAC) and enhance the activity of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and paraoxonase (PON) (21).

Furthermore, AST suppresses the expression of scavenger receptors, controlled by NF−κB, the main mediator of the inflammatory response through inhibiting IκB-α degeneration and the NF-κB nuclear translocation (22). As a result, the expression of downstream pro-inflammatory genes, including IL−1β, IL-6, TNF-α, chemokines, cyclooxygenase−2 (COX−2), inducible nitric oxide synthase (iNOS), and matrix metalloproteinases (MMPs) in macrophages and other cell types are downregulated by AST (20). Furthermore, AST causes a decrease in cytokines *via* inhibition of the MAPK signaling pathway by reducing the induction and expression of extracellular-signal-regulated kinase (ERK1/2), c-Jun N-terminal kinases (JNK), and p38 MAP Kinase (19, 23).

Fertility-enhancing properties of AST have been established in various *in vitro* and *in vivo* studies. In previous experimental studies (24–27), the protective role of AST against intracellular ROS levels has been demonstrated in mice and human granulosa cells and oocytes by up-regulating the phase II enzymes caused by Nrf2/HO-1 activation. In a previous clinical trial (28), the positive effects of AST supplementation were reported on OS and ART outcomes in infertile women with polycystic ovary syndrome (PCOS). However, no previous study has investigated the effect of AST on infertile endometriosis patients. We conducted a randomized placebo-controlled clinical trial (RCT) to study the effect of AST supplementation on OS, inflammation, and reproductive outcomes in endometriosis patients undergoing ART.

Materials and methods

Patient enrollment and study design

This was a prospective, parallel, randomized, triple-blind, placebo-controlled clinical trial (RCT) with a 1:1 ratio. We enrolled 50 infertile endometriosis patients presenting for ART at Omid Fertility Clinic, Tehran, Iran, between December 2021 and September 2022. Endometriosis patients classified as moderate (stage III) or severe (stage IV) according to the American Society of Reproductive Medicine criteria, 1997 (29) who met the inclusion criteria were randomly assigned to AST (n = 25) and placebo groups (n = 25). Eligible trial participants included patients 20 to 40 years old suffering from infertility caused by stage III/IV endometriosis confirmed by video laparoscopy and histopathological tests, with 18.5 < BMI < 30 kg/m^2, regular menstrual cycles, and no history of surgical treatments. Pregnant women, breast-feeders, or women using hormones or IUDs in the last 3 months prior to the first intervention, and those taking antioxidant drug treatments were not included. Patients with concomitant male factor infertility (especially non-obstructive azoospermia) or gynecological diseases leading to infertility, such as PCOS and tubal factor were excluded prior to randomization. Patients with a history of autoimmune disorders, abnormalities in the uterine cavity, such as fibroids and polyps, pelvic inflammatory disease, cancer, diabetes, chronic infectious diseases, or any other immune-affecting exposure were also excluded. Participants who became pregnant or those who started using hormonal drugs during the intervention were also excluded from the trial. Patients' demographic and clinical data were documented, including age, BMI, type of infertility (primary or secondary), and duration of infertility. Their follicular phase hormonal profile (serum levels of anti-Müllerian hormone [AMH], follicle-stimulating hormone [FSH], luteinizing hormone [LH], estradiol [E2], and progesterone [P]), ovarian stimulation parameters and ART outcomes were also recorded and analyzed.

Treatment randomization and blinding

Eligible patients were randomly assigned to the AST or placebo groups (1:1) using the balanced block randomization method within a block size of 4 by an independent statistician. This trial was a triple-blind study. All patients, researchers, statisticians, embryologists, and laboratory personnel were blinded to the individual treatment allocation. The Consolidated Standards of Reporting Trials (CONSORT) diagram (Figure 1) shows the distribution of participants through the trial.

Trial procedures

All infertile endometriosis patients candidate for ICSI protocol who visited Omid Fertility Clinic and met the inclusion criteria were recruited. Before and alongside the routine gonadotropin-releasing hormone (GnRH) antagonist ovarian stimulation protocol, 6 mg daily of oral AST or placebo capsules (AstaReal® astaxanthin; AstaReal Co., Ltd., Tokyo, Japan) were administered to the treatment and placebo groups, respectively for 12 weeks (from Day 1 of two menstrual cycles before starting controlled ovarian stimulation [COS] until oocyte pick-up [OPU]). Placebo capsules (produced by the same company) were identical to AST capsules in color, size, shape, packaging, and taste. The dose and duration of the intervention were determined according to the previous studies, which showed that AST in a daily dose of 2 to 24 mg exhibits antioxidant properties with no safety concerns and side effects after

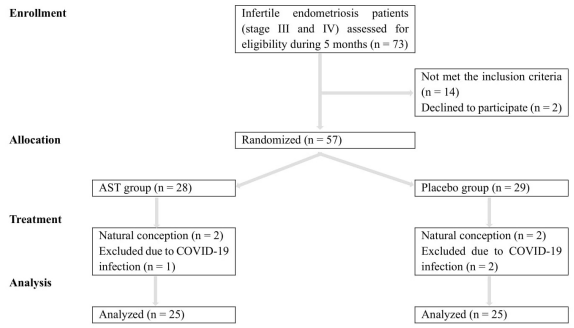


FIGURE 1
Patient enrolment and CONSORT diagram.

at least a 3-week intervention (30–32). Weekly phone calls and monthly visits were used to check on the proper use of medications and their possible side effects. Patients were instructed to maintain their usual lifestyle and to refrain from taking nutritional supplements except for prenatal folic acid (Apovital®, Folsäure 800, Germany; 800 μg/day). All subjects were asked to return any remaining capsules on the day of the OPU to assess the adherence rate.

Blood and FF collection and biochemical measurements

Sample collections were carried out following the guidelines suggested by the World Endometriosis Research Foundation (33). Blood samples (10 ml venous blood) were obtained from all patients before and after the intervention (on the day of OPU) to evaluate OS markers (the serum levels of TAC, MDA, SOD, and CAT activities) and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α). The blood samples were centrifuged immediately (1500 rpm for 10 min, 4°C), and serum was separated and stored in aliquots at − 80°C until further assessment. FF was aspirated only from the first follicle during the OPU to minimize blood contamination. The samples were centrifuged (3000 rpm for 10 min), and the clarified supernatants were collected. The FF aliquots were also promptly stored at − 80°C until further analysis.

OS markers and cytokines were measured in duplicate, in a blinded manner, in pairs (pre/post-intervention) simultaneously, and in the same analytical run to reduce systematic error and inter-assay variations. Measurements of serum and FF levels of OS markers were performed using the human enzyme-linked immunosorbent assay (ELISA) kits (Zellbio, GmbH, Germany), according to the manufacturer's instructions. The kit sensitivity was 0.5 U/mL for CAT, 1 U/mL for SOD, 0.1 μM for MDA, and 0.1 mM for TAC detection. A microplate reader (BioTek, Winooski, USA) was used to measure absorbance at wavelengths specific to each marker. Pro-inflammatory cytokine concentrations were determined in serum and FF by high sensitivity human ELISA system using commercially available human kits (Karmania Pars Gene company; KPG, Iran), following the manufacturer's instruction. The kit sensitivity was 2 pg/mL, and the absorbance was measured at 420 nm *via* a microplate reader (BioTek, Winooski, USA).

COS protocol

The GnRH antagonistic protocol combined with the whole embryo freezing strategy is more efficient for ovulation induction in the Iranian endometriosis population. Thus, patients were submitted to a flexible antagonist protocol. Recombinant follicle-stimulating hormone (rFSH) (150–300 IU/day, Gonal-F®, Merck Serono SA, Switzerland) was administered at the beginning of the cycle and proceeded until the human chorionic gonadotropin (hCG) trigger for pituitary down-regulation. Serial transvaginal sonography (TVS) was conducted to evaluate the ovarian response. The GnRH (gonadotropin-releasing hormone) antagonist, Cetrorelix acetate (Cetrotide 0.25 mg, Merck Serono SA, Switzerland), was administered when 2 or more follicles reached ≥ 14 mm in diameter. Cetrotide was discontinued when at least 2 follicles achieved a diameter of ≥ 18 mm, and 10,000 IU hCG (Ovitrelle, Merck Serono SA, Switzerland) was administered for the ultimate oocyte maturation. The OPU was performed under an ultrasound guide 36 h (± 2 h) following the trigger. The participants were all subjected to the standard intracytoplasmic sperm injection (ICSI) protocol. In line with local clinical practice, to prevent ovarian hyperstimulation syndrome (OHSS) and as a strategy to improve clinical outcomes, all embryos were cryopreserved on Day 3/5, and 2 or 3 embryos were transferred 2 cycles later. Single embryo transfer (SET) was performed if 2 embryos were unavailable.

ART outcomes

Two hours following OPU, hyaluronidase enzyme (Sigma®, USA) was used to denude cumulus-oocyte complexes (COCs). The retrieved oocytes were assessed for quality and maturity under a stereo microscope (Olympus SZX7, Tokyo, Japan) and were classified as germinal vesicle (GV), metaphase I (MI), and metaphase II (MII). ICSI was performed on injectable MII oocytes, and fertilization [the presence of 2 pronuclei (2 PN) and 2 polar bodies (2 PBs)] was assessed 16-18 hours following ICSI.

Reproductive outcomes, including the number of oocytes retrieved, the number of MII oocytes, oocyte maturity rate (the number of normal MII oocytes per the total number of normal oocytes retrieved, expressed as a percentage (34)), fertilization rate (the number of oocytes with 2PN/2PB, 16-18 h post-insemination ×100 per the number of MII oocytes injected (35)), high-quality embryos (grade A and B cleavage embryos based on the ASEBIR criteria (36)), chemical pregnancy rate (the number of pregnancies with a positive serum β-hCG test 14 days after ET per the number of ET cycles, expressed as a percentage (37)), clinical pregnancy rate (the number of pregnancies with a definitive clinical sign in ultrasound (gestational sac, heart rate, etc.) 5-6 weeks after ET per the number of ET cycles, expressed as a percentage (38)), and multiple pregnancy rate (the number of multiple pregnancies ×100 per the number of clinical pregnancies) were also collected.

Sample size and statistical analysis

The serum level of TAC was the primary outcome. According to Choi et al.'s study (39) and a 15% loss-to-follow-up, a sample size of 50 (25 in each study group) would yield 80% power and a significance level of 0.05 to detect a difference in the mean serum TAC. We tested the data for normality using the Kolmogorov–Smirnov test. AST and placebo groups were compared using an independent sample t-test. Student's paired t-test was used to compare pre- and post-intervention markers in each group. Mann–Whitney U test was used to investigate data without normal distribution. Pearson's correlation was used to evaluate the correlations between the parameters. Chemical, clinical, and multiple pregnancy rates were

compared using Fisher's exact and Pearson's chi-squared tests. The correlations between the serum and FF levels of the OS markers and cytokines with the ART outcomes were investigated using Pearson's correlation coefficient. All statistical analyses were performed in SPSS (SAS Institute Inc, version 22, Cary, NC, USA). Data were presented as mean ± standard deviation (SD). A P value of less than 0.05 was considered significant.

Ethical approval

The trial was approved by the Deputy of the Research and Ethics Committee of TUMS (approval date: 2021, December 19; code: IR.TUMS.MEDICINE.REC.1400.1085) and conducted following the Declaration of Helsinki, the International Council for Harmonisation Guidelines for Good Clinical Practice and applicable regulatory requirements. The study protocol was also registered in the Iranian Registry of Clinical Trials (registration code: IRCT20220625055274N1). Here we present a part of the RCT registered in the IRCT. All participants signed written informed consent to use the capsules and provide serum and discarded follicular fluid (FF) samples collected during OPU for research purposes. Before obtaining informed consent, the researchers explained the antioxidant properties, effects, and consumption instructions.

Results

Baseline characteristics

As depicted in the CONSORT flowchart, a total of 57 patients were randomized and exposed, of whom 50 (25 in each group) were enrolled and treated from December 2021 to May 2022 (Figure 1). Demographic and clinical data are presented in Table 1. There were no statistically significant differences in age (33.33 ± 4.97 vs. 32.08 ± 5.09; P = 0.242), BMI (23.17 ± 1.82 vs. 24.31 ± 2.60; P = 0.961), and duration of infertility (3.56 ± 3.19 vs. 2.96 ± 1.54; P = 0.102), AMH (2.72 ± 2.02 vs. 2.40 ± 1.50; P = 0.368), FSH (3.20 ± 2.27 vs. 4.56 ± 2.79; P = 0.371), LH (5.19 ± 3.60 vs. 8.86 ± 10.21; P = 0.777), estradiol (93.22 ± 68.16 vs. 88.21 ± 45.26; P = 0.776), and progesterone (2.84 ± 5.77 vs. 2.22 ± 3.75; P = 0.302) at the onset of the study between the two groups. Most patients in both groups (76% of the AST group (n = 19) and 64% of the placebo group (n = 16)) were diagnosed with primary infertility. All 50 patients completed the study. According to pill count-back on returned bottles, adherence rates were 91% and 94% in the AST and placebo groups, respectively. The patients reported no adverse effects or toxicity during the intervention.

Serum and FF antioxidants and OS profile

Pre- and post-intervention concentrations of OS markers and cytokine levels results are shown in Table 2. There were no statistically significant differences in the serum and FF levels of the markers between the two groups before AST supplementation. Comparing baseline values, no statistically significant difference was seen in the serum or FF levels of the markers in the placebo group after treatment. Increased serum levels of TAC (398.661 ± 57.686 vs. 364.746 ± 51.569; P = 0.004) and SOD (13.458 ± 7.276 vs. 9.040 ± 5.155; P = 0.010) were observed after AST therapy in the treatment group compared to the baseline values. Serum MDA (14.619 ± 2.505 vs. 15.939 ± 1.512; P = 0.031) also decreased significantly following antioxidant treatment. At the same time, the concentration of CAT remained unchanged after AST administration (11.338 ± 4.778 vs. 13.420 ± 9.535; P = 0.304). No significant differences were seen in TAC (P=0.118), MDA (P = 0.662), SOD (P = 0.908), or CAT (P = 0.993) levels in the FF between AST and placebo groups.

Serum and FF cytokine parameters

Among cytokine parameters, significantly lower serum levels of IL-1β (4.515 ± 0.907 vs. 6.8760 ± 0.8478; P = 0.000) and TNF-α (2.520 ± 0.525 vs. 2.968 ± 0.548; P = 0.038) were observed after AST treatment (Table 2). However, no significant effect was observed on serum IL-6 concentrations (5.5165 ± 0.6466 vs. 5.0543 ± 0.7099; P = 0.042). There were no significant differences in the FF IL-1β levels between the AST and the placebo group after the intervention (all P > 0.05). Nevertheless, FF IL-6 (P = 0.024) and TNF-α (P = 0.016) were significantly lower in the AST group than in the placebo group.

TABLE 1 Demographic characteristics, clinical data, and endocrine profile of the participants.

	AST (n = 25)	Placebo (n = 25)	P-value
Age (years)	33.33±4.97	32.08±5.09	0.242
BMI (kg/m^2)	23.17±1.82	24.31±2.60	0.961
Duration of infertility (years)	3.56±3.19	2.96±1.54	0.102
AMH (μg/mL)	2.72±2.02	2.40±1.50	0.368
FSH (mIU/mL)	3.20±2.27	4.56±2.79	0.371
LH (mIU/mL)	5.19±3.60	8.86±10.21	0.777
E2 (pg/mL)	93.22±68.16	88.21±45.26	0.776
P (ng/mL)	2.84±5.77	2.22±3.75	0.302

AST, Astaxanthin group; BMI, Body Mass Index [weight (kg)/height (m2)]; AMH, Anti-müllerian Hormone; FSH, Follicle-stimulating Hormone; LH, Luteinizing Hormone; E2, Estradiol; P, Progesterone. Data are presented as mean ± SD. A P-value less than 0.05 is considered significant.

10.3389/fendo.2023.1144323

TABLE 2 OS markers and cytokine levels in two groups.

	AST, pre-intervention serum levels	AST, post-intervention serum levels	Paired P-value	Placebo, pre-intervention serum levels	Placebo, post-intervention serum levels	Paired P-value	P-value between groups	AST, FF levels	Placebo, FF levels	t-test P-value
MDA	15.939821 ±1.512291	14.619420 ±2.505294	0.031*	19.568685 ±3.464909	21.392248 ±4.035089	0.059	0.000****	0.132324 ±0.030408	0.136272 ±0.28587	0.662
SOD	9.040516 ±5.155055	13.458493 ±7.276019	0.010*	9.391183 ±5.007821	9.077471 ±4.587139	0.817	0.032*	9.820102 ±5.462483	9.660697 ±4.204471	0.908
CAT	13.420228 ±9.535983	11.338269 ±4.778701	0.304	10.830017 ±8.545939	13.272669 ±8.355258	0.279	0.321	0.648546 ±0.082670	0.648880 ±0.171007	0.993
TAC	364.746301 ±51.569541	398.661250 ±57.686710	0.004***	393.730496 ±55.267005	406.175507 ±64.271907	0.341	0.666	314.443782 ±73.355726	287.858695 ±38.991069	0.118
IL-1β	6.8760±0.8478	4.5152±0.9078	0.000****	6.8045±1.6090	6.4580±0.9847	0.326	0.000****	4.2228 ±0.8870	4.2410 ±1.1094	0.324
IL-6	5.5165±0.6466	5.0543±0.7099	0.042*	5.1905±0.7789	5.5208±0.5218	0.063	0.98	4.0231 ±1.3622	4.8346 ±2.1064	0.113
TNF-α	2.9688±0.5487	2.5200±0.5255	0.038*	2.8750±0.6267	3.0102±0.7495	0.367	0.062	2.7352 ±0.0645	3.0887 ±0.7425	0.016*

AST, Astaxanthin group; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; TAC, total antioxidant capacity; IL-1β, interleukin 1β; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α. Data are presented as mean ± SD. A P-value less than 0.05 is considered significant (*P < 0.05, ***P < 0.001, and ****P < 0.0001).

Comparison of ovarian stimulation parameters and ART outcomes

Comparing ovarian stimulation parameters and ART outcomes, the number of oocytes retrieved (14.60 ± 7.79 vs. 9.84 ± 6.44; P = 0.043), the number of MII oocytes (10.48 ± 6.665 vs. 6.72 ± 4.3; P = 0.041), and high-quality embryos (4.52 ± 2.41 vs. 2.72 ± 2.40; P = 0.024) improved significantly after AST therapy. The number of transferred embryos was similar in the two groups (2.24 ± 0.43 vs. 2.04 ± 0.73; P = 0.203). The fertilization rate (P = 0.382) and multiple pregnancy rate (P = 0.741) were also similar (Table 3). No significant difference was detected in chemical (14/25 (56%) vs. 11/25 (44%); two-tailed P = 0.414), clinical (11/25 (44%) vs. 10/25 (40%); two-tailed P = 0.241), or multiple pregnancy rates (4/25 (16%) vs. 3/25 (12%); two-tailed P = 1.000) between the two groups.

Correlation analysis

Finally, as shown in Tables 4 and 5, TAC, MDA, SOD, and CAT concentrations in serum and FF were analyzed for correlations. Correlation analysis showed that serum IL-1β correlated negatively with fertilization rate (r_s = -0.416; P = 0.039). Serum TNF-α also correlated negatively with MII rate (r_s = -0.680; P = 0.000), fertilization rate (r_s = -0.446; P = 0.025), and high-quality embryos (r_s=-0.582; P = 0.002). There were positive correlations

TABLE 3 ART outcomes between AST and placebo groups.

	AST (n = 25)	Placebo (n = 25)	P-value
Number of oocytes	14.60±7.79	9.84±6.44	0.043*
GV	1.36±1.52	1.20±1.29	0.692
MI	1.08±1.28	1.20±1.25	0.664
MII	10.48±6.665	6.72±4.32	0.041*
Oocyte maturity rate (MII %)	75.52±13.54	64.64±20.40	0.055
Fertilized oocytes	8.48±5.76	5.40±3.90	0.059
Fertilization rate (%)	79.64±16.67	73.61±28.07	0.382
Number of frozen embryos	7.52±4.98	4.88±3.45	0.060
High-quality embryos	4.52±2.41	2.72±2.40	0.024*
Frozen embryos	7.52±4.98	4.88±3.45	0.060
Number of transferred embryos	2.24±0.436	2.04±0.735	0.203

AST, Astaxanthin group; Oocyte maturity rate, number of MII oocytes/ number of oocytes retrieved × 100; Fertilization rate, number of fertilized oocytes/ numbers of injected MII oocytes × 100; high-quality embryos, grade A/B cleavage embryos; Fertilization rate, number of injected oocytes with 2 pronuclei (2 PN) and 2 polar bodies (2 PBs) 16-18 h post-insemination/ number of MII oocytes injected ×100. Data are presented as mean ± SD. A P-value less than 0.05 is considered significant (*P < 0.05).

TABLE 4 Correlations between OS markers and ART outcomes.

	Total oocytes		MII rate		Fertilization rate		Number of frozen embryos		High-quality embryos		Chemical pregnancies		Clinical pregnancies	
	r_s	P	r_s	P	r_s	P	r_s	P	r_s	P	r_s	P	r_s	P
Serum MDA	-0.85	0.686	-0.254	0.220	-0.380	0.061	0.021	0.921	-0.357	0.080	-0.253	0.222	-0.217	0.296
Serum SOD	0.056	0.792	0.339	0.098	0.245	0.238	0.041	0.846	0.245	0.238	-0.121	0.564	-0.133	0.527
Serum CAT	0.006	0.978	-0.077	0.716	0.161	0.441	0.029	0.891	0.164	0.435	-0.082	0.697	-0.108	0.608
Serum TAC	0.345	0.091	0.305	0.138	0.317	0.122	0.387	0.056	0.323	0.116	0.165	0.430	0.048	0.819
FF MDA	0.115	0.585	0.278	0.179	0.420	0.036	0.052	0.803	0.449	0.025	-0.224	0.282	-0.386	0.056
FF SOD	0.258	0.213	0.181	0.358	0.239	0.249	0.101	0.630	0.243	0.242	-0.107	0.612	-0.267	0.197
FF CAT	0.291	0.158	0.744	0.000	0.843	0.000	-0.213	0.306	0.679	0.000	-0.173	0.408	-0.145	0.489
FF TAC	0.587	0.002	0.812	0.000	0.979	0.000	-0.105	0.618	0.976	0.000	-0.047	0.823	-.0185	0.375

MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; TAC, total antioxidant capacity; MII rate (oocyte maturity rate), number of MII oocytes/ number of oocytes retrieved × 100; Fertilization rate, number of fertilized oocytes/ numbers of injected MII oocytes × 100; High-quality embryos, grade A/B cleavage embryos; Chemical pregnancies, pregnancies with a positive serum β-hCG; Clinical pregnancies, pregnancies with a definitive clinical sign. r_s, Pearson correlation coefficient.

between FF MDA and fertilization rate (r_s = 0.420; P = =0.036) and high-quality embryos (r_s = 0.449; P = 0.025). There were strong positive correlations between FF CAT and MII rate (r_s = 0.744; P = 0.000), fertilization rate (r_s = 0.843; P = 0.000), and high-quality embryos (r_s = 0.679; P = 0.000). FF TAC and total oocytes retrieved (r_s = 0.587; P = 0.002), MII rate (r_s = 0.812; P = 0.000), fertilization rate (r_s = 0.979; P=0.000), and high-quality embryos (r_s = 0.976; P = 0.000) correlated positively as well. Surprisingly, we found a positive correlation between FF IL-6 and total oocytes retrieved (r_s = 0.414; P = 0.040). Eventually, FF TNF-α and MII rate (r_s = -0.723; P = 0.000), fertilization rate (r_s = -0.818; P = 0.000) and high-quality embryos (r_s = -0.683; P = 0.000) correlated strongly and negatively.

Discussion

Since OS and inflammation are interrelated and involved in the pathophysiology of endometriosis-associated infertility, in a randomized, triple-blind, placebo-controlled clinical trial on 50 infertile endometriosis patients candidate for ART, we investigated the antioxidative, anti-inflammatory, and fertility-enhancing effects of AST supplementation. We indicated that a 12-week supplementation with 6 mg/day of AST could effectively alleviate OS and inflammation and enhance ART outcomes.

This trial showed that women using AST for 12 weeks achieved significantly higher serum levels of TAC and SOD. Serum MDA was also decreased significantly following antioxidant treatment. At the same time, the concentration of CAT remained unchanged after AST administration. However, no significant changes were detected in the CAT levels. This is consistent with published RCTs performed in Korea (39, 40), which aimed to assess the effect of supplementary AST on lipid profiles and OS in overweight and obese subjects and showed a significantly lower MDA and ISP but a remarkably higher TAC after a 12-week AST supplementation. Lower MDA levels after 45 days of AST supplementation were also reported by Baralic et al. (41). They investigated the effect of AST on the OS status of young soccer players. A previous RCT (28) was conducted to investigate the effect of a 40-day AST administration

TABLE 5 Correlations between pro-inflammatory cytokines and ART outcomes.

	Total oocytes		MII rate		Fertilization rate		Number of frozen embryos		High-quality embryos		Chemical pregnancies		Clinical pregnancies	
	r_s	P	r_s	P	r_s	P	r_s	P	r_s	P	r_s	P	r_s	P
Serum IL-1β	-0.147	0.483	-0.093	0.658	-0.416	0.039	0.181	0.387	-0.356	0.081	-0.193	0.355	-0.011	0.960
Serum IL-6	-0.125	0.551	0.000	0.999	0.027	0.900	-0.46	0.826	-0.007	0.975	0.029	0.890	0.069	0.745
Serum TNF-α	-0.0219	0.294	-0.680	0.000	-0.446	0.025	-0.263	0.204	-0.582	0.002	0.260	0.210	0.210	0.314
FF IL-1β	0.171	0.413	0.121	0.564	-0.234	0.261	0.124	0.554	-0.204	0.329	-0.197	0.345	-0.087	0.679
FF IL-6	0.414	0.040	0.241	0.246	0.251	0.226	0.284	0.170	0.339	0.097	0.225	0.280	0.202	0.332
FF TNF-α	-0.308	0.134	-0.723	0.000	-0.818	0.000	0.127	0.545	-0.683	0.000	0.086	0.684	-0.009	0.965

IL-1β, interleukin 1β; IL-6, interleukin IL-6; TNF-α, tumor necrosis factor-α. MII rate (oocyte maturity rate), number of MII oocytes/ number of oocytes retrieved × 100; Fertilization rate, number of fertilized oocytes/ numbers of injected MII oocytes × 100; High-quality embryos, grade A/B cleavage embryos; Chemical pregnancies, pregnancies with a positive serum β-hCG; Clinical pregnancies, pregnancies with a definitive clinical sign. r_s, Pearson correlation coefficient.

on OS markers in PCOS patients and showed a significant increase in the serum TAC and CAT levels in the intervention group. No differences were observed in MDA and SOD serum levels.

A statistically significant decrease was observed in the FF TNF-α and IL-6 levels between the AST and the placebo group. However, dietary AST did not affect OS markers in the FF of endometriosis patients. Although AST supplementation led to a higher TAC level, it was not statistically significant. A previous study on PCOS patients undergoing ART (28) also showed no improvement in the FF levels of OS markers, except CAT, after AST supplementation. This is also in line with Gong et al. (42) who reported no significant changes in the FF TAC, MDA, and SOD levels in the FF of PCOS patients treated with growth hormone. However, the total oxidant status (TOS) and the OS Index (OSI) of the FF reduced. Thus, further studies are warranted to measure TOS, OSI, total antioxidant status (TAS), pro-oxidant-antioxidant balance (PAB), and other biomarkers of OS (8-OHdG, GSH-Px, PON, etc.) after antioxidant therapy. Moreover, in future studies, the presence of AST and its oxidation fragments should be analyzed in the FF.

Significantly lower serum levels of IL-1β and TNF-α, but no considerable changes in serum IL-6, were observed following the intervention. In an RCT by Park et al. (2010) (43) on healthy women, plasma IFN-γ and IL-6, but not TNF, increased after 8 weeks of AST supplementation. An increase in the circulating total T and B cells was observed as a result of enhanced humoral immune responses, including IFN-γ and IL-6 production. Cai et al. (2019) (44) also showed that AST treatment could attenuate inflammatory factors (TNF-α and IL-6) significantly by inhibiting MAPK/NF-κB signaling pathway in a mouse model of lipopolysaccharide (LPS)-induced sepsis and acute lung injury *in vitro* and *in vivo*. According to Wan et al. (45), treating bovine endometrial epithelial cell line with AST could alleviate LPS-induced production of IL-6 and TNF-α, improve the cellular activity of SOD and CAT, and decrease apoptosis.

Endometrial explants potentially expose the developing follicle, the oocyte, sperm, and embryos to high levels of ROS generated by inflammation. The damage to gametes and embryos can be prevented by antioxidant supplementation (15). Although limited by sample size, in this study the number of oocytes, mature oocytes, and high-quality embryos improved significantly after AST therapy. Thus, AST could promisingly protect oocytes and embryos from oxidative damage. Fertilization rate, not significantly different between the two groups, is informative of gamete quality and maturity and reflects operator competence. Moreover, although we excluded male factor patients, fertilization may be influenced by sperm quality. We indicated no significant intergroup difference in the number of fertilized oocytes on Day 1. However, the fertilized oocytes in the AST group contributed to a higher number of good-quality (grade A and B) embryos. Thus, AST may enhance the function of oocytes and improve their capability to produce higher-quality embryos. However, no significant difference was detected in ET outcomes, chemical and clinical pregnancies, between the two groups. These results are compatible with our previous clinical trial on PCOS patients (28). In addition to our small sample size, various potential variables affect pregnancy outcomes, such as male gamete status, embryologist and clinician skills in ET, potential effects of cryopreservation in frozen ET cycles, and endometrial receptivity (38). Further studies with larger sample size comparing outcomes in fresh and frozen cycles may be required.

Correlation analysis showed that serum IL-1β correlated negatively with fertilization rate. Nevertheless, increased IL-1β concentrations have been previously reported to be beneficial as high IL-1β in patients presenting male or unexplained infertility correlated with higher fertilization, implantation, and pregnancy rates (46, 47). However, they concluded that the positive association should be confirmed in patients who present different obstetric disorders leading to infertility, including endometriosis. Serum TNF-α also correlated negatively with MII rate, fertilization rate, and high-quality embryos. Promising results of treating endometriosis-associated infertility with TNF-α blockers supports our findings (48). There were positive correlations between FF MDA and fertilization rate and high-quality embryos. This finding is in line with Liu et al. (49), who showed a significant association between FF MDA and embryo quality indicators in PCOS patients. Prieto et al. (50) also found a positive correlation between levels of FF MDA and the implantation rate. There were strong positive correlations between FF CAT and MII rate, fertilization rate, and high-quality embryos. FF TAC and total oocytes retrieved, MII rate, fertilization rate, and high-quality embryos correlated positively as well. In Jana et al.'s study (51), decreased TAC in FF correlated with poor oocyte and embryo quality and low fertilization rate. Pasqualotto et al. (52) also found a positive correlation between FF TAC and pregnancy outcomes. We found a positive correlation between FF IL-6 and total oocytes retrieved. Bedaiwy et al. (53) also reported a significantly higher FF IL-6 in pregnant cycles. However, no correlation was shown between FF IL-6 and oocyte yield, embryo parameters, and fertilization rate according in a study by Altun et al. (54). Since they observed a negative correlation between FF IL-6 levels with increased chance of clinical pregnancy, they suggested endometrial receptivity as the main target for adverse effects of elevated IL-6 levels. Eventually, FF TNF-α and MII rate, fertilization rate, and high-quality embryos correlated strongly and negatively. This is in line with Lee et al. (55), who found higher FF TNF-α concentrations in poor-quality oocytes.

To the best of our knowledge, this is the first randomized, triple-blind, placebo-controlled clinical trial evaluating the effect of AST supplementation on OS markers, inflammatory cytokines, and ART outcomes in infertile endometriosis patients. Furthermore, a homogenous study population from the whole country undergoing uniform treatment practices was included. It is important to note, however, that our study had some limitations. Our study only includes patients undergoing assisted reproduction, so the results may not be generalizable to the entire endometriosis patient population. A larger sample size is required to analyze pregnancy outcomes. Besides, time constraints prevented us from evaluating late pregnancy outcomes, including live birth rate, the most relevant standard of success in ART. In addition, our analysis was limited to frozen ICSI cycles without assessing pregnancy outcomes in fresh cycles.

Conclusion

In conclusion, AST administration could be a promising supplement to combat the oxidative stress and inflammatory reaction associated with endometriosis. This pharmacological agent may also enhance oocyte and embryo quality in endometriosis patients presenting to ART clinics.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The trial was approved by the Deputy of the Research and Ethics Committee of TUMS (approval date: 2021, December 19; code: IR.TUMS.MEDICINE.REC.1400.1085). The patients/participants provided their written informed consent to participate in this study.

Author contributions

FA and AA designed the experiments and contributed reagents/materials/analysis tools. SR collected the data and performed the experiments. SN performed the statistical analysis, critically appraised the data, and reviewed the manuscript. SR and MS wrote the first draft of the manuscript. MK and AM contributed to manuscript revision and editing. All authors contributed to the article and approved the submitted version.

Acknowledgments

We would like to thank the peer reviewers for their time and effort in reviewing the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Smolarz B, Szyłło K, Romanowicz H. Endometriosis: Epidemiology, classification, pathogenesis, treatment and genetics (review of literature). *Int J Mol Sci* (2021) 22 (19):10554. doi: 10.3390/ijms221910554

2. De Ziegler D, Borghese B, Chapron CJTL. Endometriosis and infertility: Pathophysiology and management. *The Lancet* (2010) 376(9742):730–8. doi: 10.1016/S0140-6736(10)60490-4

3. Gupta S, Goldberg JM, Aziz N, Goldberg E, Krajcir N, Agarwal AJF, et al. Pathogenic mechanisms in endometriosis-associated infertility. *Fertil. Steril.* (2008) 90 (2):247–57. doi: 10.1016/j.fertnstert.2008.02.093

4. Bulletti C, Coccia ME, Battistoni S, Borini A. Endometriosis and infertility. *JJoar Genet* (2010) 27(8):441–7. doi: 10.1007/s10815-010-9436-1

5. Yland J, Carvalho LFP, Beste M, Bailey A, Thomas C, Abrão MS, et al. Endometrioma, the follicular fluid inflammatory network and its association with oocyte and embryo characteristics. *Reprod. Biomed. Online* (2020) 40(3):399–408. doi: 10.1016/j.rbmo.2019.12.005

6. Yamaguchi K, Mandai M, Toyokuni S, Hamanishi J, Higuchi T, Takakura K, et al. Contents of endometriotic cysts, especially the high concentration of free iron, are a possible cause of carcinogenesis in the cysts through the iron-induced persistent oxidative stress. (2008) 14(1):32–40. doi: 10.1158/1078-0432.CCR-07-1614

7. Hayashi S, Nakamura T, Motooka Y, Ito F, Jiang L, Akatsuka S, et al. Novel ovarian endometriosis model causes infertility *via* iron-mediated oxidative stress in mice. (2020) 37:101726. doi: 10.1016/j.redox.2020.101726

8. Clower L, Fleshman T, Geldenhuys WJ, Santanam NJB. Targeting oxidative stress involved in endometriosis and its pain. *Biomolecules* (2022) 12(8):1055. doi: 10.3390/biom12081055

9. Cacciottola L, Donnez J, Dolmans M-M. Can endometriosis-related oxidative stress pave the way for new treatment targets? *JIJoMS* (2021) 22(13):7138. doi: 10.3390/ijms22137138

10. González-Ramos R, Van Langendonckt A, Defrère S, Lousse J-C, Colette S, Devoto L, et al. Involvement of the nuclear factor-κB pathway in the pathogenesis of endometriosis. *Fertil. Steril.* (2010) 94(6):1985–94. doi: 10.1016/j.fertnstert.2010.01.013

11. Opøien HK, Fedorcsak P, Polec A, Stensen MH, Åbyholm T, Tanbo T. Do endometriomas induce an inflammatory reaction in nearby follicles? *JHum Reprod* (2013) 28(7):1837–45. doi: 10.1093/humrep/det087

12. Halis G, Arici A. Endometriosis and inflammation in infertility. *JAotNYAoS* (2004) 1034(1):300–15. doi: 10.1196/annals.1335.032

13. Opøien HK, Fedorcsak P, Omland AK, Åbyholm T, Bjercke S, Ertzeid G, et al. *In vitro* fertilization is a successful treatment in endometriosis-associated infertility. *Fertil. Steril.* (2012) 97(4):912–8. doi: 10.1016/j.fertnstert.2012.01.112

14. Santulli P, Bourdon M, Presse M, Gayet V, Marcellin L, Prunet C, et al. Endometriosis-related infertility: Assisted reproductive technology has no adverse impact on pain or quality-of-life scores. *Fertil. Steril.* (2016) 105(4):978–87.e4. doi: 10.1016/j.fertnstert.2015.12.006

15. Máté G, Bernstein LR, Török AL. Endometriosis is a cause of infertility. does reactive oxygen damage to gametes and embryos play a key role in the pathogenesis of infertility caused by endometriosis? *JFie* (2018) 9:725. doi: 10.3389/fendo.2018.00725

16. Rogers PA, D'Hooghe TM, Fazleabas A, Giudice LC, Montgomery GW, Petraglia F, et al. Defining future directions for endometriosis research: Workshop report from the 2011 world congress of endometriosis in montpellier, France. *Reprod. Sci.* (2013) 20(5):483–99. doi: 10.1177/1933719113477495

17. Baboo KD, Chen Z-Y, Zhang X-MJR, Medicine D. Role of oxidative stress and antioxidant therapies in endometriosis. *Reprod. Dev. Med.* (2019) 3(03):170–6. doi: 10.4103/2096-2924.268154

18. Yousry M, Chemistry F. Antioxidant activities of astaxanthin and related carotenoids. *JJoA* (2000) 48(4):1150–4. doi: 10.1021/jf991106k

19. Kohandel Z, Farkhondeh T, Aschner M, Pourbagher-Shahri AM, Samarghandian SJB. Anti-inflammatory action of astaxanthin and its use in the treatment of various diseases. *Pharmacotherapy* (2022) 145:112179. doi: 10.1016/j.biopha.2021.112179

20. Pereira CPM, Souza ACR, Vasconcelos AR, Prado PS. Antioxidant and anti –inflammatory mechanisms of action of astaxanthin in cardiovascular diseases. *JIJoMM* (2021) 47(1):37–48. doi: 10.3892/ijmm.2020.4783

21. Patil AD, Kasabe PJ, Dandge PBJNP. Pharmaceutical and nutraceutical potential of natural bioactive pigment: astaxanthin. *Nat. Prod. Bioprospect* (2022) 12(1):1–27. doi: 10.1007/s13659-022-00347-y

22. Wu H, Niu H, Shao A, Wu C, Dixon BJ, Zhang J, et al. Astaxanthin as a potential neuroprotective agent for neurological diseases. *Mar. Drugs* (2015) 13(9):5750–66. doi: 10.3390/md13095750

23. Faraone I, Sinisgalli C, Ostuni A, Armentano MF, Carmosino M, Milella L, et al. Astaxanthin anticancer effects are mediated through multiple molecular mechanisms: A systematic review. *Mar. Drugs* (2020) 155:104689. doi: 10.1016/j.phrs.2020.104689

24. Eslami M, Esfandyari S, Aghahosseini M, Rashidi Z, Hosseinishental S, Brenjian S, et al. Astaxanthin protects human granulosa cells against oxidative stress through activation of NRF2/ARE pathway and its downstream phase II enzymes. *Cell J. (Yakhteh)* (2021) 23(3):319. doi: 10.22074/cellj.2021.7222

25. Ebrahimi F, Rostami S, Nekoonam S, Rashidi Z, Sobhani A, Amidi F. The effect of astaxanthin and metformin on oxidative stress in granulosa cells of BALB c mouse model of polycystic ovary syndrome. *Reprod Sci* (2021) 28(10):2807–15. doi: 10.1007/s43032-021-00577-4

26. Abdel-Ghani M, Yanagawa Y, Balboula A, Sakaguchi K, Kanno C, Katagiri S, et al. Astaxanthin improves the developmental competence of *in vitro*-grown oocytes and modifies the steroidogenesis of granulosa cells derived from bovine early antral follicles. *Reprod. Fertil. Dev.* (2019) 31(2):272–81. doi: 10.1071/RD17527

27. Jia B-Y, Xiang D-C, Shao Q-Y, Zhang B, Liu S-N, Hong Q-H, et al. Inhibitory effects of astaxanthin on postovulatory porcine oocyte aging. *vitro* (2020) 10(1):1–11. doi: 10.1038/s41598-020-77359-6

28. Gharaei R, Alyasin A, Mahdavinezhad F, Samadian E, Ashrafnezhad Z, FJJoAR A, et al. Randomized controlled trial of astaxanthin impacts on antioxidant status and assisted reproductive technology outcomes in women with polycystic ovarian syndrome. *J. Assist. Reprod. Genet.* (2022) 39(4):995–1008. doi: 10.1007/s10815-022-02432-0

29. Canis M, Donnez JG, Guzick DS, Halme JK, Rock JA, Schenken RS, et al. Revised american society for reproductive medicine classification of endometriosis: 1996. *Fertil. Steril.* (1997) 67(5):817–21. doi: 10.1016/S0015-0282(97)81391-X

30. Brendler T, Williamson EM. Astaxanthin: How much is too much? a safety review. *Phytotherapy Res* (2019) 33(12):3090–111. doi: 10.1002/ptr.6514

31. Williamson EM, Liu X, Izzo AA. Trends in use, pharmacology, and clinical applications of emerging herbal nutraceuticals. *JBJoP.* (2020) 177(6):1227–40. doi: 10.1111/bph.14943

32. Spiller GA, Dewell A. Safety of an astaxanthin-rich haematococcus pluvialis algal extract: A randomized clinical trial. *JJomf* (2003) 6(1):51–6. doi: 10.1089/109662003765184741

33. Rahmioglu N, Fassbender A, Vitonis AF, Tworoger SS, Hummelshoj L, D'Hooghe TM, et al. World endometriosis research foundation endometriosis phenome and biobanking harmonization project: III. fluid biospecimen collection, processing, and storage in endometriosis research. *Fertil. Steril.* (2014) 102(5):1233–43. doi: 10.1016/j.fertnstert.2014.07.1208

34. Ozgur K, Bulut H, Berkkanoglu M, Coetzee K, Ay S. Oocyte maturation-index as measure of oocyte cohort quality; a retrospective analysis of 3135 ICSI cycles. *JMEFSJ* (2015) 20(1):37–42. doi: 10.1016/j.mefs.2014.04.005

35. ESIG of Embryology, ESHRE Special Interest Group. The Vienna consensus: report of an expert meeting on the development of ART laboratory performance indicators. *Reprod. Biomed. Online* (2017) 35(5):494–510. doi: 10.1016/j.rbmo.2017.06.015

36. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Hum. Reprod.* (2011) 26(6):1270–83.

37. Bassiouny YA, Dakhly DMR, Bayoumi YA, Hashish NMJF. Does the addition of growth hormone to the *in vitro* fertilization/intracytoplasmic sperm injection antagonist protocol improve outcomes in poor responders? A randomized, controlled trial. *Fertil. Steril.* (2016) 105(3):697–702. doi: 10.1016/j.fertnstert.2015.11.026

38. ESHRE Clinic PI Working Group, Vlaisavljevic V, Apter S, Capalbo A, D'Angelo A, Gianaroli L, et al. The maribor consensus: Report of an expert meeting on the development of performance indicators for clinical practice in ART. *Hum. Reprod. Open* (2021) 2021(3):hoab022.

39. Choi HD, Youn YK, Shin WG. Positive effects of astaxanthin on lipid profiles and oxidative stress in overweight subjects. *Plant foods Hum Nutr* (2011) 66(4):363–9. doi: 10.1007/s11130-011-0258-9

40. Choi HD, Kim JH, Chang MJ, Kyu-Youn Y, Shin WG. Effects of astaxanthin on oxidative stress in overweight and obese adults. *Phytotherapy Res* (2011) 25(12):1813–8. doi: 10.1002/ptr.3494

41. Baralic I, Djordjevic B, Dikic N, Kotur-Stevuljevic J, Spasic S, Jelic-Ivanovic Z, et al. Effect of astaxanthin supplementation on paraoxonase 1 activities and oxidative stress status in young soccer players. *Phytotherapy Res* (2013) 27(10):1536–42. doi: 10.1002/ptr.4898

42. Gong Y, Luo S, Fan P, Jin S, Zhu H, Deng T, et al. Growth hormone alleviates oxidative stress and improves oocyte quality in Chinese women with polycystic ovary syndrome: a randomized controlled trial. *Sci. Rep.* (2020) 10(1):18769. doi: 10.1038/s41598-020-75107-4

43. Park JS, Chyun JH, Kim YK, Line LL, Chew BPJN. Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans. *metabolism* (2010) 7(1):1–10. doi: 10.1186/1743-7075-7-18

44. Cai X, Chen Y, Xie X, Yao D, Ding C, Chen M. Astaxanthin prevents against lipopolysaccharide-induced acute lung injury and sepsis *via* inhibiting activation of MAPK/NF-κB. *Am J Trans Res* (2019) 11(3):1884–94.

45. Wan F-C, Zhang C, Jin Q, Wei C, Zhao H-B, Zhang X-L, et al. Protective effects of astaxanthin on lipopolysaccharide-induced inflammation in bovine endometrial epithelial cells†. *Biol Reproduction* (2019) 102(2):339–47. doi: 10.1093/biolre/ioz187

46. Zollner K-P, Hofmann T, Zollner U. Good fertilization results associated with high IL-1beta concentrations in follicular fluid of IVF patients. *JTJorm.* (2013) 58(11-12):485–90.

47. Asimakopoulos B, Demirel C, Felberbaum R, Waczek S, Nikolettos N, Koester F, et al. Concentrations of inflammatory cytokines and the outcome in ICSI cycles. *In Vivo* (2010) 24(4):495–500.

48. Alijotas-Reig J, Esteve-Valverde E, Ferrer-Oliveras R, Llurba E, JMJCria G. Tumor necrosis factor-alpha and pregnancy: Focus on biologics. an updated and comprehensive review. *immunology* (2017) 53(1):40–53. doi: 10.1007/s12016-016-8596-x

49. Liu Y, Yu Z, Zhao S, Cheng L, Man Y, Gao X, et al. Oxidative stress markers in the follicular fluid of patients with polycystic ovary syndrome correlate with a decrease in embryo quality. *J. Assist. Reprod. Genet.* (2021) 38(2):471–7. doi: 10.1007/s10815-020-02014-y

50. Prieto L, Quesada JF, Cambero O, Pacheco A, Pellicer A, Codoceo R, et al. Analysis of follicular fluid and serum markers of oxidative stress in women with infertility related to endometriosis. *Fertil. Steril.* (2012) 98(1):126–30. doi: 10.1016/j.fertnstert.2012.03.052

51. Jana SK, Babu N, Chattopadhyay R, Chakravarty B, Chaudhury KJRT. Upper control limit of reactive oxygen species in follicular fluid beyond which viable embryo formation is not favorable. *Reprod. Toxicol* (2010) 29(4):447–51. doi: 10.1016/j.reprotox.2010.04.002

52. Pasqualotto EB, Agarwal A, Sharma RK, Izzo VM, Pinotti JA, Joshi NJ, et al. Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. *Fertil. Steril.* (2004) 81(4):973–6. doi: 10.1016/j.fertnstert.2003.11.021

53. Bedaiwy M, Shahin AY, AbulHassan AM, Goldberg JM, Sharma RK, Agarwal A, et al. Differential expression of follicular fluid cytokines: Relationship to subsequent pregnancy in IVF cycles. *Reprod biomed online* (2007) 15(3):321–5. doi: 10.1016/S1472-6483(10)60346-X

54. Altun T, Jindal S, Greenseid K, Shu J, L P. Low follicular fluid IL-6 levels in IVF patients are associated with increased likelihood of clinical pregnancy. *JJoar Genetics* (2011) 28(3):245–51. doi: 10.1007/s10815-010-9502-8

55. Lee KS, Joo BS, Na YJ, Yoon MS, Choi OH, WW K, et al. Relationships between concentrations of tumor necrosis factor-α and nitric oxide in follicular fluid and oocyte quality. *JJoar* (2000) 17(4):222–8. doi: 10.1023/A:1009495913119

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 20 June 2023
DOI 10.3389/fendo.2023.1171778

OPEN ACCESS

EDITED BY
Iveta Yotova,
Medical University of Vienna, Austria

REVIEWED BY
Quanah Hudson,
Medical University of Vienna, Austria
Katharina Proestling,
Medical University of Vienna, Austria
Irene Velasco,
University of Alicante, Spain

*CORRESPONDENCE
Lei Jin
leijintongjih@qq.com
Cong Sui
csui0904@163.com

†These authors share senior authorship

RECEIVED 22 February 2023
ACCEPTED 23 May 2023
PUBLISHED 20 June 2023

CITATION
Li Y, Cai L, Guo N, Liu C, Wang M, Zhu L, Li F, Jin L and Sui C (2023) Oviductal extracellular vesicles from women with endometriosis impair embryo development. *Front. Endocrinol.* 14:1171778. doi: 10.3389/fendo.2023.1171778

Oviductal extracellular vesicles from women with endometriosis impair embryo development

Yuehan Li[1], Lei Cai[1], Na Guo[1], Chang Liu[2], Meng Wang[1], Lixia Zhu[1], Fei Li[1], Lei Jin[1*†] and Cong Sui[1*†]

[1]Reproductive Medicine Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2]Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, China

Objective: To investigate the influence of oviductal extracellular vesicles from patients with endometriosis on early embryo development.

Design: *In vitro* experimental study

Setting: University-affiliated hospital.

Patients: Women with and without endometriosis who underwent hysterectomy (n = 27 in total).

Interventions: None.

Main outcome measures: Oviductal extracellular vesicles from patients with endometriosis (oEV-EMT) or without endometriosis (oEV-ctrl) were isolated and co-cultured with two-cell murine embryos for 75 hours. Blastocyst rates were recorded. RNA sequencing was used to identify the differentially expressed genes in blastocysts cultured either with oEV-EMT or with oEV-ctrl. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed to identify potential biological processes in embryos that oEV-EMT affects. The functions of oEV on early embryo development were determined by reactive oxygen species (ROS) levels, mitochondrial membrane potentials (MMP), total cell numbers, and apoptotic cell proportions.

Results: Extracellular vesicles were successfully isolated from human Fallopian tubal fluid, and their characterizations were described. The blastocyst rates were significantly decreased in the oEV-EMT group. RNA sequencing revealed that oxidative phosphorylation was down-regulated in blastocysts cultured with oEV-EMT. Analysis of oxidative stress and apoptosis at the blastocysts stage showed

that embryos cultured with oEV-EMT had increased ROS levels, decreased MMP, and increased apoptotic index. Total cell numbers were not influenced.

Conclusion: Oviductal extracellular vesicles from patients with endometriosis negatively influence early embryo development by down-regulating oxidative phosphorylation.

KEYWORDS

endometriosis, extracellular vesicles (EVs), fallopian tube, embryo development, oxidative phosphorylation

1 Introduction

Endometriosis is a complex clinical syndrome with a prevalence of 5% to 15% in women of reproductive age, characterized by the presence of endometrial tissue outside the uterus (1). The symptoms include chronic pelvic pain, dyspareunia, and dysmenorrhea. Infertility occurs most frequently among all clinical manifestations of endometriosis, and approximately 50% of patients present with decreased fertility (2).

The reason why endometriosis impairs women's fertility hasn't been fully characterized. Several mechanisms have been raised to explain the phenomenon, including an inflammatory pelvic environment, ovulatory abnormalities, and altered functions of the endometrium (3). Inflammatory microenvironment was found to negatively influence gamete and embryo in endometriosis, as it may impair sperm function, decrease oocyte fertilization rate and harm early embryo development (4–6). Toxic factors are identified in peritoneal fluid and may enter the lumen of Fallopian tubes (7). In women who undergo assisted reproductive technology (ART), there is no difference in pregnancy outcomes in patients with endometriosis compared with patients undergoing ART for male factor infertility and non-infertile patients after transferring euploid embryos (8), and endometriosis does not affect blastocyst rates (9) or the live birth rates (10). These results indicate that ART might be the hope for patients with endometriosis-associated infertility who wish to conceive. However, how endometriosis affects fertility in natural conception is still poorly understood.

After fertilization, embryos undergo a series of cleavages within the maternal oviduct (or Fallopian tube in humans). Ciliated cells and secretory cells from the epithelial line in the oviduct, together with the oviduct fluid secreted by these cells, constitute the environment for early embryo development (11–14).

Extracellular vesicles (EVs) have been recognized to mediate intercellular interactions by transferring cargo, including RNAs and proteins (15), and they are transferrable even between species (16). EVs have been isolated from various biofluids, including follicular, oviduct, and uterine fluid (17). EVs from the reproductive system have been reported to influence embryo viability and development capacity *in vitro* (18). For instance, researchers showed that EVs secreted by endometrial cells of patients suffering from recurrent implantation failure is detrimental to the embryos (19). Also, it has been reported that oviductal EVs play an essential role in oviduct-embryo interactions (18, 20, 21).

In the current study, we hypothesize that the EVs within the Fallopian tube in patients with endometriosis (oEV-EMT) might contribute to the impaired development of embryos. Subsequently, we tried to discover the influence EVs may exert on embryo development respecting blastocyst rate and the transcriptome of the embryos.

2 Methods

2.1 Ethics approval, participants, and sample acquisition

The current study was approved by the Ethics Committee of Tongji Hospital (No. TJ-IRB20210838). The EMT patients were indicated for the surgery because of severe pelvic pain or adenomyosis by ultrasound, and were all diagnosed with peritoneal endometriosis by laparoscopic inspection. Pelvic superficial endometriosis and deep endometriosis were both included. The control patients had at least given one full-term live birth and had no history of endometriosis, and they underwent laparoscopic hysterectomy because of cervical intraepithelial neoplasia (CIN), and no pelvic pathophysiologic conditions were reported before or after the surgery inspection. The exclusion criteria for all groups were as follows: received hormone treatment three months before the surgery; pregnancy; any indication for malignant diseases (including previous history or other tests showing signs). Cycle phases were recorded according to the patient's reports. Between August 2021 and February 2022, 27 women aged between 34 to 43 were recruited for the study. The samples were collected in the operation room, immediately after the Fallopian tube and the uterus were resected. We used a 50 mL syringe that contained sterile PBS (Servicebio, Wuhan, China) to flush the lumen of the Fallopian tubes. Then we collected the flushing fluid (Fallopian tube fluid) into sterile centrifuge tubes for further analysis as reported (22). The Fallopian tube tissue was collected at the same time, and was conserved in cold PBS. The Fallopian tube fluid and tissue samples were transferred to the laboratory immediately and stored for later processing.

2.2 Isolation of oviductal extracellular vesicles

Extracellular vesicles (EVs) were isolated by the ultracentrifugation method, as described before (21). In brief, the Fallopian tubal fluid was centrifuged at 1,500 × g for 15 minutes twice, and the supernatant was centrifuged at 16,000 × g for 30 minutes at 4 °C. Then the sediment was discarded, and the supernatant was filtered through a 0.22-μm filter. Afterward, the fluid was ultracentrifuged at 120,000 × g for 90 minutes at 4 °C. The pellet was washed with clean PBS and then ultracentrifuged at 120,000 × g for 90 minutes at 4 °C. The pellets were resuspended in 30 μL PBS and stored at -80 °C.

2.3 Western blotting

EVs or tissue samples were lysed with radioimmunoprecipitation assay (RIPA) and proteinase inhibitor cocktail (Servicebio, Wuhan, China) at 4°C for 30 minutes. Then the lysates were centrifuged at 12,000 g for 20 minutes at 4°C. The protein concentration of EVs and Fallopian tube tissue samples were measured using a BCA Protein Assay Kit (Servicebio, Wuhan, China). 5μg of each sample was loaded for electrophoresis, using SDS/PAGE (10% gel), followed by transferring to PVDF membrane (Merck Millipore, Burlington, MA, USA). Then, the membrane was blocked using 5% skimmed milk for 1 h at room temperature, followed by washing with TBS for 15 min. Afterward, the membrane was incubated with the primary antibodies at 4 °C overnight. The primary antibodies used for immunostaining were TSG101 (1:1000; Abclonal, Woburn, MA, USA) and CD9 (1:1000; Abcam, Cambridge, UK), and the secondary antibodies were goat anti-rabbit labeled by horseradish peroxidase (1:2000; Servicebio, Wuhan, China). The membrane was incubated with the secondary antibodies for 1h at 37 °C and then was immersed in an electrochemiluminescence (ECL; Absin, Shanghai, China). The signals were detected using a Gene Gnome XRQ chemiluminescence imaging system (Syngene, Bengaluru, India).

2.4 Transmission electron microscopy

Each EV sample was deposited on a precoated carbon electron microscopy grid for TEM. Then the grids were labeled with 2% uranyl acetate (in double-distilled water). Grids were examined and pictured using a transmission electron microscope (Carl Zeiss, Oberkochen, Germany).

2.5 Nanoparticle tracking analysis

Samples were analyzed using an NTA instrument, zetaview (Particle Metrix, Inning am Ammersee, Germany). EV samples were diluted with PBS at a ratio of 1:1000 to reach the concentration recommended for the measurement (3×10^8 to 1×10^9 particles/mL). For each sample, three videos of 60 seconds were recorded and analyzed, and the particle concentration and sizes were recorded.

2.6 Animals and treatment

All experiments followed the Tongji Hospital Guide for the Care and Use of Laboratory Animals (approval number: TJ-202111004). Sixty female (6-8 weeks of age) and ten male (8-10 weeks of age) mice were used in the study. Female mice were intraperitoneally injected with 10 IU of pregnant mare serum gonadotropin (PMSG; Solarbio, Beijing, China), followed by 10 IU of human chorionic gonadotropin (hCG; Livzon, Zhuhai, China) 48 hours later. After the injection of hCG, two female mice were mated with one male mouse for one night. Thirty-six hours post the hCG injection, the zygotes were isolated from oviducts.

Chatot-Ziomek-Bavister (CZB; AIBI bio, Nanjing, China) medium was used as basic embryo culture medium in the current study. oEV-EMT or oEV-ctrl were added to CZB medium to obtain the culture media for the treated groups. As oEVs were suspended in PBS after the ultracentrifugation, a small volume of this primed PBS was mixed with CZB medium to obtain the final concentration of 1×10^{10} particles/mL. The culture media of the two groups(CZB with oEV-EMT, CZB with oEV-ctrl) were divided into 20-μL droplets covered with mineral oil, and each droplet contained ten zygotes, and the embryos were subjected to further study. The embryo culture media were prepared and equilibrated for 2 hours before the embryos were loaded. And the embryos were cultured at 37°C under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 75 hours. For all following experiments, at least 30 embryos per group were used in each experiment, and the experiments were repeated at least three times.

2.7 Endocytosis of extracellular vesicles by embryos

To investigate whether human Fallopian tube-derived oEVs could be taken up by murine embryos, we used 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO; Beyotime, Shanghai, China) to dye the membrane of oEVs (19). In brief, EMT-oEVs, ctrl-oEVs, or PBS (negative control) were incubated with DiO (10 μM) for 30 minutes. Then the dyed oEVs or negative control were resuspended in PBS and ultracentrifuged at 120,000 g for 80 minutes. Labeled oEVs or negative control were co-cultured with 2-cell embryos for 4 hours. oEVs uptake was observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.8 RNA extraction, library construction, and RNA sequencing procedures

Total RNA was isolated from three replicates of 10 blastocysts using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and concentration of the RNA samples were determined by 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). Transcriptome library preparation was done following the TruSeq™ RNA sample preparation Kit from Illumina (San Diego, CA, USA). In brief, messenger RNA was isolated using the polyA selection method by

oligo (dT) beads, followed by fragmentation in the buffer. Afterward, double-stranded cDNA was synthesized by a SuperScript double-stranded cDNA synthesis kit (Invitrogen, Waltham, MA, USA) with random hexamer primers (Illumina, San Diego, CA, USA). Then the synthesized cDNA was subjected to end-repair, phosphorylation, and 'A' base addition according to Illumina's library construction protocol. Libraries were selected for cDNA target fragments of 300 bp on 2% Low Range Ultra Agarose followed by PCR amplified using Phusion DNA polymerase (NEB, Ipswich, MA, USA) for 15 PCR cycles. After quantified by TBS380, the paired-end RNA-seq sequencing library was sequenced with the Illumina HiSeq X ten/NovaSeq 6000 sequencer.

The raw reads were aligned to genome sequences, and the mapped reads of each sample were assembled by StringTie (https://ccb.jhu.edu/software/stringtie/index.shtml?t=example) in a reference-based approach (23). To identify the differentially expressed genes (DEGs) between the groups of samples, the expression level of each transcript was calculated according to the transcripts per million reads (TPM) method. Moreover, differential expression analysis was performed using the DESeq2, and genes were considered significantly differentially expressed if the Benjamini-Hochberg adjusted *p*-value was ≤ 0.05 and the absolute value of the $\log_2$ foldchange was ≥ 1. In addition, functional-enrichment analysis, including GO and KEGG, was performed to identify which DEGs were significantly enriched at Bonferroni-corrected *p* value ≤ 0.05 compared with the whole-transcriptome background. GO functional enrichment and KEGG pathway analysis were carried out by Goatools (https://github.com/tanghaibao/Goatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do) (24).

2.9 Real-time-PCR

Blastocysts were collected and immediately reverse-transcribed using Single-Cell Sequence-Specific Amplification Kit (Vazyme, Nanjing, China) according to the manufacturer's protocol. Subsequent real-time RT-PCR was performed on a Roche Light Cycler 480 Instrument II PCR System with TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, Kusatsu, Japan). The expression level of each gene was normalized by *Gapdh* and was calculated using the $2^{-\triangle\triangle Cq}$ method. Five blastocysts per group were used for each reaction, and each experiment was repeated at least three times. The primer sequence is provided in Supplementary Table 1.

2.10 ROS measurement

To measure ROS levels by fluorescence, the stock solution of 2', 7' - dichlorodihydro-fluorescein diacetate (DCHF-DA; Sigma, St. Louis, MO, USA) was used to dye the embryos. DCHF-DA was diluted to 10 μM with CZB medium, and embryos in each group (n = 10 - 15) were dyed at 37°C for 20 minutes. The embryos were washed three times in CZB and immediately observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). All images were analyzed using Image J software.

2.11 Measurement of MMP

To investigate the mitochondrial membrane potential (MMP), living blastocysts were incubated with CZB supplemented with 2 μg/L JC-1 Dye (Service bio, Wuhan, China) for 30 min at 37 °C according to the manufacturer's instructions. After washing in CZB twice, embryos were observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Each blastocyst was observed through the TRITC channel (red fluorescence) and the FITC channel (green fluorescence). All images were analyzed using Image J software, and the ratio of aggregated JC-1 (red fluorescence) to monomeric JC-1 (green fluorescence) was calculated to reflect changes in MMP.

2.12 TUNEL assay

After co-cultured with oEVs-EMT, oEVs-ctrl, or PBS for 75h, the blastocysts were fixed with 4% paraformaldehyde (Servicebio, Wuhan, China). To investigate the proportion of the apoptotic cells in blastocysts, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using the TMR (red) Cell Apoptosis Detection Kit (Servicebio, Wuhan, China) according to the manufacturer's instructions. Then the embryos were dyed with diamidino-2-phenylindole (DAPI; Servicebio, Wuhan, China). Dyed blastocysts were mounted on a glass slide and observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The cell numbers were counted, and the percentage of apoptosis cells was calculated as the TUNEL-positive cell number divided by the total cell number of the blastocyst.

2.13 Statistical analysis

Experiments were performed at least three times. Statistical tests were performed using Graph Pad Prism 8.0 (San Diego, CA, USA). Quantitative variables were shown as mean ± SEM. Blastocyst rates were analyzed using the chi-square test. To determine statistical differences in maternal age and body mass index (BMI), student t-tests were performed. For relative gene expression, relative expression level of ROS, MMP, total cell number, and apoptotic cell proportion, one-way analysis of variance (ANOVA) was performed. $p < 0.05$ was considered to be significantly different.

3 Results

3.1 Clinical characteristics and the characterization of oEVs from endometriosis patients and controls

Demographics of the patients are characterized in Supplementary Table 2. The morphology and distribution of oEVs were detected using NTA and TEM. NTA profiles showed peaks at around 144.4

nm in the control group and 148.4 nm in the EMT group (Figure 1A). There's no difference in the diameters between the groups (Figure 1B). Western blotting showed the presence of classic EV protein markers in samples from both groups, including CD9 and TGS 101. Fallopian tube epithelial cells from endometriosis patients and controls were used as control samples, and CD9 and TSG101 were almost undetectable in the control samples (Figure 1C). TEM images showed the typical bilayer structure of the EVs from both the groups at the expected size (Figure 1D).

3.2 oEV-EMT impairs the development of embryos

oEVs were confirmed to be taken up by murine embryos, as green fluorescence signals could only be seen in oEV-EMT- or oEV-ctrl-co-cultured embryos, not in the blank control group (Figure 1E). Blastocyst rate is lower in the oEV-EMT group comparing to the oEV-ctrl group (65.59% vs. 85.71%) (Figure 1F). Notably, oEV-ctrl significantly improves the blastocyst rate from 73.54% to 85.72%, compared to the blank control group. This result is consistent with our previous study, which showed that oEV-ctrl is beneficial for embryo quality *in vitro*.

3.3 oEV-EMT supplementation during *in vitro* culture altered embryonic transcriptome

The principal component analysis showed blastocysts treated with oEV-EMT or oEV-ctrl display distinct linear distribution constructed by the first component (Figure 2A). Using DESeq2; we have found expression profiles of blastocysts from the two groups which are significantly different from one another. A total of 197 differentially expressed genes (DEGs) were identified; 111 were upregulated, and 85 were downregulated (Figure 2B, Supplementary Table 3). The heatmap showed the top 29 most upregulated and the top 21 most down-regulated genes (Figure 2C). To investigate the biological processes that contribute most to the differentially expressed gene profiles between the blastocysts treated with oEV-ctrl or oEV-EMT, we performed KEGG and GO enrichment on the 197 differentially expressed genes. KEGG analysis showed the most enriched pathway was oxidative phosphorylation (Figure 3A). Also, pathways of human neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease, and Huntington's disease, were highlighted. Enriched GO terms include metabolic process, developmental process, cell proliferation, transporter activity, cargo receptor

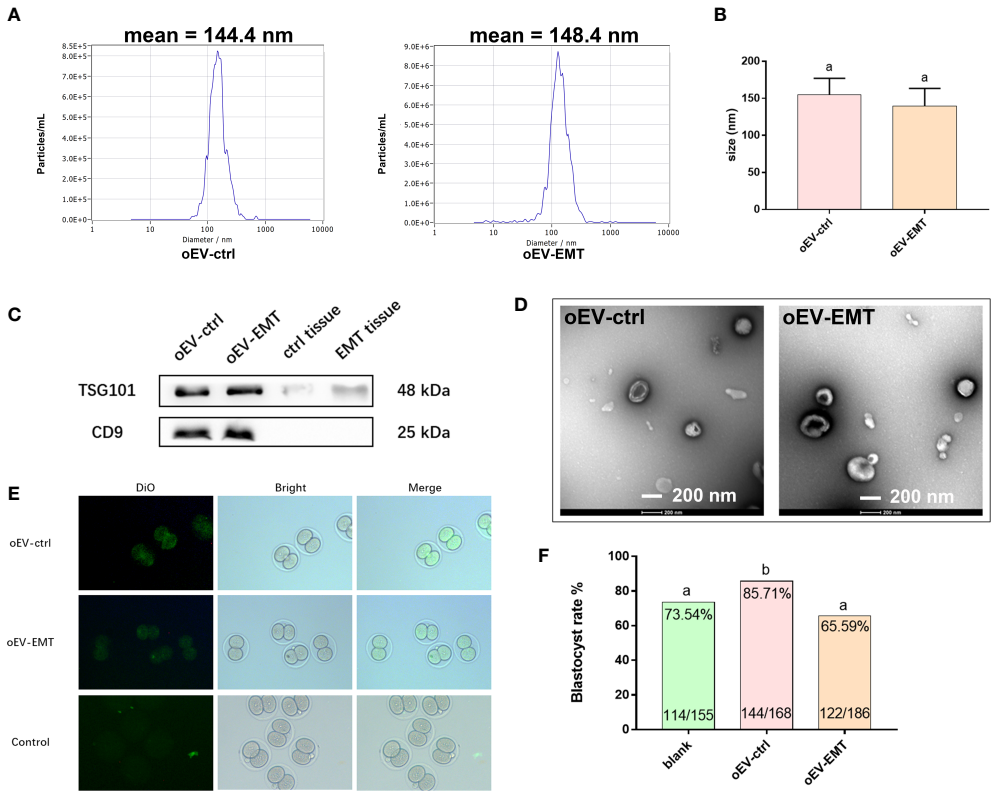


FIGURE 1
Characterization of oEV-EMT and oEV-ctrl. **(A)** The sizes of oEV-EMT and oEV-ctrl were evaluated using NTA. **(B)** The diameters of oEV-EMT and oEV-ctrl samples are similar. **(C)** The expression of EV markers was detected by Western blotting, and Fallopian tube tissue was used as a control. **(D)** Representative TEM images of oEV-EMT and oEV-ctrl are shown. The typical bilayer of the EV structure can be seen. **(E)** Pictures of DiO-labelled oEV-EMT, oEV-ctrl, or blank control being taken in by two-cell murine embryos. **(F)** Blastocyst rate in each group and the number of embryos were given. oEV, oviductal extracellular vesicles; EMT, endometriosis; ctrl, control; NTA, nanoparticle tracking analysis; TEM, transmission electronic microscopy, DiO, 3,3'-dioctadecyloxacarbocyanine perchlorates. Different superscripts per column **(A, B)** represent statistical differences ($p < 0.05$) between groups.

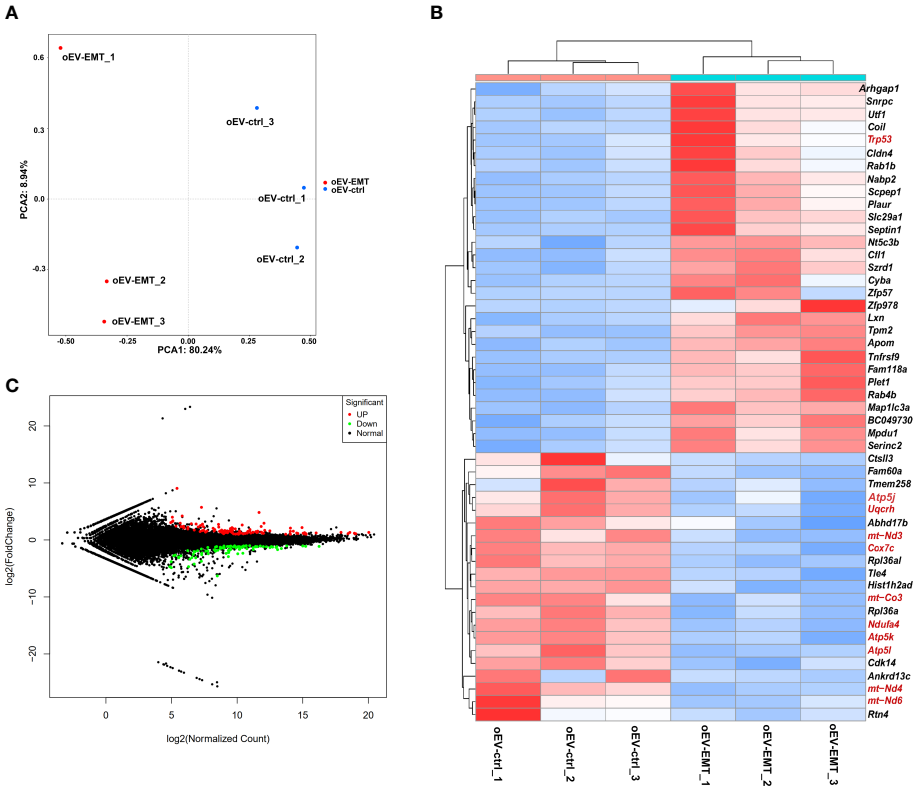


FIGURE 2
High-throughput sequencing showed differentially expressed genes between blastocysts treated with oEV-EMT or oEV-ctrl. **(A)** Principal component analysis. **(B)** MA plot shows all the genes in blastocysts from the two groups. Green and red dots denote down- and upregulated genes with FDR < 0.05. **(C)** Heatmap shows 29 most upregulated and 21 most downregulated genes. MA, M-versus-A plot. FDR, false discovery rate; oEV, oviductal extracellular vesicles; EMT, endometriosis; ctrl, control.

activity, and antioxidant activity (Figure 3B). Using real-time RT-PCR, we confirmed that in the oEV-EMT-treated embryos, *Ndufa4* and *Cox7c* were significantly down-regulated, and *Trp53* and *Fasl* were significantly upregulated (Figure 3C). *Uqcrh* and *Atp5l* did not show a statistical difference.

3.4 Oxidative stress, mitochondrial membrane potential, and apoptotic analysis in oEV-treated blastocysts

We checked the effects of oEV-EMT on oxidative stress and apoptosis in blastocysts. The ROS level in blastocysts treated with oEV-EMT was significantly higher than those treated with oEV-ctrl (1.556 ± 0.051 vs. 1.025 ± 0.056, p=0.039) (Figure 4A). MMP level was evaluated by staining blastocysts with JC-1. The MMP (referring to the ratio of red to green fluorescence) of oEV-EMT-treated blastocysts was significantly lower than oEV-ctrl treated blastocysts (2.846 ± 0.099 vs. 3.972 ± 0.096, p=0.028) (Figure 4B). Furthermore, as shown in Figure 4C, though the total cell number in blastocysts was not affected by oEV-EMT (87.769 ± 3.461 vs. 88.077 ± 5.523, p=0.963), oEV-EMT significantly increased the proportion of apoptotic cells in blastocysts (11.075 ± 1.391 vs. 5.475 ± 1.112, p= 0.003).

4 Discussion

This study provides evidence that EVs in the Fallopian tubes of women with endometriosis negatively affect embryonic development by down-regulating oxidative phosphorylation. Endometriosis is strongly associated with infertility. Several women with endometriosis would seek ART, including *in vitro* fertilization (IVF), for conception. Several studies have shown that endometriosis does not impair IVF outcomes in terms of live birth and clinical pregnancy, though women with endometriosis have lower oocyte yield in each cycle than women without endometriosis (9, 25). These results suggested embryo quality might be affected in women with endometriosis by the harmful environment in the Fallopian tube where fertilization and early embryo development happen. By circumventing the tubal microenvironment, the *in-vitro*-fertilized embryos can develop as well as in women without endometriosis (8). EVs have been recognized to contribute to disease pathophysiology in endometriosis (26–28), and whether EVs in endometriosis affect embryo development has not been studied. In this study, we constructed a co-cultured system using two-cell murine embryos and oEV-EMT or oEV-ctrl, as previously described (19). This co-culture system is reasonable because oviductal EVs are demonstrated to be conserved between humans and mice (22), and cargos in EVs are identified as transferrable

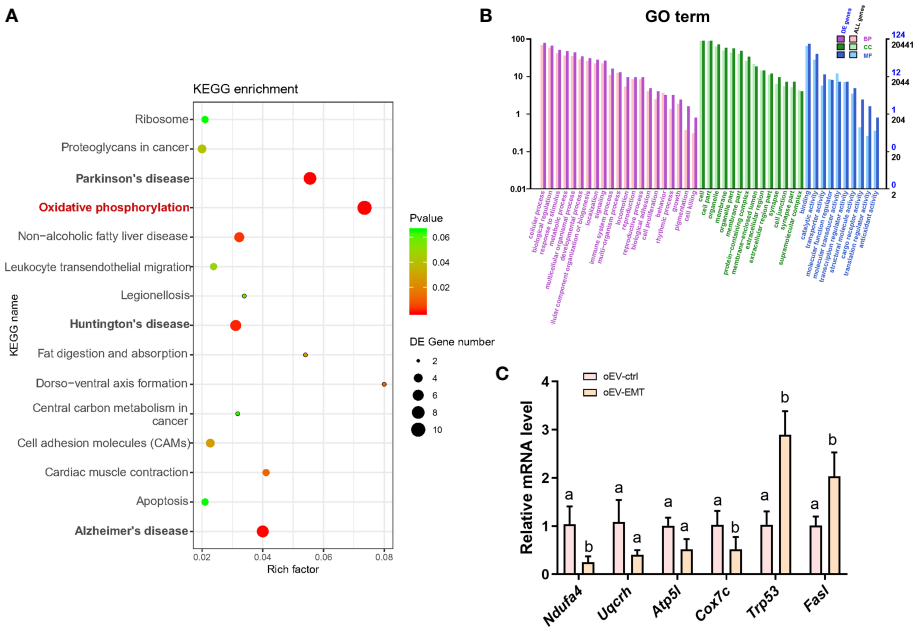


FIGURE 3
KEGG pathway enrichment and GO terms analysis of the differentially expressed genes. **(A)** The top 15 significant KEGG pathways are shown. Rich factor = (the number of DEGs in some KEGG pathway/the number of all DEGs that can be assigned to the KEGG database)/(the number of genes contained in a KEGG pathway/the total number of genes that can be assigned to the KEGG database). The greater the rich factor, the greater the degree of enrichment. **(B)** Bar plot showing GO terms with the number of differentially expressed genes for BP (biological process), CC (cell component), and MF (molecular function). **(C)** Validation of selected differentially expressed mRNAs by real-time RT-PCR. Different superscripts per column (a, b) represent statistical differences ($p < 0.05$) between groups. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; DEG, differentially expressed genes; oEV, oviductal extracellular vesicles; EMT, endometriosis; ctrl, control.

between species, which means EVs produced by one species can be taken up by other species and function in the recipient cells subsequently (16).

First, we confirmed that murine embryos could take human-derived oEVs. We observed that oEV-EMT significantly decreased the blastocyst rate, compared to the oEV-ctrl group, though the characterizations of oEVs are not different between the groups. Significantly, the blastocyst rate in the oEV-ctrl group was significantly higher than in the control group. This result is consistent with our previous study, which suggested that oEVs from women without endometriosis or other pelvic inflammation improve embryo quality *in vitro* (29). Previous literature demonstrated that *in vitro* fertilized embryos show poorer developmental competence than their *in vivo* counterparts (30, 31). Also, as conventional *in vitro* culture media might epigenetically affect embryos (32), the addition of healthy oEVs that contain RNAs and proteins associated with chromatin modification would partly compensate for the alterations (33). On the other hand, oEVs from pathological conditions might negatively affect embryos. As EVs were not recognized as one of the main elements of oviductal fluid until recently, their roles as regulators of oviduct-embryo communications are mostly mysterious (34). In this study, the difference in blastocyst rate between the oEV-EMT group and the oEV-ctrl group showed oEVs in patients with endometriosis impaired embryo development compared with women without this condition. However, the blastocyst rates in the oEV-EMT group and the blank control group were not statistically different.

Further, high-throughput sequencing on blastocysts and subsequent KEGG analysis revealed decreased expression levels of genes related to oxidative phosphorylation in blastocysts cultured with oEV-EMT. Moreover, analysis of oxidative stress and apoptosis at the blastocysts stage showed that embryos cultured with oEV-EMT had decreased JC-1 ratio, increased ROS level, and increased apoptotic index.

During development, embryonic metabolism shifts from predominantly glycolytic to predominantly oxidative phosphorylation (35), and enhanced oxidative phosphorylation is critical for supporting high energy needs in preimplantation embryos. Our KEGG analysis showed that 'oxidative phosphorylation' is disturbed in oEV-EMT-treated blastocysts. Oxidative phosphorylation is one of the most indispensable metabolic pathways in which cells produce the majority of the adenosine triphosphate (ATP), and take place inside mitochondria. Pathways of neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease, and Huntington's disease, were also enriched, due to DEGs in these pathways are associated with oxidative phosphorylation and apoptosis, which are central aspects of these neurodegenerative diseases (36). We validated that some genes responsible for the electron transport chain were significantly downregulated in oEV-EMT-treated embryos, including *Ndufa4* and *Cox7c*, at the blastocyst stage. *Ndufa4* encodes a subunit of complex IV of the electron transport chain and was reported to promote oxidative metabolism and MMP and could inhibit ROS levels and promote tumor cells (37, 38). *Uqcrh* encodes a subunit of the mitochondrial complex III and is responsible for the electron transfer between cytochrome *C* and cytochrome *C*1 during oxidative phosphorylation

10.3389/fendo.2023.1171778

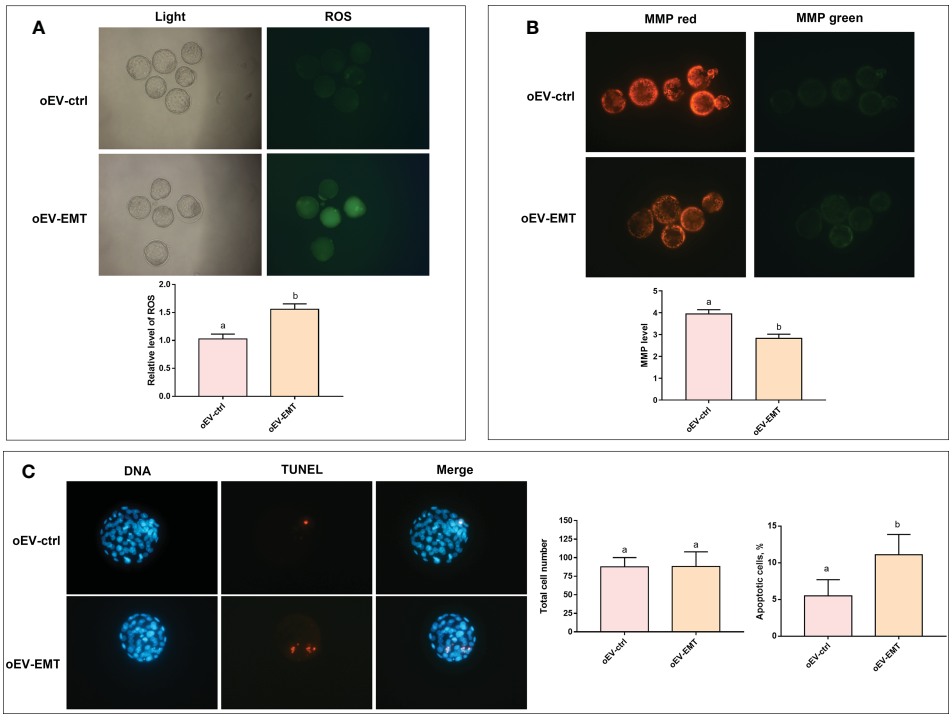


FIGURE 4
Effect of oEV-EMT on the relative level of ROS, MMP, and apoptotic cell numbers. **(A)** The fluorescence pictures and the relative levels of ROS (green fluorescence intensity value) were shown. **(B)** MMP, as measured by JC-1 staining, was shown as red/green fluorescence. **(C)** Representative images of apoptosis detected by TUNEL in blastocysts and the comparison of total cell number and apoptosis rates in blastocysts treated with oEV-EMT or oEV-ctrl are provided. Values indicated by different superscripts (a, b) are significantly different ($p < 0.05$). ROS, reactive oxygen species; MMP, mitochondrial membrane potential; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; oEV, oviductal extracellular vesicles; EMT, endometriosis; ctrl, control.

(39). *Atp5l*, *Atp5j*, and *Atp5k* encode the components of mitochondrial ATP synthase. ATP synthase affects embryo development using catalyzing ATP synthesis through an electrochemical gradient of protons across the mitochondrial inner membrane during oxidative phosphorylation (35). *Cox7c* encodes a long-lived mitochondrial protein that forms a stable contact between complex I complex II and is required complex IV (40). Altogether, oEV-EMT-treated embryos showed a down-regulation of a gene series that encodes the proteins involved in mitochondrial transmembrane transport, which may debilitate general mitochondrial function, resulting in attenuated embryo development. Supplementing the transcriptomic analysis, we observed functional abnormalities on oEV-EMT-treated blastocysts, including decreased MMP and higher ROS levels. Several studies observed a similar phenomenon in murine embryos when co-cultured with peritoneal fluid from patients with endometriosis (6, 41) and suggested an impairment in embryo viability. Also, some studies found embryos would show a higher ROS level and decreased MMP after being cultured in less optimal conditions, like higher oxygen concentrations (42), or with lipotoxic gradients in culture media which simulated maternal metabolic disorders (43). The latter study proposed that mitochondrial-targeted antioxidants might help rescue development competence in embryos.

'Apoptosis' is another significantly enriched pathway closely relevant to embryo development. We validated that the expression level of *Trp53* and *Fasl* was upregulated in oEV-EMT-treated blastocysts, and the TUNEL assay showed an increased apoptotic index in oEV-EMT-treated blastocysts. Embryonic quality is generally associated with lower apoptotic rates, though apoptosis plays an essential role during early mammalian embryo development under physiological conditions (44, 45). Previous literature reported that animal-derived oEV from the normal condition would be beneficial for improving the quality of the *in-vitro*-fertilized embryos, appearing as increased total cell number and lower ROS level, and fewer apoptotic cells (45, 46). Our results suggested that oEV in pathological conditions, specifically endometriosis, may play a harmful role through similar mechanisms in an opposite way. Intriguingly, considering mitochondria-ROS crosstalk is pivotal for apoptosis induction (47), and apoptosis would result in MMP dissipation and excessive ROS formation, it is not clear whether the increased apoptosis in the oEV-EMT-treated embryos was caused by the excessive ROS or represented a direct effect of oEV-EMT, which requires further investigation.

We have shown no difference in oEV characterizations between the two groups, including vesicle size and ultrastructure. However, they cause different results in embryos. Several studies deciphered oEV cargo in animals, proved they carry various bioactive molecules, including proteins, lipids, microRNAs, and DNAs, and proved they hold the possibility of affecting the gene expression and behavior of gamete and embryos (18, 33, 48). Studies on endometriosis have recognized EV contents from patients' plasma and peritoneal fluid (26, 27) and showed that the protein and RNA

contents are associated with numerous biological processes and might contribute to disease pathophysiology. In our case, it is difficult to determine the critical factor or factors that are accountable for the results because any differentially expressed proteins, lipids, or nucleic acids might be the answer, and it requested numerous amounts of efforts involving high-throughput sequencing workload in the future.

The disease process of endometriosis can impair the oocyte quantity and quality for inflammatory conditions in the peritoneum. Proinflammatory factors that harm gametes, including cytokines and interleukin, have been identified in the peritoneal and Fallopian tubal fluid (49, 50). We showed for the first time that EVs also constitute bioactive factors in the tubal fluid which might negatively affect embryo development in endometriosis.

A particular strength of the current study is that the oEVs are directly isolated from human Fallopian tubal fluid instead of cultured cells. One possible limitation is that we were not able to conduct high-throughput sequencing in the blastocysts which had been cultured without oEVs. Blastocysts might show altered gene expression even in the oEV-ctrl group compared to the embryos cultured with solely culture media. Additionally, due to methodological and ethical limitations, we could not utilize human embryos as the study object, and oEVs may have different effects on human embryos. For a similar reason, the control group was CIN III patients, rather than healthy women. CIN is a precancerous disease, in which affected cells are considered restricted to the uterine cervix. Previous studies indicated that CIN history or treatments for CIN do not affect fecundability or obstetric outcomes (51, 52). Therefore, given ethical and logistical constraints, we believe that they provide the best available controls, although we cannot completely exclude that oEVs from CIN patients may show some differences with disease-free women. Notably, the clinical characteristics in this study showed no difference in gravidity and parity between EMT patients and controls, and this result seems to contradict the consensus that EMT patients have decreased fertility. We surmised that the small patient cohort in this study could explain this. Furthermore, cargos in oEVs may be under hormonal regulation, which is observed both in cows and pigs (33, 53). Though the fluctuation patterns and the content compositions are different among species (54), the situation might be much more complicated in humans. These conditions should be noticed when a more general conclusion is about to be drawn.

5 Conclusion

The present study compared the characterizations of the oEVs from women with or without endometriosis and showed that oEV-EMT negatively influences early embryo development. Altered transcriptome and decreased MMP, increased ROS levels, and apoptotic cell numbers were observed in blastocysts. Our results call for further effort in deciphering the protein and nucleic acid content in oEV-EMT and the mechanisms they affect embryos and gamete development *in vivo*, which would be meaningful for unrevealing the mystery of how endometriosis impairs human fertility.

Data availability statement

The transcriptomics datasets generated during the current study were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database with accession number GSE 225323. The data will be kept private until Aug 31, 2023, and the reviewer token is cvangueqnjoblgt.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Tongji Hospital (No. TJ-IRB20210838). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Tongji Hospital Guide for the Care and Use of Laboratory Animals (approval number: TJ-202111004).

Author contributions

YL conducted the experiment and drew the manuscript. LC and NG conducted the animal experiment. CL isolated extracellular vesicles from the oviductal fluid. MW helped with bioinformatical analysis. LZ and FL helped to collect oviductal fluid samples. CS and LJ designed the experiment. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the National Key Research and Development Project of China (2021YFC2700603).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1171778/full#supplementary-material

References

1. Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. *Fertility sterility* (2012) 98(3):511–9. doi: 10.1016/j.fertnstert.2012.06.029

2. Practice committee of the American society for reproductive medicine. endometriosis and infertility: a committee opinion. *Fertility sterility* (2012) 98 (3):591–8. doi: 10.1016/j.fertnstert.2012.05.031

3. Santulli P, Lamau MC, Marcellin L, Gayet V, Marzouk P, Borghese B, et al. Endometriosis-related infertility: ovarian endometrioma per Se is not associated with presentation for infertility. *Hum Reprod (Oxford England)* (2016) 31(8):1765–75. doi: 10.1093/humrep/dew093

4. Mansour G, Aziz N, Sharma R, Falcone T, Goldberg J, Agarwal A. The impact of peritoneal fluid from healthy women and from women with endometriosis on sperm DNA and its relationship to the sperm deformity index. *Fertility Sterility* (2009) 92 (1):61–7. doi: 10.1016/j.fertnstert.2008.05.048

5. Chen Y-J, Wu H-H, Liau W-T, Tsai C-Y, Tsai H-W, Chao K-C, et al. A tumor necrosis factor-α inhibitor reduces the embryotoxic effects of endometriotic peritoneal fluid. *Fertility sterility* (2013) 100(5):1476–85. doi: 10.1016/j.fertnstert.2013.07.1985

6. Ding GL, Chen XJ, Luo Q, Dong MY, Wang N, Huang HF. Attenuated oocyte fertilization and embryo development associated with altered growth Factor/Signal transduction induced by endometriotic peritoneal fluid. *Fertil Steril* (2010) 93(8):2538–44. doi: 10.1016/j.fertnstert.2009.11.011

7. Hunter RHF, Cicinelli E, Einer-Jensen N. Peritoneal fluid as an unrecognised vector between female reproductive tissues. *Acta Obstet Gynecol Scand* (2007) 86 (3):260–5. doi: 10.1080/00016340601155098

8. Bishop LA, Gunn J, Jahandideh S, Devine K, Decherney AH, Hill MJ. Endometriosis does not impact live-birth rates in frozen embryo transfers of euploid blastocysts. *Fertil Steril* (2021) 115(2):416–22. doi: 10.1016/j.fertnstert.2020.07.050

9. Hamdan M, Dunselman G, Li TC, Cheong Y. The impact of endometrioma on Ivf/Icsi outcomes: a systematic review and meta-analysis. *Hum Reprod Update* (2015) 21(6):809–25. doi: 10.1093/humupd/dmv035

10. Sanchez AM, Pagliardini L, Cermisoni GC, Privitera L, Makieva S, Alteri A, et al. Does endometriosis influence the embryo quality and/or development? insights from a Large retrospective matched cohort study. *Diagnostics (Basel)* (2020) 10(2). doi: 10.3390/diagnostics10020083

11. Li S, Winuthayanon W. Oviduct: roles in fertilization and early embryo development. *J Endocrinol* (2017) 232(1):R1–R26. doi: 10.1530/JOE-16-0302

12. Al-Dossary AA, Strehler EE, Martin-Deleon PA. Expression and secretion of plasma membrane Ca2+-atpase 4a (Pmca4a) during murine estrus: association with oviductal exosomes and uptake in sperm. *PloS One* (2013) 8(11):e80181. doi: 10.1371/journal.pone.0080181

13. Lai YM, Wang HS, Lee CL, Lee JD, Huang HY, Chang FH, et al. Insulin-like growth factor-binding proteins produced by vero cells, human oviductal cells and human endometrial cells, and the role of insulin-like growth factor-binding protein-3 in mouse embryo Co-culture systems. *Hum Reprod (Oxford England)* (1996) 11 (6):1281–6. doi: 10.1093/oxfordjournals.humrep.a019372

14. Leese HJ, Hugentobler SA, Gray SM, Morris DG, Sturmey RG, Whitear S-L, et al. Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. *Reproduction fertility Dev* (2008) 20(1):1–8. doi: 10.1071/rd07153

15. Mathieu M, Martin-Jaular L, Lavieu G, Théry C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-Cell communication. *Nat Cell Biol* (2019) 21(1):9–17. doi: 10.1038/s41556-018-0250-9

16. Woith E, Fuhrmann G, Melzig MF. Extracellular vesicles-connecting kingdoms. *Int J Mol Sci* (2019) 20(22):5695. doi: 10.3390/ijms20225695

17. Machtinger R, Laurent LC, Baccarelli AA. Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. *Hum Reprod Update* (2016) 22 (2):182–93. doi: 10.1093/humupd/dmv055

18. Alminana C, Corbin E, Tsikis G, Alcantara-Neto AS, Labas V, Reynaud K, et al. Oviduct extracellular vesicles protein content and their role during oviduct-embryo cross-talk. *Reproduction* (2017) 154(3):153–68. doi: 10.1530/REP-17-0054

19. Liu C, Yao W, Yao J, Li L, Yang L, Zhang H, et al. Endometrial extracellular vesicles from women with recurrent implantation failure attenuate the growth and invasion of embryos. *Fertility sterility* (2020) 114(2):416–25. doi: 10.1016/j.fertnstert.2020.04.005

20. Lopera-Vásquez R, Hamdi M, Fernandez-Fuertes B, Maillo V, Beltrán-Breña P, Calle A, et al. Extracellular vesicles from boec in *in vitro* embryo development and quality. *PloS One* (2016) 11(2):e0148083. doi: 10.1371/journal.pone.0148083

21. Mazzarella R, Bastos NM, Bridi A, Del Collado M, Andrade GM, Pinzon J, et al. Changes in oviductal cells and small extracellular vesicles mirnas in pregnant cows. *Front veterinary Sci* (2021) 8:639752. doi: 10.3389/fvets.2021.639752

22. Bathala P, Fereshteh Z, Li K, Al-Dossary AA, Galileo DS, Martin-DeLeon PA. Oviductal extracellular vesicles (Oviductosomes, ovs) are conserved in humans: murine ovs play a pivotal role in sperm capacitation and fertility. *Mol Hum Reprod* (2018) 24 (3):143–57. doi: 10.1093/molehr/gay003

23. Pertea M, Pertea GM, Antonescu CM, Chang T-C, Mendell JT, Salzberg SL. Stringtie enables improved reconstruction of a transcriptome from rna-seq reads. *Nat Biotechnol* (2015) 33(3):290–5. doi: 10.1038/nbt.3122

24. Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, et al. Kobas 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res* (2011) 39(Web Server issue):W316–W22. doi: 10.1093/nar/gkr483

25. Hamdan M, Omar SZ, Dunselman G, Cheong Y. Influence of endometriosis on assisted reproductive technology outcomes: a systematic review and meta-analysis. *Obstet Gynecol* (2015) 125(1):79–88. doi: 10.1097/AOG.0000000000000592

26. Khalaj K, Miller JE, Lingegowda H, Fazleabas AT, Young SL, Lessey BA, et al. Extracellular vesicles from endometriosis patients are characterized by a unique mirna-lncrna signature. *JCI Insight* (2019) 4(18):e128846. doi: 10.1172/jci.insight.128846

27. Nazri HM, Imran M, Fischer R, Heilig R, Manek S, Dragovic RA, et al. Characterization of exosomes in peritoneal fluid of endometriosis patients. *Fertil Steril* (2020) 113(2):364–73.e2. doi: 10.1016/j.fertnstert.2019.09.032

28. Li W-N, Hsiao K-Y, Wang C-A, Chang N, Hsu P-L, Sun C-H, et al. Extracellular vesicle-associated vegf-c promotes lymphangiogenesis and immune cells infiltration in endometriosis. *Proc Natl Acad Sci United States America* (2020) 117(41):25859–68. doi: 10.1073/pnas.1920037117

29. Li Y, Liu C, Guo N, Cai L, Wang M, Zhu L, et al. Extracellular vesicles from human fallopian tubal fluid benefit embryo development *in vitro*. *Hum Reprod Open* (2023) 2023(2):hoad006. doi: 10.1093/hropen/hoad006

30. Rizos D, Clemente M, Bermejo-Alvarez P, de la Fuente J, Lonergan P, Gutiérrez-Adán A. Consequences of *in vitro* culture conditions on embryo development and quality. *Reprod Domest Anim* (2008) 43 Suppl 4:44–50. doi: 10.1111/j.1439-0531.2008.01230.x

31. Duranthon V, Chavatte-Palmer P. Long term effects of art: what do animals tell us? *Mol Reprod Dev* (2018) 85(4):348–68. doi: 10.1002/mrd.22970

32. Lazaraviciute G, Kauser M, Bhattacharya S, Haggarty P, Bhattacharya S. A systematic review and meta-analysis of DNA methylation levels and imprinting disorders in children conceived by Ivf/Icsi compared with children conceived spontaneously. *Hum Reprod Update* (2014) 20(6):840–52. doi: 10.1093/humupd/dmu033

33. Almiñana C, Tsikis G, Labas V, Uzbekov R, da Silveira JC, Bauersachs S, et al. Deciphering the oviductal extracellular vesicles content across the estrous cycle: implications for the gametes-oviduct interactions and the environment of the potential embryo. *BMC Genomics* (2018) 19(1):622. doi: 10.1186/s12864-018-4982-5

34. Fu B, Ma H, Liu D. Extracellular vesicles function as bioactive molecular transmitters in the mammalian oviduct: an inspiration for optimizing *in vitro* culture systems and improving delivery of exogenous nucleic acids during preimplantation embryonic development. *Int J Mol Sci* (2020) 21(6):2189. doi: 10.3390/ijms21062189

35. Ren L, Wang Z, An L, Zhang Z, Tan K, Miao K, et al. Dynamic comparisons of high-resolution expression profiles highlighting mitochondria-related genes between *in vivo* and *in vitro* fertilized early mouse embryos. *Hum Reprod (Oxford England)* (2015) 30(12):2892–911. doi: 10.1093/humrep/dev228

36. Cai Q, Jeong YY. Mitophagy in alzheimer's disease and other age-related neurodegenerative diseases. *Cells* (2020) 9(1):150. doi: 10.3390/cells9010150

37. Balsa E, Marco R, Perales-Clemente E, Szklarczyk R, Calvo E, Landázuri MO, et al. Ndufa4 is a subunit of complex iv of the mammalian electron transport chain. *Cell Metab* (2012) 16(3):378–86. doi: 10.1016/j.cmet.2012.07.015

38. Xu W, Lai Y, Pan Y, Tan M, Ma Y, Sheng H, et al. M6a rna methylation-mediated Ndufa4 promotes cell proliferation and metabolism in gastric cancer. *Cell Death Dis* (2022) 13(8):715. doi: 10.1038/s41419-022-05132-w

39. Song L, Yu J, Zhang D, Li X, Chen L, Cai Z, et al. Androgen excess induced mitochondrial abnormality in ovarian granulosa cells in a rat model of polycystic ovary syndrome. *Front In Endocrinol* (2022) 13:789008. doi: 10.3389/fendo.2022.789008

40. Krishna S, Arrojo E Drigo R, Capitanio JS, Ramachandra R, Ellisman M, Hetzer MW. Identification of long-lived proteins in the mitochondria reveals increased stability of the electron transport chain. *Dev Cell* (2021) 56(21):2952–2965.e9. doi: 10.1016/j.devcel.2021.10.008

41. Shu J, Xing L, Ding G, Luo Q, Liu X, Yan Q, et al. The effect of peritoneal fluid from patients with endometriosis on mitochondrial function and development of early mouse embryos. *PloS One* (2013) 8(12):e82334. doi: 10.1371/journal.pone.0082334

42. Belli M, Zhang L, Liu X, Donjacour A, Ruggeri E, Palmerini MG, et al. Oxygen concentration alters mitochondrial structure and function in *in vitro* fertilized preimplantation mouse embryos. *Hum Reprod (Oxford England)* (2019) 34(4):601–11. doi: 10.1093/humrep/dez011

43. Marei WFA, Van den Bosch L, Pintelon I, Mohey-Elsaeed O, Bols PEJ, Leroy JLMR. Mitochondria-targeted therapy rescues development and quality of embryos derived from oocytes matured under oxidative stress conditions: a bovine *in vitro* model. *Hum Reprod (Oxford England)* (2019) 34(10):1984–98. doi: 10.1093/humrep/dez161

44. Ramos-Ibeas P, Gimeno I, Cañón-Beltrán K, Gutiérrez-Adán A, Rizos D, Gómez E. Senescence and apoptosis during embryo development in a bovine model. *Front Cell Dev Biol* (2020) 8:619902. doi: 10.3389/fcell.2020.619902

45. Qu P, Luo S, Du Y, Zhang Y, Song X, Yuan X, et al. Extracellular vesicles and melatonin benefit embryonic develop by regulating reactive oxygen species and 5-methylcytosine. *J Pineal Res* (2020) 68(3):e12635. doi: 10.1111/jpi.12635

46. Qu P, Zhao Y, Wang R, Zhang Y, Li L, Fan J, et al. Extracellular vesicles derived from donor oviduct fluid improved birth rates after embryo transfer in mice. *Reproduction fertility Dev* (2019) 31(2):324–32. doi: 10.1071/RD18203

47. Marchi S, Giorgi C, Suski JM, Agnoletto C, Bononi A, Bonora M, et al. Mitochondria-ros crosstalk in the control of cell death and aging. *J Signal Transduct* (2012) 2012:329635. doi: 10.1155/2012/329635

48. Lee SH, Oh HJ, Kim MJ, Lee BC. Exosomes derived from oviduct cells mediate the Egfr/Mapk signaling pathway in cumulus cells. *J Cell Physiol* (2020) 235(2):1386–404. doi: 10.1002/jcp.29058

49. Vallvé-Juanico J, Houshdaran S, Giudice LC. The endometrial immune environment of women with endometriosis. *Hum Reprod Update* (2019) 25(5):564–91. doi: 10.1093/humupd/dmz018

50. Gupta S, Goldberg JM, Aziz N, Goldberg E, Krajcir N, Agarwal A. Pathogenic mechanisms in endometriosis-associated infertility. *Fertility Sterility* (2008) 90(2):247–57. doi: 10.1016/j.fertnstert.2008.02.093

51. Wise LA, Willis SK, Perkins RB, Wesselink AK, Klann A, Crowe HM, et al. A prospective study of treatments for cervical intraepithelial neoplasia and fecundability. *Am J Obstet Gynecol* (2020) 223(1):96.e1–96.e15. doi: 10.1016/j.ajog.2019.12.017

52. Kyrgiou M, Mitra A, Arbyn M, Stasinou SM, Martin-Hirsch P, Bennett P, et al. Fertility and early pregnancy outcomes after treatment for cervical intraepithelial neoplasia: systematic review and meta-analysis. *BMJ* (2014) 349:g6192. doi: 10.1136/bmj.g6192

53. Laezer I, Palma-Vera SE, Liu F, Frank M, Trakooljul N, Vernunft A, et al. Dynamic profile of evs in porcine oviductal fluid during the periovulatory period. *Reproduction* (2020) 159(4):371–82. doi: 10.1530/REP-19-0219

54. Harris EA, Stephens KK, Winuthayanon W. Extracellular vesicles and the oviduct function. *Int J Mol Sci* (2020) 21(21):8280. doi: 10.3390/ijms21218280

frontiers | Frontiers in Endocrinology

TYPE Review
PUBLISHED 26 June 2023
DOI 10.3389/fendo.2023.1199569

OPEN ACCESS

EDITED BY
Achmad Kemal Harzif,
University of Indonesia, Indonesia

REVIEWED BY
James Arthur MacLean,
Washington State University, United States
Mila Maidarti,
University of Indonesia, Indonesia

*CORRESPONDENCE
Shuli Yang
yangsl@jlu.edu.cn

RECEIVED 03 April 2023
ACCEPTED 05 June 2023
PUBLISHED 26 June 2023

CITATION
Wang M, Zheng L, Lin R, Ma S, Li J and Yang S (2023) A comprehensive overview of exosome lncRNAs: emerging biomarkers and potential therapeutics in endometriosis. *Front. Endocrinol.* 14:1199569. doi: 10.3389/fendo.2023.1199569

A comprehensive overview of exosome lncRNAs: emerging biomarkers and potential therapeutics in endometriosis

Min Wang[1], Lianwen Zheng[1], Ruixin Lin[2], Shuai Ma[1], Jiahui Li[1] and Shuli Yang[1*]

[1]Department of Obstetrics and Gynecology, The Second Hospital of Jilin University, Changchun, China, [2]Department of Hepato-Biliary-Pancreatic Surgery, The Second Hospital of Jilin University, Changchun, China

Endometriosis is a gynecological condition that significantly impacting women's daily lives. In recent years, the incidence of endometriosis has been rising yearly and is now an essential contributor to female infertility. Exosomes are extracellular vesicles (EVs) that carry long noncoding RNA (lncRNA) and shield lncRNA from the outside environment thanks to their vesicle-like structure. The role of exosome-derived lncRNAs in endometriosis is also receiving more study as high-throughput sequencing technology develops. Several lncRNAs with variable expression may be crucial to the emergence and growth of endometriosis. The early diagnosis of endometriosis will be considerably improved by further high specificity and sensitivity Exosome lncRNA screening. Exosomes assist lncRNAs in carrying out their roles, offering a new target for creating endometriosis-specific medications. In order to serve as a reference for clinical research on the pathogenesis, diagnosis, and treatment options of endometriosis, this paper covers the role of exosome lncRNAs in endometriosis and related molecular mechanisms.

KEYWORDS

exosome lncRNA, expression, biomarkers, therapeutics, endometriosis

1 Introduction

Exosomes are microsomal vesicles with a diameter of 30–160 nm produced by cells and control the transmission of information from cells to the extracellular matrix by carrying proteins, lncRNA, DNA, and other molecules (1–3). LncRNAs are RNAs longer than 200 nucleotides without the ability to code for proteins (4). lncRNAs have a significant role in several crucial regulatory processes, including nuclear transport, chromatin silencing, genomic imprinting, chromatin remodeling, transcriptional activation, and transcriptional interference (5, 6). LncRNAs play an essential role in the development of endometriosis, an estrogen-dependent condition in which endothelial cells in ectopic

lesions are controlled by estrogen, as well as cancers, cardiovascular disorders, and hematologic diseases (7–9). It is a common gynecological, endocrine condition that harms women's physical and emotional health (10–12). Exosome lncRNAs have been demonstrated to involved in the genesis of endometriosis, govern the activity of endothelium cells, and serve as a clinical marker for endometriosis (13, 14). They have also been linked to infertility in endometriosis patients. Compared to patients with stage I/II endometriosis and non-endometriosis, patients with stage III/IV endometriosis had significantly higher levels of TC101441 expression in their serum EVs. Because TC101441 can travel through EVs and control ESC migration and invasion, its presence in serum EVs may serve as a biomarker for endometriosis. Laparoscopy is the only current confirmation available and that many women may not be able to afford the procedure and this could be a cheap screen.

2 Overview of exosome

2.1 Biogenesis of exosome

Exosomes, which can be seen as cup-shaped objects under electron microscopy, are vesicles actively produced extracellularly by various live cells (15–17). The cell membrane invaginates to generate endosomes, multiple endosomes merge to form Early-Sorting Endosomes (ESE), and then ESE invaginates once more to wrap intracellular material and form numerous vesicles, which further convert into Late-Sorting Endosomes (LSE) (18, 19). Several tightly controlled mechanisms are involved in producing, sorting, and releasing the exosome's contents. Several molecules, including the Endosomal Sorting Complex Needed for Transport (ESCRT), four transmembrane proteins (CD9, CD63, and CD81), the apoptosis-linked gene 2-interacting protein X (*Alix*), and the tumor susceptibility gene 101(*TSG101*), are involved in the intracellular transport of the exosome (20–22). The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein complex and the family of synaptic binding proteins are necessary for the production of exosomes (Figure 1) (23).

2.2 Exosome features

Exosomes are vital in transmitting materials and information between cells (24). They can reach the appropriate target cells or organs by direct fusion, endocytosis, receptor-ligand interaction, and other mechanisms (25, 26). Intercellular communication and biological processes like antigen presentation, immunological response, and cell differentiation are regulated by endometriosis in the body (27, 28). Endometriosis are involved in intercellular communication activities and govern biological processes,

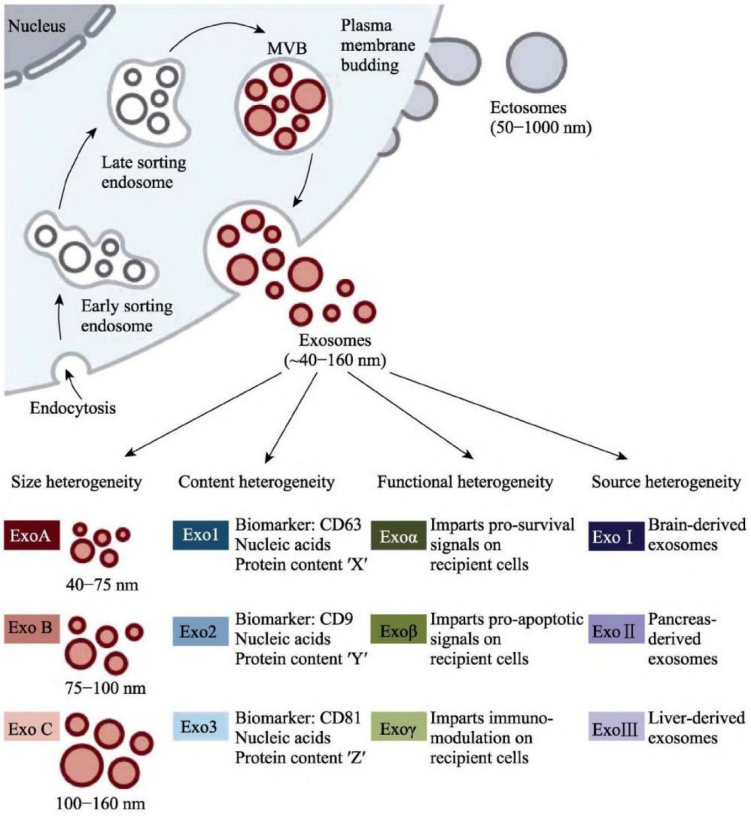


FIGURE 1
Production and secretion of exosomes (23).

10.3389/fendo.2023.1199569

including antigen presentation, immunological response, and cell differentiation, thereby partaking in the initiation and progression of diseases (2, 29, 30). Proteomics analyses have shown exosomes from different cellular origins are heterogeneous (31, 32). Exosomes can physiologically affect many cells and tissues because of their various contents (33, 34). Exosomes from a healthy microenvironment may help preserve the target cells' function. Still, exosomes from a stressful microenvironment might cause damage by sending oxidative and inflammatory signals to the target tissues (35). So, it is suggested that exosomes serve as significant intercellular communication carriers and can transport bioactive chemicals from their source cells, which may be crucial for intercellular communication (36–38).

2.3 Exosome extraction

While exosomes represent a potential tool for endometriosis diagnosis, their efficient purification without cellular contamination is a limiting factor (39). The primary techniques for isolating exosomes are gradient density centrifugation, differential ultracentrifugation, polymer immunoprecipitation, gel exclusion separation, and membrane affinity kits. One of the most popular separation techniques nowadays is ultracentrifugation, and the purest separation can be achieved using ultracentrifugation in combination with sucrose gradient density centrifugation (40). Exosomes have a low level of toxicity, are highly bioavailable, and are biologically stable (41). Consequently, it is anticipated that the identification and isolation of disease-specific exosomes free of cellular exosomes that are typically contaminated will shed new light on the advancement of precision medicine (42). Due to the size and physicochemical characteristics of exosomes, which differ from those of lipoproteins and protein complexes, as well as the fact that a variety of cells secretes exosomes, there is still much to learn about how to isolate and purify exosomes, which is a bottleneck problem in basic research and clinical applications related to exosomes.

3 Overview of lncRNA

3.1 Occurrence of lncRNA

LncRNAs are a family of RNAs with transcripts longer than 200 nucleotides frequently present in eukaryotic genomes but do not code for proteins (43, 44). Most lncRNAs can be found in the cytoplasm or nucleus of a cell and are produced from a single strand inside a protein-coding gene sequence (45). Antisense Bidirectional lncRNAs share promoters with protein-coding genes but are transcribed in the opposite direction to protein-coding genes; long intergenic ncRNAs (lincRNAs) are produced from the complementary DNA strand of the protein-coding gene, which is transcribed in the opposite direction and overlaps with at least one exon of the forward gene. Intronic lncRNAs are found in the protein-coding gene's intron region and do not overlap with its exons (46, 47).

3.2 Biological functions of lncRNA

Recent research has shown that lncRNAs are crucial for maintaining intracellular homeostasis when cells or tissues mature (48). This dispels the myth that noncoding RNAs (ncRNAs) are just "transcriptional noise" with no biological purpose (49, 50). It has been demonstrated that LncRNAs have a role in a variety of physiological and pathological processes (51, 52). *Via* epistasis, transcription, post-transcription, and acting as mediators of biological processes, lncRNAs are implicated in the regulation of apoptosis: epistasis lncRNAs can bind to Pol II to repress DNA expression, bind to DNA to form a triple helix structure to regulate, and can bind to transcription factors or recruit transcription-related factors to target genes to regulate the transcription of target genes (53–55). These lncRNA-specific structures or sequences can be specific sites on genomic DNA and can recruit the chromatin reconstruction complex (56, 57). The post-transcriptional regulation impacts the binding shear body and regulates the shearing process of mRNA by binding to antisense lncRNA at the target region of mRNA (Figure 2) (58, 59).

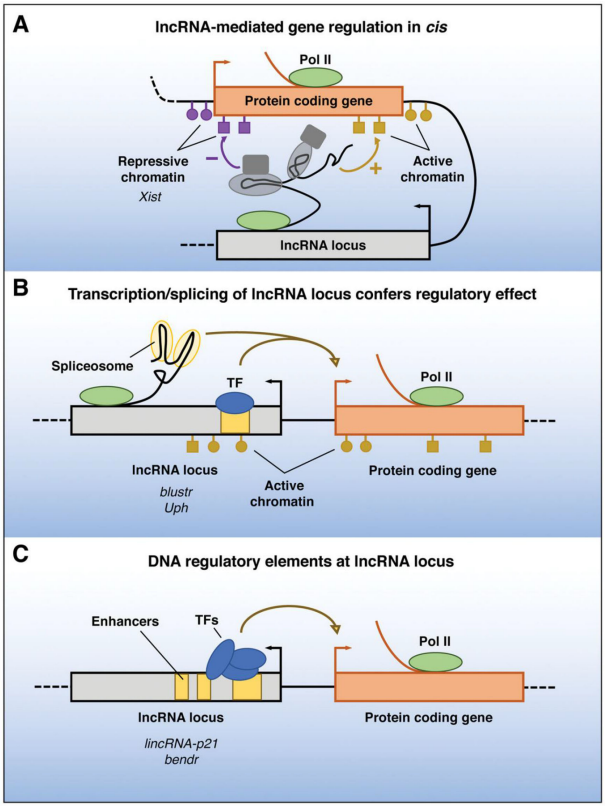


FIGURE 2
Functions of lncRNA loci in local gene regulation. The ability of a lncRNA locus to control the expression of nearby genes in cis may be due to DNA elements within the lncRNA promoter or gene body that function independently of the transcribed RNA **(A)**, may require transcription or splicing of an RNA but the lncRNA itself is not functional **(B)**, or may be due to sequence-specific functions of the mature lncRNA transcript **(C)**—Pol II, RNA polymerase II; TF, transcription factor (59).

3.3 LncRNA regulates mRNA transport and translation

Before being translated into protein, pre-mRNA is specially spliced in the nucleus to generate mature mRNA, which is subsequently translocated from the heart to the ribosome in the cytoplasm. Only when the structural gene ultimately succeeds in producing an active protein is the gene considered functional (60). Several studies have demonstrated that lncRNAs can control mRNA translation in various ways (61). mRNA transport can be impacted by lncRNAs, which in turn control adipogenesis. In addition to acting as an insulin-sensitizing hormone to lower blood sugar and encourage adipogenesis, lncRNAs are an endogenous bioactive peptide released by adipocytes during lipogenesis. AdipoQ ASlncRNA joins forces with AdipoQ mRNA to create a double-strand in the nucleus, preventing AdipoQ mRNA from moving from the core to the cytoplasm suppressing adipogenesis. In order to play a part in controlling the stability of the target genes, lncRNA often attaches to the relevant mRNA or particular protein to form an RNA-RNA or RNA-protein complex (62). Antisense lncRNAs' cytoplasmic binding to sense mRNAs is a typical example of this regulation. It has been demonstrated that lncRNAs can associate with RNA-binding proteins (RBPs) to create RNA-protein complexes that subtly control the stability of mRNA (63).

3.4 Competing endogenous RNA functions

LncRNAs can indirectly regulate the expression of target genes by competing for binding one or more miRNAs with the same response element, thereby promoting or inhibiting the onset or progression of disease (64, 65). This is a critical distinction between lncRNAs and miRNAs: the latter cannot interfere with or activate gene expression. LncRNAs can interact with miRNAs as a competitive endogenous RNA (ceRNA), functioning as a "sponge" for the miRNA molecule and easing its stress (66). This is one of the many ways lncRNAs and miRNAs function in cells. The ceRNA mechanism is a large and delicate method of gene expression control whose expression level is affected by conditions (67). LncRNAs, circRNAs, pseudogenes, synthetic miRNA inhibitors, and viral miRNA inhibitors are currently ceRNAs (68).

3.5 LncRNA controls the expression of inflammatory, immunological factors

LncRNAs can regulate gene expression and immune system responses, demonstrating biological processes' tissue specificity and complexity (69). Involved in the body's intrinsic and adaptive immunity, lncRNAs are crucial to many physical and immunological methods. The etiology of autoimmune and inflammatory illnesses is influenced by lncRNAs, such as lincRNA-Cox2 and lncRNA-dendritic cells (DC) (70). Numerous significant pathways connected to endometriosis are enriched in lncRNAs. Transient Receptor Potential (Trp) ion channels and thyroid hormone production were discovered to produce large amounts of inflammatory mediators, which suggests that lncRNAs may regulate the expression of inflammatory and immunological components linked to endometriosis (71). Inflammatory reactions may develop and be controlled in part by lncRNAs. According to immunological research, epigenetic dysregulation of B cells, which includes aberrant lncRNA expression, histone changes, and altered DNA methylation, might result in the body producing pathogenic autoantibodies. Endometriosis and autoimmune disorders share a biological mechanism with lncRNAs. Current hormonal and surgical treatments for endometriosis may be improved if the concept of immunomodulatory therapy for autoimmune illnesses is extended to the treatment of endometriosis (72, 73).

3.6 Correlation between lncRNA, miRNA, and endometriosis

Reverse regulatory RNA, siRNA competitive binding, and target gene stability regulation are techniques that LncRNAs can use to control target genes (74). LncRNAs can work as ceRNAs to control the expression of target genes, altering how well those genes function and becoming a key player in the emergence of disease (75, 76). MiR-199a can compete with vascular endothelial growth factor (VEGF)-A for the binding site on the lncRNA ENST00000465368, which reduces miR-199a's ability to bind to its target gene VEGF-A and weakens miR-199a (77). Inducing downstream signaling pathways, endothelial cell proliferation and migration, increased microvascular permeability, promotion of neovascularization, and successful ectopic endothelial implantation are all made possible by the angiogenesis-specific regulator VEGF-A, which binds to its receptor (78, 79). A decrease in the expression of Syndecan-1 (*SDC1*), a cell adhesion molecule, could result from downregulating the expression of lncRNANR 033688. This could enhance the repressive effect of miR-10b on its target gene *SDC1*, which would reduce cell-cell and cell-matrix adhesion, facilitating the invasion of ectopic endometrial glandular epithelial cells (80). The growth of endometriosis and the interactions between miRNAs and lncRNAs are tightly connected, and endometriosis proliferation, invasion, and metastasis are all significantly impacted by these factors (81, 82).

4 Exosome lncRNA has excellent potential for clinical applications

Exosome-derived lncRNAs have potential uses. lncRNAs are RNAs with strong regulatory abilities (83). lncRNAs can control the expression of mRNA through ceRNA processes, bind directly to proteins to alter or increase their functions, form complexes with DNA to affect gene transcription, and more (84). One of the critical pathways in the development of endometriosis is lncRNA

expression imbalance (85). The neural *CHL1* gene family includes the close homologue of the L1 (*CHL1*) gene, and CHL1-AS1 and CHL1-AS2 are two antisense lncRNA molecules of the CHL1 mRNA (86). Compared to *in situ* endometrial tissues, CHL1-AS1, CHL1-AS2, and *CHL1* mRNA expression was considerably higher in ectopic endometrial tissues. *CHL1* participates in the formation of endometriosis through interactions with CHL1-AS1 and CHL1-AS2. CHL1-AS1 or *CHL1* can bind to MiR-6076, which controls the expression of CHL1. Reduced expression of the CHL1-AS1 or increased miR-6076 have a protective effect on cell migration and proliferation (87).

Exosome lncRNA plays a significant role in the entire biological process of endometriosis compared to conventional endometriosis diagnosis and treatment. It exhibits the following features: Exosome lncRNA research helps ESCs proliferate, metastasize, and evolve while providing the molecular underpinnings for their targeted therapy. It is also more accessible for clinical diagnosis and relatively non-invasive (available in plasma, urine, and vaginal fluids) (88, 89). The exosome can influence the immune response in targeted therapy and release chemotherapeutic medicines and nucleic acids locally with low toxicity and high efficacy. Its contents are proteins and different nucleic acids uniquely expressed in distinct diseases. Exosome lncRNA research can investigate the pathophysiology of endometriosis, screen endometriosis for biomarkers, and offer a new fundamental framework for illness diagnosis and treatment (90).

5 Antifibrotic effects of exosome lncRNAs

The uterine cavity is invaded by endometrial cells, which then migrate to locations outside of it and undergo cyclic damage repair (91). The ability of stromal tissue to infiltrate and migrate causes a change from endometrial stromal fibrosis to fibrosis, which causes cell adhesion, collagen aggregation, and finally, fibrosis, resulting in the frequent presence of fibrous connective tissue around endometriosis lesions (92, 93). Endometrial stromal cells' ability to transition from stromal fibrosis to fibrosis and the invasion and migration of endometrial cells can be aggravated by prolonged high estrogen stimulation levels in the body (94, 95). This can cause several clinical symptoms, including chronic inflammation, progressive pelvic pain, and infertility (96). Infertility is more difficult by the fibrosis forming the walls and surrounding ovarian endometriotic cysts (97). Under estrogenic control, highly expressed H19 can enhance actin alpha 2 (ACTA2) expression by decreasing miR-216a-5p, further boosting the invasive and migratory abilities of stromal cells and ultimately leading to the development of endometriosis lesions and the formation of fibrosis at the lesions (98, 99). A lncRNA called Homeobox Gene Transcript Antisense Intergenic RNA (HOTAIR) has been linked to several fibrotic disorders. By increasing miR-326, which decreases cell proliferation, promotes fibrosis, and promotes apoptosis, HOTAIR silencing may inhibit NUS1 expression. Exosome lncRNAs may develop into novel endometriosis inhibitory vectors and present novel therapeutic approaches for managing endometriosis. To treat fibrosis and aid in the prevention and treatment of endometriosis, antifibrotic lncRNAs carried by exosomes may be exploited as therapeutic targets.

6 Exosomal lncRNAs and endometriosis

6.1 Endometrial cells with tumorigenic properties

Endometrial glands and stromal implants, known as endometriosis, are found inside the uterine cavity but develop outside (100). Even though endometriosis is a benign condition, it is frequently described as "benign cancer" due to its local infiltration, implantation, metastasis, and recurrence (101–105). Surgery is an invasive procedure with some dangers, and pathological examinations take too long to diagnose endometriosis. More significant and of theoretical relevance are future investigations into the pathogenesis of endometriosis and the hunt for novel biomarkers and molecular targets for diagnosis and therapy (106). An essential characteristic of endometriosis is the enhanced capacity of endometrial cells to infiltrate and migrate (107). Endometriosis lesions adhere extensively or densely to the tissues around them, and many different lesions exist. lncRNAs play a significant role in controlling human disease, physiology, and cancer (108, 109). As elaborated in section 6.3, more researchers are discovering that lncRNAs are abnormally expressed in endometriosis patients and play a role in controlling the progression of the illness due to the in-depth study of lncRNAs in recent years. Exosome extracellular nucleotidase was discovered to be a diagnostic sign for endometriosis in ovarian endometriosis cysts.

6.2 Immunomodulatory imbalance is closely associated with the occurrence of endometriosis

The pathogenesis and pathophysiology of endometriosis, classified as chronic inflammatory illnesses, are significantly influenced by immunological variables. However, only a tiny portion of the population has endometrial fragments that spread, grow, and eventually develop into endometriosis. This is likely because of changes in the internal environment of endometriosis patients, such as immune imbalance, and the ectopic lesions are typically accompanied by inflammatory congestion and inflammatory infiltration (110). Many women experience the "menstrual reflux" phenomenon multiple times. One of the clinical classification indicators of endometriosis is the degree of inflammation. The abnormal immune function in endometriosis patients is primarily exhibited in two ways: on the one hand, it is a compromised immunosurveillance and immunocidal cytotoxic effect, which prevents the body from effectively clearing the endothelium; and on the other hand, it is a compromised

immune environment in the abdominal cavity, which interferes with pregnancy and causes spots. Infertility, repeated implantation failure, early miscarriage, and aberrant histogenesis are related to endometriosis worsened by the peripheral circulating immune system and female endometrial autoimmune imbalance (Figures 3, 4) (111).

6.3 Exosome lncRNA regulates the development of endometriosis

6.3.1 Promotes neovascularization in endometriosis

Research has shown that neuro angiogenesis is crucial for the growth of endometriosis and that ESC-derived exosomes promote angiogenesis *in vitro* (112). Local angiogenesis and neurogenesis are encouraged, macrophages are activated, endometriosis lesions are made more extensive, and endometriosis advance when *in situ* ESC-derived exosomes are present (113). Angiogenesis-related lncRNA antisense hypoxia-inducible factor (a-HIF) is widely known (114). In the ectopic endometrium and serum of endometriosis patients, aHIF is abundantly expressed. Hence, exosome lncRNA-aHIFs are being investigated as potential molecular markers for the detection of endometriosis (115, 116). a-HIF plays a role in increasing angiogenesis in endometriosis and is strongly expressed in the endometriotic stromal cells (ECSCs) of patients with endometriosis. Another hypothesis for a molecular marker for the diagnosis of endometriosis is a-HIF. By activating VEGF-A, VEGF-D, and primary fibroblast growth factor (bFGF) to induce angiogenesis in human umbilical vein endothelial cells(HUVECs), exosomeaHIF is transported from ECSCs to HUVECs, which may have diagnostic relevance by stimulating angiogenesis in endometriosis (117, 118). The basic idea for the pathogenesis of endometriosis is the transcatheter blood flow reversal theory, and it is plausible that exosomes generated by healthy endometrium might travel through the blood to the pelvis or other places to advance endometriosis (119).

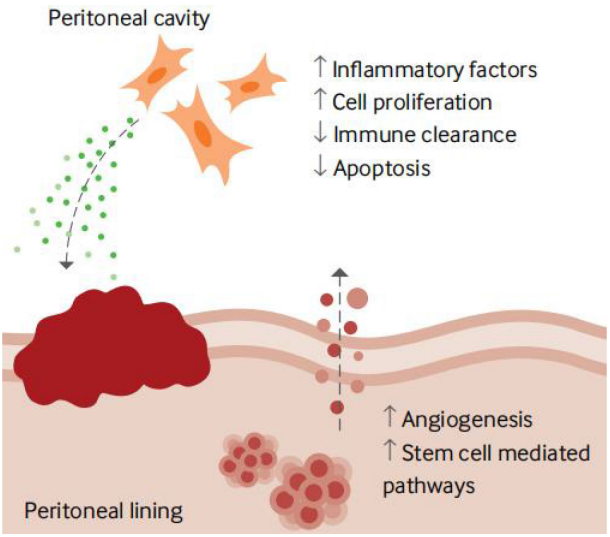


FIGURE 3
Local factors involved in developing an endometriosis lesion (111).

6.3.2 Regulating the biological behavior of endometriosis cells

The emphasis of the research is on how exosome lncRNA affects the biological function of endometriosis. LINC00261 directly binds to miR-132-3p to regulate BCL2L11 expression, which inhibits the proliferation and invasion of endometriosis cells, suggesting that LINC00261 plays an inhibitory role in the occurrence and development of endometriosis (120, 121). Expression of LINC00261 was significantly downregulated in ectopic endothelial tissues. Cell proliferation and migration decreased considerably after LINC00261 was overexpressed. Since LINC01279 expression in endometriosis tissues was markedly increased and connected with cyclin-dependent kinase 14 (CDK14) expression, LINC01279 may control endometriosis' cell cycle (122). In patients with endometriosis, lncRNACCDC144NL-AS1 showed differential expression in matched *in situ* and ectopic endothelial tissues. ESC migration and invasion were hindered by CCDC144NL-AS1 deletion, and CCDC144NL-AS1 knock-down reduced wave protein and matrix metalloprotein (MMP-9) expression, indicating that CCDC-144NL-AS1 may play a role in the pathogenesis of endometriosis (123). Application of BRAF-activated noncoding RNA (BANCR) inhibitors in a rat model of endometriosis resulted in a significant reduction in lesion size, accompanied by a decrease in mesenchymal cells and blood supply, and the BANCR inhibitors may inhibit endometriosis by inhibiting the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathway to reduce the expression of molecules associated with angiogenesis (124).

6.3.3 Correlation of cellular autophagy with the occurrence of endometriosis

Cellular autophagy is induced by hypoxia, a crucial microenvironmental element in endometriosis growth of endometriosis (125). Hypoxia caused a time-dependent induction of autophagy and the lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in cultured human ESCs (126). nuclear factor kappa-B/induciblenitricoxidesynthase (NF-κB/iNOS) co-stimulation was linked to an inflammatory response, and increased expression of iNOS promotes angiogenesis (127). MALTA1 expression was considerably raised in ectopic tissues of patients with endometriosis. MALAT1 regulated the proliferation, invasion, and death of ESCs *via* NF-B/iNOS. On the one hand, the hypoxic environment in the pelvis boosted MALAT1 expression, while on the other, it promoted autophagy in ESCs, allowing endothelium debris to survive ectopically. And by inhibiting the autophagy brought on by hypoxia, MALAT1 expression suppression in ESCs may enhance apoptosis (126). Actinfilament-associatedprotein1-antisenseRNA1 (AFAP1-AS1) was significantly up-regulated in endometriosis tissues, and lncRNA-AFAP1-AS1 significantly downregulated the expression of pGL3-P886, the promoter of the transcription factor zinc-fingerE-boxbindinghomeobox1(ZTB1), promoted epithelial-mesenchymal transition (EMT), and increased

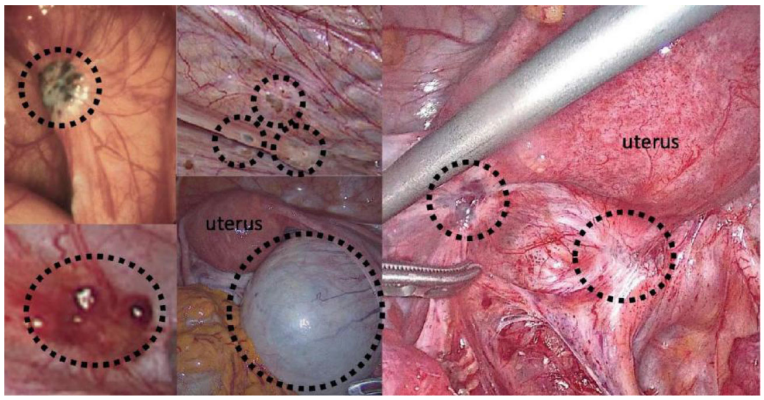


FIGURE 4
Surgical images of endometriosis sub-phenotypes (111).

ectopic endometrial invasion and implantation (128). By encouraging EMT, LINC01541 can be silenced to improve ESC invasion, whereas LINC01541 overexpression can inhibit EMT, ESC metastasis, and VEGF-A production, as well as cause apoptosis through controlling the Wnt/-catenin pathway (129). Exosome lncRNA encourages endometriosisESC invasion and proliferation, and it can act as a diagnostic marker for endometriosis to aid in the early detection of endometriosis.

6.4 Exosome lncRNA mediates infertility linked to endometriosis

Patients with endometriosis frequently experience infertility, and aberrant changes in endometrial status and uterine artery flow resistance can impact endometrial tolerance and, in turn, affect pregnancy and prognosis (130–132). Studies have revealed that the *in situ* endometrium of endometriosis exhibits a marked reduction in H19 expression (133). H19 usually is abundantly expressed in late-proliferating ESCs, whereas in the situ endometrium of endometriosis, its expression is downregulated. Low levels of H19 raise the activity of miRNALet-7, which in turn suppresses *in situ* ESC proliferation and survival and inhibits the downstream target insulin-like growth factor 1 receptor (IGF1R) at the post-transcriptional level. This results *in situ* endometrial defects, which affect endometrial tolerance and obstruct embryo implantation. By interfering with the H19/Let-7/IGF1R regulation system, endometriosis patients may experience spontaneous miscarriage or infertility due to diminished endometrial readiness and pregnancy receptivity. Through controlling miR-124-3p and Integrin beta-3 (ITGB3), lncRNA-H19 downregulation may prevent the proliferation and invasion of ectopic endometrial cells. This gives the treatment of endometriosis a new target (134).

Endometriosis can hinder oocyte growth and maturation, and as endometriosis levels rise, so does the disruption of ovarian reserve function. The expression of lncRNMALAT1 is increased in ectopic endometrial tissue (135). Through increasing the EMT-related transcription factors ZEB1 and ZEB2, MALAT1, a ceRNA of miR-200C, can support ESCs, boosting proliferation, invasion, and ectopic pregnancy. In ovarian granulosa cells of endometriosis, the expression of lncRNA-MALAT1 was markedly downregulated and positively associated with the number of sinus follicles (136). Further *in vitro* research supported the hypothesis that MALAT1 knock-down could affect follicular granulosa cell proliferation by activating the ERK/MAPK signaling pathway and up-regulating the expression of cell cycle regulatory proteins and that up-regulating the lncRNAENST00000433673 could affect embryo implantation and impair female fertility (137–139).

6.5 A potential diagnostic sign for endometriosis is exosome lncRNA

Exosome lncRNAs have proven to offer promise as non-invasive biomarkers for determining the presence of illness. In endometriosis, lncRNA AC002454.1 is considerably overexpressed, and the expression level is closely associated with cyclin-dependent kinase6(CDK6) levels (140). The biological behavior of endometrial cells may be cooperatively influenced by AC002454.1 and CDK6, which may work in concert to encourage the growth of endometriosis (141). In the endometrial tissues of endometriosis patients and healthy individuals, 86 lncRNAs showed differential expression. KEGG pathway analysis revealed that these lncRNAs were connected to various biological functions and signaling pathways in endometriosis (142). The lncRNA ENST00000482343 in the serum of endometriosis patients could be used as a potential molecular marker, according to earlier studies that examined the differential expression profiles of lncRNAs in 110 serum samples and 24 tissue samples and assessed their diagnostic value using the receiver operating characteristic curve. In ovarian endometriotic tissues, Urothelial Cancer Associated 1 (UCA1) expression was markedly downregulated, and the extent of UCA1 downregulation was positively connected with the severity of ovarian endometriosis (143). UCA1 might be used as a biomarker to evaluate the prognosis and staging of ovarian endometriosis (143). HOXA11-AS1 and *HOXA* gene mRNA fragments Patients with endometriosis had considerably lower expression levels in their *in situ* endometrium than in their ectopic endometrium, which raises the possibility that

HOXA11-AS1 may be involved in the growth of peritoneal endometriosis (144).

In comparison to *in situ* and normal endometrium, ectopic endometrium expressed TC0101441 at a greater level. High expression of TC0101441 in ECSCs produced EVs, which were subsequently internalized and absorbed by low-expression ECSCs to achieve TC0101441 transfer in ECSCs and eventually encourage endometriosis migration and invasion (145). Women with endometriosis had less maternally expressed gene 3 (MEG3)-210 in their ectopic endometrium (146). ESC migration and invasion are encouraged by the downregulation of MEG3-210. Through interacting with Galectin-1 *via* p38MAPK and cyclic AMP-dependent protein kinase A/sarco-endoplasmic reticulum ATPase (PKA/SERCA) signaling, mEG3-210 promotes ESC migration and invasion (147). In endometriosis, miR-145 reduces tumor growth, invasiveness, and stem cell characteristics. Prostate cancer-associated transcript-1 (PCAT-1) lncRNA and siRNA dramatically boosted miR-145 expression, reducing endometriotic stem cell proliferation and invasiveness (148). Normal endometrial tissues had a lower ratio of lncRNA steroid receptor activator (SRA) to steroid receptor activating protein (SRAP) than endometriosis tissues. In contrast to normal endometrial tissue, the expression of SRA and ER-lncRNA in endometriotic ovarian tissue was lower than that of SRAP and ER-lncRNA. Through controlling ER, SRA in ovarian endometriosis may play a significant regulatory function in the development of ESCs (149).

7 Conclusion

Exosome lncRNA can be a valuable biomarker for endometriosis, a prevalent gynecological, endocrine disorder that harms women's reproductive health and significantly burdens society. The present diagnostic indicators for endometriosis have low specificity and sensitivity. Hence, alternative diagnostic indicators must be discovered to increase the diagnostic effectiveness of endometriosis. lncRNAs are crucial for epigenetic control, transcription, translation, RNA metabolism regulation, cell autophagy, and death, among other processes. LncRNAs are quickly disturbed by other components in the circulation; however, because of the protection provided by the EVs structure, lncRNAs encapsulated in exosomes are not as easily destroyed once they have entered the circulation. Exosome lncRNA is stably expressed in serum or bodily fluids and can be employed as a disease biomarker to create a diagnostic model that will direct early diagnosis and postoperative follow-up of endometriosis, enable early disease treatment, and avoid recurrence. Exosome lncRNA research in endometriosis is still in its infancy, and there are still a lot of unexplored frontiers. The issues mentioned above can be resolved piecemeal with the rapid advancement of proteomics, transcriptomics, and bioinformatics analysis. Researchers will have a better knowledge of the role of exosome lncRNA in the genesis of endometriosis and its therapeutic utility.

Author contributions

MW, LZ, RL, SM, JL, and SY performed literature searches and selected the studies and reviews discussed in the manuscript. The first draft of the manuscript was prepared by MW. LZ, RL, SM, and JL made subsequent amendments. SY revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was provided by Project of Jilin Provincial Education Department and Jilin Provincial Health and Family Planning Commission Project (No.JJKH20201049KJ and No.2018J059).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Nojima T, Proudfoot NJ. Mechanisms of lncRNA biogenesis as revealed by nascent transcriptomics. *Nat Rev Mol Cell Biol* (2022) 23(12):853–. doi: 10.1038/s41580-022-00551-1

2. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* (2018) 19(4):213–28. doi: 10.1038/nrm.2017.125

3. Wang Y, Liu Q, Wang F. Potential roles of exosome noncoding RNAs in cancer chemoresistance. *Oncol Rep* (2021) 45(2):439–47. doi: 10.3892/or.2020.7887

4. Wu P, Mo Y, Peng M, Tang T, Zhong Y, Deng X, et al. Emerging role of tumor-related functional peptides encoded by lncRNA and circRNA. *Mol Cancer* (2020) 19 (1):22. doi: 10.1186/s12943-020-1147-3

5. Tsagakis I, Douka K, Birds I, Aspden JL. Long noncoding RNAs in development and disease: conservation to mechanisms. *J Pathol* (2020) 250(5):480–95. doi: 10.1002/path.5405

6. Zubrzycka A, Zubrzycki M, Perdas E, Zubrzycka M. Genetic, epigenetic, and steroidogenic modulation mechanisms in endometriosis. *J Clin Med* (2020) 9(5):1309. doi: 10.3390/jcm9051309

7. Lan X, Wu N, Wu LT, Qu K, Osoro EK, Guan DX, et al. The human novel gene LNC-HC inhibits hepatocellular carcinoma cell proliferation by sequestering has-miR-183-5p. *Mol Therapy-Nucleic Acids* (2020) 20:468–79. doi: 10.1016/j.omtn.2020.03.008

8. Zhao JL, Chen FD, Ma W, Zhang P. Suppression of long noncoding RNA NEAT1 attenuates hypoxia-induced cardiomyocytes injury by targeting miR-378a-3p. *Gene* (2020) 731:144324. doi: 10.1016/j.gene.2019.144324

9. Kar R, Dhar R, Mukherjee S, Nag S, Gorai S, Mukerjee N, et al. Exosome-based smart drug delivery tool for cancer theranostics. *ACS Biomater Sci Engineering* (2023) 9 (2):577–94. doi: 10.1021/acsbiomaterials.2c01329

10. Saunders PTK, Horne AW. Endometriosis: etiology, pathobiology, and therapeutic prospects. *Cell* (2021) 184(11):2807–24. doi: 10.1016/j.cell.2021.04.041

11. Xiang DF, Zhao M, Cai XF, Wang YX, Zhang L, Yao HL, et al. Betulinic acid inhibits endometriosis through suppression of estrogen receptor beta signaling pathway. *Front Endocrinol* (2020) 11. doi: 10.3389/fendo.2020.604648

12. Ceccaroni M, Bounous VE, Clarizia R, Mautone D, Mabrouk M. Recurrent endometriosis: a battle against an unknown enemy. *Eur J Contraception Reprod Health Care* (2019) 24(6):464–74. doi: 10.1080/13625187.2019.1662391

13. Hudson QJ, Proestling K, Perricos A, Kuessel L, Husslein H, Wenzl R, et al. The role of long noncoding RNAs in endometriosis. *Int J Mol Sci* (2021) 22(21):11425. doi: 10.3390/ijms222111425

14. Ahmed W, Liu ZF. Long noncoding RNAs: novel players in regulation of immune response upon herpesvirus infection. *Front Immunol* (2018) 9. doi: 10.3389/fimmu.2018.00761

15. Han J, Zhang Y, Ge P, Dakal TC, Wen H, Tang S, et al. Exosome-derived CIRP: an amplifier of inflammatory diseases. *Front Immunol* (2023) 14. doi: 10.3389/fimmu.2023.1066721

16. Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes *in vitro*: selective externalization of the receptor. *Cell* (1983) 33 (3):967–78. doi: 10.1016/0092-8674(83)90040-5

17. Khazaei F, Rezakhani L, Alizadeh M, Mahdavian E, Khazaei M. Exosomes and exosome-loaded scaffolds: characterization and application in modern regenerative medicine. *Tissue Cell* (2023) 80:102007. doi: 10.1016/j.tice.2022.102007

18. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science* (2020) 367(6478):640. doi: 10.1126/science.aau6977

19. Nafar S, Nouri N, Alipour M, Fallahi J, Zare F, Tabei SMB. Exosome as a target for cancer treatment. *J Invest Med* (2022) 70(5):1212–8. doi: 10.1136/jim-2021-002194

20. Henne WM, Stenmark H, Emr SD. Molecular mechanisms of the membrane sculpting ESCRT pathway. *Cold Spring Harbor Perspect Biol* (2013) 5(9):a016766. doi: 10.1101/cshperspect.a016766

21. Gall AR, Amoah S, Kitase Y, Jantzie LL. Placental mediated mechanisms of perinatal brain injury: evolving inflammation and exosomes. *Exp Neurol* (2022) 347:113914. doi: 10.1016/j.expneurol.2021.113914

22. Pegtel DM, Gould SJ. Exosomes. *Annu Rev Biochem* (2019) 88(1):487–514. doi: 10.1146/annurev-biochem-013118-111902

23. Tang YF, Long JQ. Placenta-derived exosomes and their role in the pathogenesis of adverse pregnancies. *Chin J Birth Health Heredity* (2023) 31(03):649–54. doi: 10.13404/j.cnki.cjbhh.2023.03.030

24. Xia X, Wang Y, Qin Y, Zhao S, Zheng JC. Exosome: a novel neurotransmission modulator or non-canonical neurotransmitter? *Ageing Res Rev* (2022) 74:101558. doi: 10.1016/j.arr.2021.101558

25. Hannafon BN, Gin AL, Xu YF, Bruns M, Calloway CL, Ding WQ. Metastasis-associated protein 1 (MTA1) is transferred by exosomes and contributes to the regulation of hypoxia and estrogen signaling in breast cancer cells. *Cell Communication Signaling: CCS* (2019) 17(1):13. doi: 10.1186/s12964-019-0325-7

26. Saheera S, Potnuri AG, Krishnamurthy P. Nano-vesicle (Mis)Communication in senescence-related pathologies. *Cells* (2020) 9(9):1974. doi: 10.3390/cells9091974

27. Guo M, Hao Y, Feng Y, Li H, Mao Y, Dong Q, et al. Microglial exosomes in neurodegenerative disease. *Front Mol Neurosci* (2021) 14. doi: 10.3389/fnmol.2021.630808

28. Sun X, Ding T, Wang B, Chang Z, Fei H, Geng L, et al. Identification of lncRNA-miRNA-mRNA networks in circulating exosomes as potential biomarkers for systemic sclerosis. *Front Med* (2023) 10. doi: 10.3389/fmed.2023.1111812

29. Negahdaripour M, Vakili B, Nezafat N. Exosome-based vaccines and their position in next-generation vaccines. *Int Immunopharmacol* (2022) 113(Pt A):109265. doi: 10.1016/j.intimp.2022.109265

30. Roy A, Girija As S, Sankar Ganesh P, Saravanan M, Sunny B. Exosome mediated cancer therapeutic approach: present status and future prospectives. *Asian Pacific J Cancer prevention: APJCP* (2023) 24(2):363–73. doi: 10.31557/APJCP.2023.24.2.363

31. Abak A, Abhari A, Rahimzadeh S. Exosomes in cancer: small vesicular transporters for cancer progression and metastasis, biomarkers in cancer therapeutics. *Peerj* (2018) 6:e4763. doi: 10.7717/peerj.4763

32. Wen SW, Lima LG, Lobb RJ, Norris EL, Hastie ML, Krumeich S, et al. Breast cancer-derived exosomes reflect the cell-of-Origin phenotype. *Proteomics* (2019) 19(8): e1800180. doi: 10.1002/pmic.201800180

33. Simona F, Laura S, Simona T, Riccardo A. Contribution of proteomics to understanding the role of tumor-derived exosomes in cancer progression: state of the art and new perspectives. *Proteomics* (2013) 13(10-11):1581–94. doi: 10.1002/pmic.201200398

34. Zhu L, Sun H-T, Wang S, Huang S-L, Zheng Y, Wang C-Q, et al. Isolation and characterization of exosomes for cancer research. *J Hematol Oncol* (2020) 13(1):152. doi: 10.1186/s13045-020-00987-y

35. Schmitz SU, Grote P, Herrmann BG. Mechanisms of long noncoding RNA function in development and disease. *Cell Mol Life Sci* (2016) 73(13):2491–509. doi: 10.1007/s00018-016-2174-5

36. Hu Y, Rao SS, Wang ZX, Cao J, Tan YJ, Luo J, et al. Exosomes from human umbilical cord blood accelerate cutaneous wound healing through the miR-21-3p-mediated promotion of angiogenesis and fibroblast function. *Theranostics* (2018) 8 (1):169–84. doi: 10.7150/thno.21234

37. Ghafourian M, Mahdavi R, Akbari Jonoush Z, Sadeghi M, Ghadiri N, Farzaneh M, et al. The implications of exosomes in pregnancy: emerging as new diagnostic markers and therapeutics targets. *Cell Communication Signaling* (2022) 20(1):51. doi: 10.1186/s12964-022-00853-z

38. Hade MD, Suire CN, Suo Z. Mesenchymal stem cell-derived exosomes: applications in regenerative medicine. *Cells* (2021) 10(8):1959. doi: 10.3390/cells10081959

39. Shi Y, Du L, Lv D, Li Y, Zhang Z, Huang X, et al. Emerging role and therapeutic application of exosome in hepatitis virus infection and associated diseases. *J Gastroenterol* (2021) 56(4):336–49. doi: 10.1007/s00535-021-01765-4

40. Yang H, Zhang H, Gu H, Wang J, Zhang J, Zen K, et al. Comparative analyses of human exosome proteomes. *Protein J* (2023). doi: 10.1007/s10930-023-10100-0

41. Jia R, Rotenberg SA V, Mirkin M. Electrochemical resistive-pulse sensing of extracellular vesicles. *Anal Chem* (2022) 94(37):12614–20. doi: 10.1021/acs.analchem.2c01216

42. Ye Z, Wang S, Huang X, Chen P, Deng L, Li S, et al. Plasma exosomal miRNAs associated with metabolism as early predictor of gestational diabetes mellitus. *Diabetes* (2022) 71(11):2272–83. doi: 10.2337/db21-0909

43. Bonasio R, Shiekhattar R. Regulation of transcription by long noncoding RNAs. *Annu Rev Genet* (2014) 48:433–55. doi: 10.1146/annurev-genet-120213-092323

44. Chen A, Yu R, Jiang S, Xia Y, Chen Y. Recent advances of MicroRNAs, long noncoding RNAs, and circular RNAs in preeclampsia. *Front Physiol* (2021) 12. doi: 10.3389/fphys.2021.659638

45. Yang J, Yang FJ, Wang YG, Su GF, Mao X. LncRNA MIR497HG inhibits proliferation and migration of retinal endothelial cells under high-level glucose treatment *via* miRNA-128-3p/SIRT1 axis. *Eur Rev Med Pharmacol Sci* (2020) 24 (11):5871–7. doi: 10.26355/eurrev_202006_21479

46. St Laurent G, Wahlestedt C, Kapranov P. The landscape of long noncoding RNA classification. *Trends Genet* (2015) 31(5):239–51. doi: 10.1016/j.tig.2015.03.007

47. Wang Y, Nicholes K, Shih IM. The origin and pathogenesis of endometriosis. *Annu Rev Pathol* (2020) 15:71–95. doi: 10.1146/annurev-pathmechdis-012419-032654

48. Maligianni I, Yapijakis C, Nousia K, Bacopoulou F, Chrousos GPP. Exosomes and exosomal noncoding RNAs throughout human gestation (Review). *Exp Ther Med* (2022) 24(3):582. doi: 10.3892/etm.2022.11518

49. Jain N, Gupta P, Sahoo S, Mallick B. Noncoding RNAs and their cross-talks impacting reproductive health of women. *Wiley Interdiscip Reviews-Rna* (2022) 13(3): e1695. doi: 10.1002/wrna.1695

50. Bridges MC, Daulagala AC, Kourtidis A. LNCcation: lncRNA localization and function. *J Cell Biol* (2021) 220(2):e202009045. doi: 10.1083/jcb.202009045

51. Zhang T, Tang X, Zhu Y, Wang C, Jiang Z, Yang N, et al. IGF2BP2 enhances LincRNA01116 stability *via* m(6)A: a potential biomarker and therapeutic target for patients with preeclampsia. *J Cell Biochem* (2023) 124(2):239–53. doi: 10.1002/jcb.30358

52. Ghafouri-Fard S, Shoorei H, Taheri M. Role of noncoding RNAs in the pathogenesis of endometriosis. *Front Oncol* (2020) 10. doi: 10.3389/fonc.2020.01370

53. Nunez-Martinez HN, Recillas-Targa F. Emerging functions of lncRNA loci beyond the transcript itself. *Int J Mol Sci* (2022) 23(11):6258. doi: 10.3390/ijms23116258

54. Yang L, Li L-P, Yi H-C. DeepWalk-based method to predict lncRNA-miRNA associations *via* lncRNA-miRNA-disease-protein-drug graph. *BMC Bioinf* (2022) 22 (SUPPL 12):621. doi: 10.1186/s12859-022-04579-0

55. Zhao W, Liu Y, Zhang C, Duan C. Multiple roles of exosomal long noncoding RNAs in cancers. *BioMed Res Int* (2019) 2019:1–12. doi: 10.1155/2019/1460572

56. Huang W, Li H, Yu Q, Xiao W, Wang DO. LncRNA-mediated DNA methylation: an emerging mechanism in cancer and beyond. *J Exp Clin Cancer Res* (2022) 41(1):100. doi: 10.1186/s13046-022-02319-z

57. Herman AB, Tsitsipatis D, Gorospe M. Integrated lncRNA function upon genomic and epigenomic regulation. *Mol Cell* (2022) 82(12):2252–66. doi: 10.1016/j.molcel.2022.05.027

58. Sanbonmatsu K. Getting to the bottom of lncRNA mechanism: structure-function relationships. *Mamm Genome* (2022) 33(2):343–53. doi: 10.1007/s00335-021-09924-x

59. Kopp F, Mendell JT. Functional classification and experimental dissection of long noncoding RNAs. *Cell* (2018) 172(3):393–407. doi: 10.1016/j.cell.2018.01.011

60. An B, Kameda T, Imamura T. The evolutionary acquisition and mode of functions of promoter-associated noncoding RNAs (pancRNAs) for mammalian development. *Essays Biochem* (2021) 65(4):697–708. doi: 10.1042/EBC20200143

61. Tripathi V, Ellis JD, Shen Z, Song DY, Prasanth KV. The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. *Mol Cell* (2010) 39(6):925–38. doi: 10.1016/j.molcel.2010.08.011

62. Wang X, Yu Q. Endometriosis-related ceRNA network to identify predictive biomarkers of endometrial receptivity. *Epigenomics* (2019) 11(2):147–67. doi: 10.2217/epi-2018-0190

63. Brandenburger T, Somoza AS, Devaux Y, Lorenzen JM. Noncoding RNAs in acute kidney injury. *Kidney Int* (2018) 94(5):870–81. doi: 10.1016/j.kint.2018.06.033

64. Zhang D, Wang L, Guo HL, Zhang ZW, Wang C, Chian RC, et al. MicroRNA-202 inhibits endometrial stromal cell migration and invasion by suppressing the K-Ras/Raf1/MEK/ERK signaling pathway. *Int J Mol Med* (2020) 46(6):2078–88. doi: 10.3892/ijmm.2020.4749

65. Sabaie H, Amirinejad N, Asadi MR, Jalaiei A, Daneshmandpour Y, Rezaei O, et al. Molecular insight into the therapeutic potential of long noncoding RNA-associated competing endogenous RNA axes in alzheimer's disease: a systematic scoping review. *Front Aging Neurosci* (2021) 13. doi: 10.3389/fnagi.2021.742242

66. Liu LY, Zheng B, Wang ZX. Protective effects of the knock-down of lncRNA AK139328 against oxygen-glucose deprivation/reoxygenation-induced injury in PC12 cells. *Mol Med Rep* (2021) 24(3):621. doi: 10.3892/mmr.2021.12260

67. Schuler-Toprak S, Skrzypczak M, Weber F, Ortmann O, Treeck O. Long non-coding RNA CCAT1 is overexpressed in endometrial cancer and regulates growth and transcriptome of endometrial adenocarcinoma cells. *Geburtshilfe Und Frauenheilkunde* (2020) 80(10):E206–E. doi: 10.1055/s-0040-1718183

68. Cao DW, Liu MM, Duan R, Tao YF, Zhou JS, Fang WR, et al. The lncRNA Malat1 functions as a ceRNA to contribute to berberine-mediated inhibition of HMGB1 by sponging miR-181c-5p in poststroke inflammation. *Acta Pharmacologica Sinica* (2020) 41(1):22–33. doi: 10.1038/s41401-019-0284-y

69. Ohkura N, Yasumizu Y, Kitagawa Y, Tanaka A, Nakamura Y, Motooka D, et al. Regulatory T cell-specific epigenomic region variants are a key determinant of susceptibility to common autoimmune diseases. *Immunity* (2020) 52(6):1119. doi: 10.1016/j.immuni.2020.04.006

70. Mariotti B, Servaas NH, Rossato M, Tamassia N, Cassatella MA, Cossu M, et al. The long non-coding RNA NRIR drives IFN-response in monocytes: implication for systemic sclerosis. *Front Immunol* (2019) 10. doi: 10.3389/fimmu.2019.00100

71. Craciunas L, Gallos I, Chu J, Bourne T, Quenby S, Brosens JJ, et al. Conventional and modern markers of endometrial receptivity: a systematic review and meta-analysis. *Hum Reprod Update* (2019) 25(2):202–23. doi: 10.1093/humupd/dmy044

72. Santoso B, Sa'adi A, Dwiningsih SR, Tunjungseto A, Widyanugraha MYA, Mufid AF, et al. Soluble immune checkpoints CTLA-4, HLA-G, PD-1, and PD-L1 are associated with endometriosis-related infertility. *Am J Reprod Immunol* (2020) 84(4):e13296. doi: 10.1111/aji.13296

73. Oksasoglu B, Hepokur C, Misir S, Yildiz C, Sonmez G, Yanik A. Determination of PD-1 expression in peripheral blood cells in patients with endometriosis. *Gynecological Endocrinol* (2021) 37(2):157–61. doi: 10.1080/09513590.2020.1821358

74. Cheng D, Deng JG, Zhang B, He XY, Meng Z, Li GL, et al. LncRNA HOTAIR epigenetically suppresses miR-122 expression in hepatocellular carcinoma *via* DNA methylation. *Ebiomedicine* (2018) 36:159–70. doi: 10.1016/j.ebiom.2018.08.055

75. Goodall GJ, Wickramasinghe VO. RNA In cancer. *Nat Rev Cancer* (2021) 21(1):22–36. doi: 10.1038/s41568-020-00306-0

76. Zafari N, Bahramy A, Zolbin MM, Allahyari SE, Farazi E, Hassannejad Z, et al. microRNAs as novel diagnostic biomarkers in endometriosis patients: a systematic review and meta-analysis. *Expert Rev Mol Diagn* (2022) 22(4):479–95. doi: 10.1080/14737159.2021.1960508

77. Wang WT, Sun YM, Huang W, He B, Zhao YN, Chen YQ. Genome-wide long noncoding RNA analysis identified circulating LncRNAs as novel non-invasive diagnostic biomarkers for gynecological disease. *Sci Rep* (2016) 6:23343. doi: 10.1038/srep23343

78. Huang YZ, Zhang T, Chen LQ, Yu MH, Liu Q, Zhou CY, et al. Elevated expressions of SHP2 and GAB2 correlated with VEGF in eutopic and ectopic endometrium of women with ovarian endometriosis. *Gynecological Endocrinol* (2020) 36(9):813–8. doi: 10.1080/09513590.2020.1787378

79. Wang L, Yang JJ, Xiao X, Zheng CG, Ming D. VEGF modulates the neural dynamics of hippocampal subregions in chronic global cerebral ischemia rats. *Neuromolecular Med* (2021) 23(3):416–27. doi: 10.1007/s12017-020-08642-y

80. Schneider C, Kassens N, Greve B, Hassan H, Schuring AN, Starzinski-Powitz A, et al. Targeting of syndecan-1 by micro-ribonucleic acid miR-10b modulates invasiveness of endometriotic cells via dysregulation of the proteolytic milieu and interleukin-6 secretion. *Fertility Sterility* (2013) 99(3):871. doi: 10.1016/j.fertnstert.2012.10.051

81. Zhang L, Li HH, Yuan M, Li D, Sun C, Wang GY. Serum exosomal MicroRNAs as potential circulating biomarkers for endometriosis. *Dis Markers* (2020) 2020:2456340. doi: 10.1155/2020/2456340

82. Wang XQ, Wu PL, Zeng C, Zhu JW, Zhou YF, Lu Y, et al. Long intergenic non-protein coding RNA 02381 promotes the proliferation and invasion of ovarian endometrial stromal cells through the miR-27b-3p/CTNNB1 axis. *Genes* (2022) 13(3):433. doi: 10.3390/genes13030433

83. Zhan Y, Du L, Wang L, Jiang X, Zhang S, Li J, et al. Expression signatures of exosomal long noncoding RNAs in urine serve as novel non-invasive biomarkers for diagnosis and recurrence prediction of bladder cancer. *Mol Cancer* (2018) 17(1):142. doi: 10.1186/s12943-018-0893-y

84. Zhang L, Li HH, Yuan M, Li D, Wang GY. Exosomal miR-22-3p derived from peritoneal macrophages enhances proliferation, migration, and invasion of ectopic endometrial stromal cells through regulation of the SIRT1/NF-kappa b signaling pathway. *Eur Rev Med Pharmacol Sci* (2020) 24(2):571–80. doi: 10.26355/eurrev_202001_20033

85. Wang Y, Hu SG, Yao GX, Zhu QL, He YQ, Lu Y, et al. A novel molecule in human cyclic endometrium: lncRNA TUNAR is involved in embryo implantation. *Front Physiol* (2020) 11. doi: 10.3389/fphys.2020.587448

86. Zhang C, Wu W, Ye X, Ma RQ, Luo JJ, Zhu HL, et al. Aberrant expression of CHL1 gene and long noncoding RNA CHL1-AS1, CHL1-AS2 in ovarian endometriosis. *Eur J Obstetrics Gynecol Reprod Biol* (2019) 236:177–82. doi: 10.1016/j.ejogrb.2019.03.020

87. Shi YM, Zha JF, Zuo MZ, Yan Q, Song HM. Long noncoding RNA CHL1-AS1 promotes cell proliferation and migration by sponging miR-6076 to regulate CHL1 expression in endometrial cancer. *J Cell Biochem* (2020) 121(3):2655–63. doi: 10.1002/jcb.29486

88. Farzaneh M, Najafi S, Anbiyaee O, Azizidoost S, Khoshnam SE. LncRNA MALAT1-related signaling pathways in osteosarcoma. *Clin Trans Oncol* (2023) 25(1):21–32. doi: 10.1007/s12094-022-02876-x

89. Martins TS, Vaz M, Henriques AG. A review on comparative studies addressing exosome isolation methods from body fluids. *Analytical Bioanalytical Chem* (2023) 415(7):1239–63. doi: 10.1007/s00216-022-04174-5

90. Wu JN, Huang HY, Huang W, Wang L, Xia XM, Fang XL. Analysis of exosomal lncRNA, miRNA and mRNA expression profiles and ceRNA network construction in endometriosis. *Epigenomics* (2020) 12(14):1193–213. doi: 10.2217/epi-2020-0084

91. Filip L, Duicc F, Pradatu A, Creoiu D, Suciu N, Cretoiu SM, et al. Endometriosis associated infertility: a critical review and analysis on etiopathogenesis and therapeutic approaches. *Medicina-Lithuania* (2020) 56(9):460. doi: 10.3390/medicina56090460

92. Sciarrillo R, Wojtuszkiewicz A, Assaraf YG, Jansen G, Kaspers GJL, Giovannetti E, et al. The role of alternative splicing in cancer: from oncogenesis to drug resistance. *Drug Resistance Updates* (2020) 53:100728. doi: 10.1016/j.drup.2020.100728

93. Agarwal SK, Chapron C, Giudice LC, Laufer MR, Leyland N, Missmer SA, et al. Clinical diagnosis of endometriosis: a call to action. *Am J Obstetrics Gynecol* (2019) 220(4):354.e1–e12. doi: 10.1016/j.ajog.2018.12.039

94. Tarumi Y, Mori T, Shimura K, Izumi Y, Okimura H, Kataoka H, et al. Progesterone receptor status of epithelial cells as a predictive marker for postoperative recurrence of endometriosis. *J Clin Endocrinol Metab* (2022) 107(6):1552–9. doi: 10.1210/clinem/dgac118

95. Delbandi AA, Mahmoudi M, Shervin A, Heidari S, Kolahdouz-Mohammadi R, Zarnani AH. Evaluation of apoptosis and angiogenesis in ectopic and eutopic stromal cells of patients with endometriosis compared to non-endometriotic controls. *BMC Womens Health* (2020) 20(1):3. doi: 10.1186/s12905-019-0865-4

96. Bafort C, Beebeejaun Y, Tomassetti C, Bosteels J, Duffy JMN. Laparoscopic surgery for endometriosis. *Cochrane Database Systematic Rev* (2020) 10(10):CD011031. doi: 10.1002/14651858.CD011031.pub3

97. Li WN, Hsiao KY, Wang CA, Chang N, Hsu PL, Sun CH, et al. Extracellular vesicle-associated VEGF-c promotes lymphangiogenesis and immune cell infiltration in endometriosis. *Proc Natl Acad Sci United States America* (2020) 117(41):25859–68. doi: 10.1073/pnas.1920037117

98. Xu Z, Zhang LP, Yu Q, Zhang YN, Yan L, Chen ZJ. The estrogen-regulated lncRNA H19/miR-216a-5p axis alters stromal cell invasion and migration *via* ACTA2 in endometriosis. *Mol Hum Reproduction* (2019) 25(9):550–61. doi: 10.1093/molehr/gaz040

99. Liu SP, Xin WJ, Tang XY, Qiu JJ, Zhang YJ, Hua KQ. LncRNA H19 overexpression in endometriosis and its utility as a novel biomarker for predicting recurrence. *Reprod Sci* (2020) 27(9):1687–97. doi: 10.1007/s43032-019-00129-x

100. Asghari S, Valizadeh A, Aghebati-Maleki L, Nouri M, Yousefi M. Endometriosis: perspective, lights, and shadows of etiology. *Biomed Pharmacother* (2018) 106:163–74. doi: 10.1016/j.biopha.2018.06.109

101. Gao XY, Zhang YS, Xu XX, Lu SM, Yan L. Effects of ovarian endometrioma aspiration on *in vitro* fertilization-intracytoplasmic sperm injection and embryo transfer outcomes: a systematic review and meta-analysis. *Arch Gynecol Obstetrics* (2022) 306(1):17–28. doi: 10.1007/s00404-021-06278-2

102. Taylor HS, Kotlyar AM, Flores VA. Endometriosis is a chronic systemic disease: clinical challenges and novel innovations. *Lancet* (2021) 397(10276):839–52. doi: 10.1016/S0140-6736(21)00389-5

103. Leenen S, Hermens M, van Steenwijk PJD, Bekkers RLM, van Esch EMG. Immunologic factors involved in the malignant transformation of endometriosis to endometriosis-associated ovarian carcinoma. *Cancer Immunol Immunother* (2021) 70(7):1821–9. doi: 10.1007/s00262-020-02831-1

104. Murakami K, Kotani Y, Shiro R, Takaya H, Nakai H, Matsumura N. Endometriosis-associated ovarian cancer occurs early during follow-up of endometrial cysts. *Int J Clin Oncol* (2020) 25(1):51–8. doi: 10.1007/s10147-019-01536-5

105. Kralickova M, Lagana AS, Ghezzi F, Vetvicka V. Endometriosis and risk of ovarian cancer: what do we know? *Arch Gynecol Obstetrics* (2020) 301(1):1–10. doi: 10.1007/s00404-019-05358-8

106. Wang HX, Ni CX, Xiao W, Wang SL. Role of lncRNA FTX in invasion, metastasis, and epithelial-mesenchymal transition of endometrial stromal cells caused by endometriosis by regulating the PI3K/Akt signaling pathway. *Ann Trans Med* (2020) 8(22):1504. doi: 10.21037/atm-20-6810

107. Wuyung PE, Rahadiati FB, Tjahjadi H, Salinah S, Kusmardi K, Kodariah R, et al. Histopathology and ARID1A expression in endometriosis- associated ovarian carcinoma (EAOC) carcinogenesis model with endometrial autoimplantation and DMBA induction. *Asian Pacific J Cancer Prev* (2021) 22(2):553–8. doi: 10.31557/APJCP.2021.22.2.553

108. Cai Y, Yin J, Jin Y, Li Y, Pan L. Endometriosis-associated ovarian cancer is not a distinct clinical entity among young patients: a 12-year cohort study. *Eur J Surg Oncol* (2019) 46(5):876–82. doi: 10.1016/j.ejso.2019.11.517

109. Gadducci A, Multinu F, Cosio S, Carinelli S, Ghioni M, Aletti GD. Clear cell carcinoma of the ovary: epidemiology, pathological and biological features, treatment options and clinical outcomes. *Gynecologic Oncol* (2021) 162(3):741–50. doi: 10.1016/j.ygyno.2021.06.033

110. Barra F, Lagana AS, Casarin J, Ghezzi F, Desideri LF, Scala C, et al. Molecular targets for endometriosis therapy: where we are and where we are going? *Int J Fertility Sterility* (2019) 13(2):89–92. doi: 10.22074/ijfs.2019.5736

111. Home AW, Missmer SA. Pathophysiology, diagnosis, and management of endometriosis. *BMJ-British Med J* (2022) 379:19. doi: 10.1136/bmj-2022-070750

112. Hozain S, Hernandez A, Fuller J, Sharp G, Cottrell J. Zinc chloride affects chondrogenesis *via* VEGF signaling. *Exp Cell Res* (2021) 399(2):112436. doi: 10.1016/j.yexcr.2020.112436

113. Farahani ZK, Taherianfard M, Naderi MM, Ferrero H. Possible therapeutic effect of royal jelly on endometriotic lesion size, pain sensitivity, and neurotrophic factors in a rat model of endometriosis. *Physiol Rep* (2021) 9(22):e15117. doi: 10.14814/phy2.15117

114. Dong L, Zhang L, Liu H, Xie MT, Gao J, Zhou XY, et al. Circ_0007331 knock-down suppresses the progression of endometriosis *via* miR-200c-3p/HiF-1 alpha axis. *J Cell Mol Med* (2020) 24(21):12656–66. doi: 10.1111/jcmm.15833

115. Qiu JJ, Lin XJ, Zheng TT, Tang XY, Zhang Y, Hua KQ. The exosomal long noncoding RNA aHIF is up-regulated in serum from patients with endometriosis and promotes angiogenesis in endometriosis. *Reprod Sci* (2019) 26(12):1590–602. doi: 10.1177/1933719119831775

116. Pang CH, Wu ZJ, Xu XY, Yang WX, Wang XX, Qi YH. Paeonol alleviates migration and invasion of endometrial stromal cells by reducing HIF-1 alpha-regulated autophagy in endometriosis. *Front Bioscience-Landmark* (2021) 26(9):485–95. doi: 10.52586/4961

117. Razi MH, Eftekhar M, Ghasemi N, Sheikhha MH, Firoozabadi AD. Expression levels of circulatory mir-185-5p, vascular endothelial growth factor, and platelet-derived growth factor target genes in endometriosis. *Int J Reprod Biomed* (2020) 18(5):347–58. doi: 10.18502/ijrm.v13i5.7155

118. Galano M, Li YC, Li L, Sottas C, Papadopoulos V. Role of constitutive STAR in leydig cells. *Int J Mol Sci* (2021) 22(4):2021. doi: 10.3390/ijms22042021

119. Feng Y, Zhan FL, Zhong YY, Tan BZ. Effects of human umbilical cord mesenchymal stem cells derived from exosomes on migration ability of endometrial glandular epithelial cells. *Mol Med Rep* (2020) 22(2):715–22. doi: 10.3892/mmr.2020.11137

120. Chen YH, Liu XH, He L. The value of long noncoding RNAs for predicting the recurrence of endometriosis a protocol for meta-analysis and bioinformatics analysis. *Medicine* (2021) 100(21):e26036. doi: 10.1097/MD.0000000000026036

121. Wang HC, Sha LX, Huang LX, Yang SM, Zhou QY, Luo XS, et al. LINC00261 functions as a competing endogenous RNA to regulate BCL2L11 expression by sponging miR-132-3p in endometriosis. *Am J Trans Res* (2019) 11(4):2269–79.

122. Liu J, Wang Q, Zhang RR, Zhang C, Lin JH, Huang XJ. Identification of LINC01279 as a cell cycle-associated long noncoding RNA in endometriosis with GBA analysis. *Mol Med Rep* (2018) 18(4):3850–8. doi: 10.3892/mmr.2018.9387

123. Zhang C, Wu W, Zhu HL, Yu XM, Zhang YL, Ye X, et al. Knock-down of long noncoding RNA CCDC144NL-AS1 attenuates migration and invasion phenotypes in endometrial stromal cells from endometriosis. *Biol Reproduction* (2019) 100(4):939–49. doi: 10.1093/biolre/ioy252

124. Zhu MB, Chen LP, Hu M, Shi Z, Liu YN. Effects of lncRNA BANCR on endometriosis through ERK/MAPK pathway. *Eur Rev Med Pharmacol Sci* (2019) 23(16):6806–12. doi: 10.26355/eurrev_201908_18719

125. Li WN, Wu MH, Tsai SJ. HYPOXIA AND REPRODUCTIVE HEALTH: the role of hypoxia in the development and progression of endometriosis. *Bioscientifica* (2021) 161(1):F19–F31. doi: 10.1530/REP-20-0267

126. Liu HW, Zhang ZB, Xiong WQ, Zhang L, Du Y, Liu Y, et al. Long noncoding RNA MALAT1 mediates hypoxia-induced pro-survival autophagy of endometrial stromal cells in endometriosis. *J Cell Mol Med* (2019) 23(1):439–52. doi: 10.1111/jcmm.13947

127. Yu J, Chen LH, Zhang B, Zheng QM. The modulation of endometriosis by lncRNA MALAT1 *via* NF-kappa B/iNOS. *Eur Rev Med Pharmacol Sci* (2019) 23(10):4073–80. doi: 10.26355/eurrev_201905_17908

128. Lin DC, Huang QS, Wu RF, Dai SJ, Huang ZX, Ren LL, et al. Long noncoding RNA AFAP1-AS1 promoting the epithelial-mesenchymal transition of endometriosis is correlated with transcription factor ZEB1. *Am J Reprod Immunol* (2019) 81(1):e13074. doi: 10.1111/aji.13074

129. Mai H, Wei YP, Yin Y, Huang SJ, Lin HS, Liao Y, et al. LINC01541 overexpression attenuates the 17 beta-estradiol-induced migration and invasion capabilities of endometrial stromal cells. *Syst endometriosis Biol Reprod Med* (2019) 65(3):214–22. doi: 10.1080/19396368.2018.1549290

130. Farren J, Jalmbrant M, Falconieri N, Mitchell-Jones N, Bobdiwala S, Al-Memar M, et al. Posttraumatic stress, anxiety, and depression following a miscarriage and ectopic pregnancy: a multicenter, prospective, cohort study. *Am J Obstetrics Gynecol* (2020) 222(4):367.e1–e22. doi: 10.1016/j.ajog.2019.10.102

131. Prescott J, Farland LV, Tobias DK, Gaskins AJ, Spiegelman D, Chavarro JE, et al. A prospective cohort study of endometriosis and subsequent risk of infertility. *Hum Reproduction* (2016) 31(7):1475–82. doi: 10.1093/humrep/dew085

132. Vesali S, Razavi M, Rezaeinejad M, Maleki-Hajiagha A, Maroufizadeh S, Sepidarkish M. Endometriosis fertility index for predicting non-assisted reproductive technology pregnancy after endometriosis surgery: a systematic review and meta-analysis. *Bjog-an Int J Obstetrics Gynaecol* (2020) 127(7):800–9. doi: 10.1111/1471-0528.16107

133. Ghazal S, McKinnon B, Zhou JC, Mueller M, Men Y, Yang LH, et al. H19 lncRNA alters stromal cell growth *via* IGF signaling in the endometrium of women with endometriosis. *EMBO Mol Med* (2015) 7(8):996–1003. doi: 10.15252/emmm.201505245

134. Liu SP, Qiu JJ, Tang XY, Cui HY, Zhang Q, Yang QH. LncRNA-H19 regulates cell proliferation and invasion of ectopic endometrium by targeting ITGB3 *via* modulating miR-124-3p. *Exp Cell Res* (2019) 381(2):215–22. doi: 10.1016/j.yexcr.2019.05.010

135. Du Y, Zhang ZB, Xiong WQ, Li N, Liu HW, He HT, et al. Estradiol promotes EMT in endometriosis *via* MALAT1/miR200s sponge function. *Reproduction* (2019) 157(2):179–88. doi: 10.1530/REP-18-0424

136. Liang ZW, Chen YJ, Zhao Y, Xu CY, Zhang AQ, Zhang Q, et al. miR-200c suppresses endometriosis by targeting MALAT1 in vitro and *in vivo*. *Stem Cell Res Ther* (2017) 8(1):251. doi: 10.1186/s13287-017-0706-z

137. Li Y, Liu YD, Chen SL, Chen X, Ye DS, Zhou XY, et al. Down-regulation of long noncoding RNA MALAT1 inhibits granulosa cell proliferation in endometriosis by up-regulating P21 *via* activation of the ERK/MAPK pathway. *Mol Hum Reproduction* (2019) 25(1):17–29. doi: 10.1093/molehr/gay045

138. Liu XJ, Zhang P, Li YM, Zhao N, Han HY. The AMPK-mTOR axis requires increased MALAT1 expression for promoting granulosa cell proliferation in endometriosis. *Exp Ther Med* (2021) 21(1):21. doi: 10.3892/etm.2020.9453

139. Xie QP, Chen CY, Li HY, Xu JH, Wu L, Yu Y, et al. miR-3687 overexpression promotes bladder cancer cell growth by inhibiting the negative effect of FOXP1 on cyclin E2 transcription. *Mol Ther* (2019) 27(5):1028–38. doi: 10.1016/j.ymthe.2019.03.006

140. Liu J, Wang Y, Chen P, Ma Y, Wang S, Tian Y, et al. AC002454.1 and CDK6 synergistically promote endometrial cell migration and invasion in endometriosis. *Reproduction* (2019) 157(6):535–43. doi: 10.1530/REP-19-0005

141. Wang Y, Li Y, Yang Z, Liu KR, Wang DB. Genome-wide microarray analysis of long noncoding RNAs in eutopic secretory endometrium with endometriosis. *Cell Physiol Biochem* (2015) 37(6):2231–45. doi: 10.1159/000438579

142. Sun PR, Jia SZ, Lin H, Leng JH, Lang JH. Genome-wide profiling of long noncoding ribonucleic acid expression patterns in ovarian endometriosis by microarray. *Fertility Sterility* (2014) 101(4):1038–U469. doi: 10.1016/j.fertnstert.2013.12.035

143. Huang H, Zhu ZY, Song Y. Downregulation of lncRNA uca1 as a diagnostic and prognostic biomarker for ovarian endometriosis. *Rev Da Associacao Med Brasileira* (2019) 65(3):336–41. doi: 10.1590/1806-9282.65.3.336

144. Wang MM, Hao CF, Huang X, Bao HC, Qu QL, Liu ZT, et al. Aberrant expression of lncRNA (HOXA11-AS1) and homeobox a (HOXA9, HOXA10, HOXA11, and HOXA13) genes in infertile women with endometriosis. *Reprod Sci* (2018) 25(5):654–61. doi: 10.1177/1933719117734320

145. Qiu JJ, Lin YY, Tang XY, Ding Y, Yi XF, Hua KQ. Extracellular vesicle-mediated transfer of the lncRNA-TC0101441 promotes endometriosis migration/invasion. *Exp Cell Res* (2020) 388(1):111815. doi: 10.1016/j.yexcr.2020.111815

146. Liu Y, Ma JY, Cui D, Fei XW, Lv YF, Lin J. LncRNA MEG3-210 regulates endometrial stromal cells migration, invasion, and apoptosis through p38 MAPK and PKA/SERCA2 signaling via interaction with galectin-1 in endometriosis. *Mol Cell Endocrinol* (2020) 513:110870. doi: 10.1016/j.mce.2020.110870

147. Sun KX, Wu DD, Chen S, Zhao Y, Zong ZH. LncRNA MEG3 inhibits endometrial carcinoma tumorigenesis and progression through the PI3K pathway. *Apoptosis* (2017) 22(12):1543–52. doi: 10.1007/s10495-017-1426-7

148. Wang LM, Xing Q, Feng TF, He M, Yu WX, Chen H. SNP rs710886A > G in long noncoding RNA PCAT1 is associated with the risk of endometriosis by modulating expression of multiple stemness-related genes *via* microRNA-145 signaling pathway. *J Cell Biochem* (2020) 121(2):1703–15. doi: 10.1002/jcb.29406

149. Tanikawa M, Oda K. Current status and issues of genome medicine in gynecological oncology. *Gan to kagaku ryoho Cancer chemother* (2021) 48(2):195–9.

Glossary

EVs	extracellular vesicles
lncRNA	long noncoding RNA
ESE	early-Sorting Endosomes
LSE	Late-Sorting Endosomes
ESCRT	Endosomal Sorting Complex Needed for Transport
Alix	apoptosis-linked gene 2-interacting protein X
TSG101	tumor susceptibility gene 101
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
lincRNAs	long intergenic ncRNAs
ncRNAs	noncoding RNAs
Pol II	RNA polymerase II
TF	transcription factor
RBPs	RNA-binding proteins
ceRNA	competitive endogenous RNA
DC	dendritic cells
Trp	Transient Receptor Potential
VEGF	vascular endothelial growth factor
SDC1	Syndecan-1
CHL1	close homologue of the L1
ACTA2	actin alpha 2
HOTAIR	Homeobox Gene Transcript Antisense Intergenic RNA
aHIF	antisense hypoxia-inducible factor
ECSCs	endometriotic stromal cells
bFGF	basic fibroblast growth factor
HUVECs	human umbilical vein endothelial cells
CDK14	cyclin-dependent kinase14
MMP-9	matrix metalloprotein-9
BANCR	BRAF-activated noncoding RNA
ERK/MAPK	extracellular signal-regulated kinase/mitogen-activated protein kinase
MALAT1	metastasis-associated lung adenocarcinomatranscript 1
NF-κB/iNO	nuclear factor kappa-B/inducible nitricoxide synthase
AFAP1-AS1	Actinfilament-associatedprotein1-antisenseRNA1
ZTB1	zinc-fingerE-boxbindinghomeobox1
EMT	epithelial-mesenchymal transition
IGF1R	insulin-like growth factor 1 receptor
ITGB3	Integrin beta-3
CDK6	cyclin-dependent kinase6
UCA1	Urothelial Cancer Associated 1
MEG3	maternally expressed gene 3
PKA/SERCA	cyclic AMP-dependent protein kinase A/sarco-endoplasmic reticulum ATPase
PCAT-1	Prostate cancer associated transcript-1
SRA	steroid receptor activator
SRAP	steroid receptor activating protein

frontiers | Frontiers in Endocrinology

TYPE Review
PUBLISHED 16 August 2023
DOI 10.3389/fendo.2023.1226687

OPEN ACCESS

EDITED BY
Iveta Yotova,
Medical University of Vienna, Austria

REVIEWED BY
Michele Da Broi,
University of Sao Paulo, Brazil
Abdulsamed Kükürt,
Kafkas University, Türkiye

*CORRESPONDENCE
Zheng Yuan
gyy1331128@163.com

RECEIVED 22 May 2023
ACCEPTED 01 August 2023
PUBLISHED 16 August 2023

CITATION
Fan W, Yuan Z, Li M, Zhang Y and Nan F (2023) Decreased oocyte quality in patients with endometriosis is closely related to abnormal granulosa cells. *Front. Endocrinol.* 14:1226687. doi: 10.3389/fendo.2023.1226687

Decreased oocyte quality in patients with endometriosis is closely related to abnormal granulosa cells

Weisen Fan[1], Zheng Yuan[2]*, Muzhen Li[3], Yingjie Zhang[1] and Fengjuan Nan[2]

[1]The First Clinical Medical College, Shandong University of Traditional Chinese Medicine, Jinan, Shandong, China, [2]Department of Gynecology, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, Shandong, China, [3]College of Acupuncture and Tuina, Shandong University of Traditional Chinese Medicine, Jinan, Shandong, China

Infertility and menstrual abnormalities in endometriosis patients are frequently caused by aberrant follicular growth or a reduced ovarian reserve. Endometriosis typically does not directly harm the oocyte, but rather inhibits the function of granulosa cells, resulting in a decrease in oocyte quality. Granulosa cells, as oocyte nanny cells, can regulate meiosis, provide the most basic resources required for oocyte development, and influence ovulation. Endometriosis affects oocyte development and quality by causing granulosa cells apoptosis, inflammation, oxidative stress, steroid synthesis obstacle, and aberrant mitochondrial energy metabolism. These aberrant states frequently interact with one another, however there is currently relatively little research in this field to understand the mechanism of linkage between abnormal states.

KEYWORDS

endometriosis, granulosa cells, infertility, mechanism, oocytes

1 Introduction

Endometriosis (EMS) is a common condition in reproductive-age women. Its most common clinical signs are pelvic pain, dyspareunia, a prolonged menstrual period, and a rise in menstrual volume, which can lead to infertility, anxiety, and depression. Ovarian endometriosis (OEM) has the potential to progress to ovarian cancer (1). It is concerning that the etiology of EMS is unclear and has been debated for a long time. There are numerous contentious causes of EMS at the moment, including endometrial cell implantation hypothesis, body cavity metaplasia theory, induction theory, genetic variables, immune factors, and so on (2). The prevalence of this condition has risen in recent years as contemporary medical diagnosis and treatment technology has advanced. At the moment, the primary method of treatment is surgical intervention and hormone therapy, but it is easy to relapse after surgical treatment, which not only costs more but also

causes repeated trauma to the patient, especially when patients with OEM and fertility needs are repeatedly stripped by laparoscopic surgery, which will further damage the patient's reproductive ability (3, 4). Although hormone is effective in treating this disease, it does not improve the ovarian reserve of EMS patients. As a result, we must first understand how EMS lowers patient fertility, which will allow us to develop effective therapies and preventive measures to assist EMS patients in conceiving.

Reduced ovarian reserve and follicle quality, changes in normal pelvic physical environment, decreased endometrial receptivity, and immunological dysfunction are the main reasons for EMS impeding female fertility. The most concerning aspect is that certain EMS patients are unable to generate qualifying eggs, which reduces the success rate of natural conception and assisted reproduction (5). Despite the fact that the meta-analysis found a minor variation in the success rate of assisted reproduction between patients with EMS and those without EMS. However, there are numerous risk factors for long-term pregnancy in EMS patients (6, 7). Because of the delay in diagnosing EMS, some patients with EMS are clinically diagnosed when their ovarian reserve has been compromised, and this damage is difficult to reverse (8). Oocytes are discharged during the maturity of the follicle, the ovary's most essential functional unit. Follicular granulosa cells(GCs) are the "guards" that accompany oocytes as they grow in size. During egg cell development, they rely mainly on GCs promotion and communication between oocyte and GCs (9). Recent research has demonstrated that the presence of EMS disrupts energy metabolism, apoptosis, and steroid hormone synthesis in GCs, lowering oocyte quality and limiting patients' reproductive hopes. As a result, this paper explains how EMS injury lowers oocyte quality by causing follicular GCs damage.

2 Ovarian reserve and oocyte quality are reduced in EMS patients

The essence of EMS is that endometrial cells occur in places other than the uterine cavity, with the most common locations being the surface of tissues or organs such as the ovary, utero-rectal pouch, sacral ligament, bladder, and ureter. EMS pathophysiology is comparable to tumor biology in that it involves enhanced proliferation, adhesion, and invasion, increased neovascularization in ectopic endometrial lesions, and decreased apoptosis (10). Endometrial stromal cells from ectopic lesions had significantly increased proliferation, migration, and invasion capacities as compared to normal female endometrial stromal cells, according to research (11). Endometriotic lesions, like cancers, rely on angiogenesis to proliferate. Vascular endothelial growth factor may be produced by the endometrium in the uterine cavity of EMS patients, and the amount of Vascular endothelial growth factor in peritoneal fluid of EMS patients is much higher than that of normal individuals (12). Ectopic endometrial cells respond to estrogen and progesterone in the same way as eutopic endometrial cells do. Surprisingly, some ectopic endometrial cells can also manufacture estrogen on their own (13). Estrogen production is closely related to EMS-associated inflammation. Estrogen can stimulate Cytochrome c oxidase subunit 2 synthesis, and Cytochrome c oxidase subunit 2 can enhance Prostaglandin E2 (PGE2) expression. PEG2 can increase the expression of aromatase, which in turn increases estrogen synthesis (14). Estrogen works on the highly expressed estrogen receptors in EMS lesions, promoting endometrial stromal cell survival and invasion, producing pro-inflammatory factors, and perpetuating inflammation (15, 16). The ultimate result of EMS is fibrosis, which is frequently histologically characterized as overly dense fibrous tissue around endometrial glands and stroma (17). Long-term inflammation of endometriotic lesions, which activates the Transforming growth factor beta-1 proprotein(TGF-β) signaling pathway, results in the creation of fibrotic lesions (18). Transforming growth factor beta-1 proprotein levels were observed to be higher in the serum and peritoneal fluid of EMS patients compared to healthy women (19).

Inflammatory, fibrotic, and oxidative responses caused by EMS can all harm a patient's ovarian reserve. OEM, in particular, can have a direct impact on a patient's ovarian reserve. In the ovaries afflicted by EMS cysts, the number of primordial follicles and AMH level fell, whereas the number of atretic follicles and primary follicles increased (20). This could be because EMS causes inflammation and oxidative stress, which leads to the recruitment of dormant primordial follicles into the growth and development track, while the local inflammatory response of the foci leads to ovarian fibrosis, affecting ovarian blood supply, and the growing follicles cannot get enough nutritional support and enter the atresia state (21, 22). A vicious cycle ensues, resulting in a patient's decreasing ovarian reserve and oocyte quality. The most obvious impact is EMS patients have a lower oocyte retrieval rate, a lower oocyte fertilization rate, a lower number of final available embryos and high-quality embryos, and a worse cumulative live birth rate of IVF cycles (23, 24). The ovarian damage of EMS patients is more severe as the disease progresses. According to other research, compared to normal women, the fertilization rate of oocytes in stage I/II EMS patients is around 7% lower, and the fertilization rate in stage III/IV EMS patients is even lower (25). The ultrastructure of EMS patients' oocytes revealed brown degeneration, deeper cytoplasm, larger refractive body, incomplete protrusion or separation of the first polar body, and a prolonged disintegration time of the zona pellucida (26, 27). Not only is the maturation ability of oocytes from EMS patients reduced, but also cortical granule loss, spindle fragmentation, and zona pellucis sclerosis may develop in immature oocytes following maturation and culture to meiosis II *in vitro*, which may impede with sperm penetration (28).

3 GSs' effect on oocytes

The female reproductive system's fundamental functioning component is the follicle. Oocytes, GCs, theca cells, and follicular antrum make up the mature follicle. The oocyte is surrounded by flat undifferentiated GCs at the primordial follicle stage. A zona pellucida forms around the oocyte as the follicle develops, separating the GCs. Flat, undifferentiated GCs will become cuboidal and proliferate progressively. Transzonal projections (TZPs) connect the cumulus granulosa cells to the oocyte

primarily through the zona pellucida (29). TZPS bind to the oocyte via Gap-junctions (GJs), which allow low-molecular-weight messages or nutrients to be transferred from cell to cell. Cumulus granulosa cells(CGCs) that are distant from the oocyte will form filopodia to interact with the oocyte as it develops (30). Undifferentiated GCs will gradually differentiate into mural granulosa cells(MGCs) and CGCs during the follicle development stage to preantral follicles. MGCs primarily execute endocrine tasks and are the first to accept Follicle stimulating hormone(FSH) and Luteinizing hormone(LH) action.

The function of follicular GCs is to help oocyte development and fertilization. These functions are mostly manifested in four areas (29): 1)Involved in maintaining of oocyte meiotic arrest; 2) Oocyte meiosis was induced to recover; 3)Provide the most fundamental substance needed for oocyte maturation; 4) Influences oocyte ejection from the follicle. Following female birth, the high level of Cathelicidin antimicrobial peptide(cAMP) secreted by undifferentiated GCs can keep cyclin Maturation promoting factor inactive by activating adenylate cyclase in oocytes, causing oocytes to enter the prophase stage of meiosis I (31). Following the appearance of the zona pellucida in oocytes, cyclic guanosine monophosphate(cGMP) secreted by GCs enters the oocyte via GJs, inactivating cGMP-inhibited 3',5'-cyclic phosphodiesterase 3A and maintaining a high level of cAMP in oocytes that cannot be degraded, thereby inhibiting the meiotic process (32). When women enter the period of sexual development, gonadotropins can act on the follicles, causing them to develop further. When LH secretion was at its height, it might activate mitogen-activated protein kinase3/1(MAPK3/1) in CGCs and decrease the concentration of natriuretic peptide precursor type C in MGCs, resulting in cAMP hydrolysis (33). Activated MAPK also lowered GJs permeability, limiting cAMP and cGMP transport to oocytes and resulting in meiotic recovery of oocytes (34). Furthermore, after receiving the LH signal, the CGCs depolarized, increasing the intracellular calcium ion(Ca2+)concentration (35). Elevated Ca2+ will enter the oocyte via GJs, resulting in a temporary increase in Ca2+ concentration (36). The rise in Ca2+ concentration in oocytes will facilitate meiosis recovery (37).

Because oocytes have a limited ability to utilize nutrients, CGCs provide the majority of them. Through TZPs and GJs, GCs can deliver amino acids, nucleotides, glutathione, and carbohydrate metabolites to oocytes (38). Because of the limited activity of phosphofructokinase and lactate dehydrogenase in oocytes, as well as the sluggish glycolysis process, glucose is primarily glycolyzed by CGCs to create pyruvate and lactate, which are then transported to oocyte mitochondria (39). Oocytes, in turn, can ensure pyruvate availability by increasing the expression of glycolytic genes in CGCs. In addition, sorbitol dehydrogenase in granulosa cells reduces glucose to form sorbitol, which is then fed by oocytes' indirect fructose synthesis (40). GCs not only provide necessary resources for oocyte formation, but they also control germinal vesicle rupture. FSH and LH control follicle growth, however the oocyte lacks Follicle stimulating hormone receptors (FSHR) and Luteinizing hormone receptors (LHR). As a result, FSH and LH influence follicular growth by acting on FSHR and LHR in CGCs and MGCs. FSH primarily works on MGCs, promoting estrogen synthesis in MGCs as well as GC proliferation. When GCs receive FSH signals, they produce inhibin, which inhibits FSH secretion and promotes the development of dominant and atresia non-dominant follicles (41). FSH and E2 synergistically encourage GCs to create LHR in preparation for the subsequent LH signal. FSH has the ability to stimulate CGC proliferation and hyaluronic acid synthesis. FSH stimulates CGC proliferation, hyaluronic acid synthesis, and then promotes the expansion of cumulus oocyte complex (42). Cumulus oocyte complex quality represents oocyte quality and is required for oocyte maturation and fertilization (43–45). When LH levels rise, LH and FSH work together to promote the production of progesterone and progesterone receptors in CGCs and to withdraw TZP from the oocyte membrane to the CGCs in preparation for the oocyte germinal vesicle to rupture (46, 47). Figure 1 depicts a more intuitive role for granule cells.

4 EMS causes GCs destruction

4.1 Apoptosis in GCs was triggered by EMS

When the granulosa cells die, the oocyte suffers from a quality decline or atresia due to a lack of various growth substances required for development. EMS can severely disrupt the cell cycle of follicular granulosa cells and increase the apoptotic rate of granulosa cells in patients (48, 49). Apoptotic bodies are extracellular vesicles containing nuclear and cytoplasmic debris that form as a result of apoptosis. The number of apoptotic bodies in undifferentiated GCs was considerably higher in patients with EMS compared to patients without EMS, and it was higher in patients with OEM compared to patients with EMS at other sites (50). Undifferentiated GC apoptosis can predict the result of *in vitro* fertilization (51). The more apoptotic bodies there are in EMS undifferentiated GCs, the lower the rate of recovered oocytes and the higher the rate of empty follicles.

The targets of differentially expressed CircRNAs were predominantly connected to apoptosis, Phosphatidylinositol3-kinase-RAC-beta serine/threonine-protein kinase(PI3K-AKT), and Cellular tumor antigen p53 signaling pathways by sequencing the CGCs of women with EMS and women without EMS (52). The PI3K-Akt signaling pathway is a critical regulator of cellular transcription, translation, proliferation, growth, and survival. After being phosphorylated, AKT participates in important biological processes such as apoptosis, protein synthesis, and cell cycle progressionAfter being phosphorylated, AKT participates in important biological processes such as apoptosis, protein synthesis, and cell cycle progression. Apoptosis of granulosa cells is higher in patients, which may be associated to decreased serum testosterone levels. In the CV434 granulosa cell line, testosterone can inhibit the PI3K-AKT signaling pathway and reduce cell death (49). Furthermore, the over-activation of primordial follicles in EMS patients' ovaries is linked to the activation of the PI3K-AKT pathway (53). It was discovered by sequencing the follicular GCs of OEM patients and normal female follicular GCs that the differently expressed genes were primarily abundant in the MAPK, Protein WNT(WNT) signaling pathway, apoptosis, and steroid hormone response (54). Among these, the Wnt

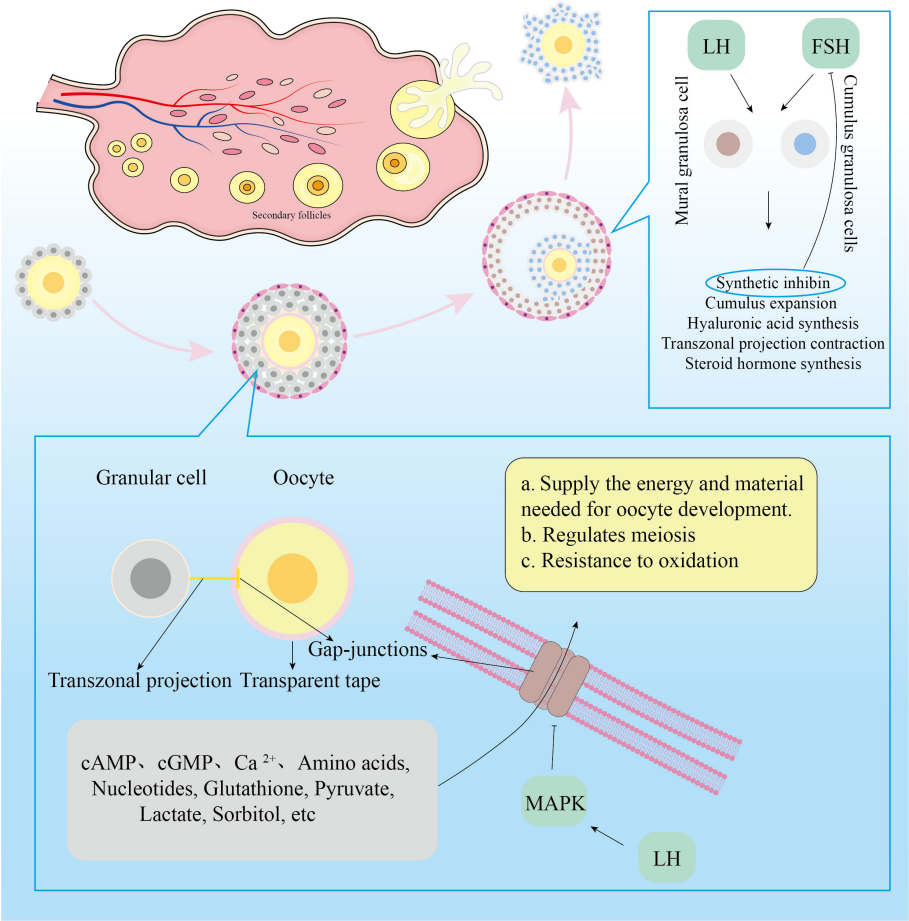


FIGURE 1
GCsls' physiological roles during follicular development.

signaling pathway is linked to cell proliferation, death, and migration (55). WNT4 and WNT5a transcription levels were significantly increased in luteinizing granulosa cells from EMS patients, while WNT1 transcription levels were significantly decreased, β-catenin and its dephosphorylated active form expression was decreased, and the expression of apoptosis inhibitor genes was decreased, while apoptosis was enhanced. This shows that Wnt signaling dysregulation is linked to granulosa cell death and follicular cell atresia (48).

4.2 EMS promotes inflammation and oxidative stress in GCs

Inflammation and oxidative stress can impair oocyte quality and potentially cause follicular atresia. EMS, especially OEM, can lead to follicular inflammation and oxidative stress. According to previous research (56), the levels of C-C motif chemokine 2(CCL2) and Interleukin(IL)-8 in the follicular fluid produced by EMS-affected ovaries are higher than those produced by normal ovaries. CCL2 is a tiny cytokine that has the ability to cause inflammation. It can not only attract inflammatory cells like neutrophils, monocytes, and lymphocytes to the lesion site, but it can also stimulate the production of additional cytokines including IL-2, IL-6, and cell adhesion molecules (57). IL-8 is also a chemokine cytokine, and its involvement and regulation of human reproductive physiological and pathological processes has been established. IL-8's primary biological activity is to recruit and activate neutrophils, hence promoting inflammation (58). The inflammatory reaction involving the follicles may become more severe as the lesions progress. IL-23 levels in follicular fluid and serum, for example, were considerably greater in individuals with III-IV EMS compared to those with I-II stages (59). In autoimmune inflammatory illnesses, IL-23 is a related factor that can mediate inflammatory and immunological responses by T cells, NK cells, and macrophages (60). When granulosa cells from EMS patients were cultivated *in vitro*, the levels of Tumor necrosis factor ligand superfamily member(TNF)-α, IL-8, and IL-1β in the cell supernatant were greater than in the control group (61). Furthermore, Nuclear Factor Kappa B(NFκB), Inhibitor of nuclear factor kappa-B kinase subunit beta, and NF-kappa-B inhibitor alpha expression was increased in granulosa cells from ovarian EMS patients, and the NFκB signaling pathway was significantly activated when granulosa cells were cultured with TNF-α (62). NFκB is an essential regulator of cellular inflammatory response and is involved in increasing the inflammatory cascade through cytokine activation.

Telomerase activity is high in healthy follicles, whereas NF-kB expression in granulosa cells is inversely associated to oocyte mass and telomerase activity, implying that post-inflammatory alterations in granulosa cells are deleterious to oocyte development (63). However, Liang found no significant differences in chemokines and inflammatory cytokines between patients with surgically removed endometrial cysts and those with untreated EMS in follicular fluid (64).

Inflammation and oxidative stress are interrelated, and increases in oxidative stress can cause acute and chronic inflammation (65). Oxidative stress can cause inflammation via a number of mechanisms, including Nucleotide-binding oligomerization domain-like receptors, TOLL receptors, and NFκB pathways (66). Simultaneously, inflammation can result in oxidative stress (67, 68). The oxidative stress level in the ovarian cortex around endometriotic cysts was much higher than in dermoid cysts (69). The composition of follicular fluid revealed that patients with EMS had higher levels of oxidative substances such as 8-hydroxy-2 deoxyguanosine, reactive oxygen species, peroxynitrite ion, Nitric oxide, and malondialdehyde, and lower levels of antioxidant substances such as peroxide dismutase, catalase, vitamin A, vitamin C, vitamin E, and reduced glutathione (70–72). This could be connected to EMS-induced senescence, endoplasmic reticulum stress, and oxidative stress in CGCs (73, 74). When mouse cumulus oocyte complexes were cultured with endometrial cyst fluid, CGC mitochondrial performance was impaired, glutathione content was reduced, reactive oxygen species levels rose, and oxidative damage to oocytes was hastened (75). This shows that EMS can impact oocyte quality by generating oxidative stress in GCs. However, Donabela's study found that the expression of superoxide dismutase 1, an antioxidant, was elevated in CGCs from individuals with moderate-to-severe EMS (76). As a result, we cannot say if the presence of EMS causes adaptive changes in CGCs, such as increased antioxidant capability. Oxidative stress may hinder ovulation in addition to lowering oocyte quality. Lin discovered that oxidative stress might decrease the expression of the histone-lysine N-methyltransferase EZH2 and the level of lysine 27 of the histone H3 protein in GCs while increasing the expression of Interleukin-1 receptor type 2 to suppress ovulation signals (77).

4.3 EMS influences GCs steroid hormone production

The synthesis and secretion of steroid hormones by GCs is critical for follicular growth and, as a result, can impact the quality of oocytes. The amount of FSHR and LHR on the surface of MGCs steadily increases as follicles expand, and estrogen released by MGCs can enhance CGCs proliferation. The aberrant follicular development in EMS patients is linked to a defective FSH signaling pathway operating on GCs, and EMS patients respond to FSH less effectively during ovulation induction (78). Although FSHR and cytochrome P450 family 19 subfamily A member 1(CYP19A1) expression levels are lower in GCs from EMS patients (77), it is unknown how EMS specifically changes FSHR signaling. According to other research (79, 80), the levels of estrogen and testosterone in the follicular fluid of patients with EMS are lower than those of patients without EMS, but the amount of progesterone is higher. When estrogen levels in the follicle are low, it frequently represents a deterioration in oocyte quality and the failure of *in vitro* fertilization (81). Follicular fluid progesterone levels rise as EMS severity rises, whereas testosterone levels fall as EMS severity rises (82, 83). However, another investigation found no change in progesterone synthesis by granulosa-lutein cells between patients with and without EMS (84). As a result, more information is required to establish the status of steroid synthesis by GCs in EMS.

The GCs produce and secrete the majority of the hormones in the follicle, and the hormone level in the follicular fluid represents the GCs' steroid hormone secretion ability. The increased progesterone release by GCs may be related to increased autophagy of GCs in EMS patients, higher expression of Beclin-1(BECN1), and greater low-density lipoprotein degradation capacity. BECN1 inhibition decreases GCs autophagy and lowers low-density lipoprotein-induced progesterone synthesis (85). Furthermore, unusually increased PGE2 levels in EMS patients' follicular fluid can enhance the expression of Steroidogenic acute regulatory protein(StAR) and the synthesis of progesterone in GCs (78). STAR may transport cholesterol from the outside mitochondrial membrane to the inner mitochondrial membrane and convert cholesterol to pregnenolone. Pregnenolone is a progestogen precursor that causes progesterone to be produced in response to 3β-HSD. However, Sreerangaraja's (79) study found that the expression of STAR and 3β-HSD was reduced in CGCs from EMS patients. As a result, the mechanism by which EMS influences progesterone production by granulosa cells is unclear and requires additional investigation.

GCs from EMS patients exhibited lower expression of not just 3β-HSD, but also CYP19, as well as impaired ability to release inhibin B and estrogen (79, 86, 87). Among them, 3β-HSD was capable of converting dehydroepiandrosterone to androstenediol.CYP19 catalyzes the conversion of androstenedione and testosterone into estrone and estradiol, respectively, and is the rate-limiting enzyme in estrogen biosynthesis. Both proteins are essential regulators of the estrogen synthesis process and have a rate-limiting effect on the synthesis of steroid hormones, which may explain why GCs produce less estrogen. The mechanism through which EMS interferes with estrogen synthesis by GCs is currently unknown. A reliable theory is that the presence of EMS activates the extracellular regulated protein kinase(ERK)1/2 signaling pathway in GCs. When the ERK1/2 signaling pathway is activated, it inhibits estrogen production by CYP19 and promotes progesterone generation by STAR (88). Elevated IL-6 levels in EMS patients' follicular fluid may activate the ERK1/2 signaling pathway in GCs. When Deura utilized IL-6 to culture granulosa tumor cell lines, it could enhance ERK1/2 phosphorylation, limit CYP19 expression, and diminish estrogen release (89). Li's research also discovered that ERK1/2 signaling is enhanced in granulosa cells from EMS patients (90).

4.4 EMS influences mitochondrial energy metabolism in GCs

Because GCs are the nanny cells that feed the oocyte, mitochondria play a vital role in energy metabolism. Therefore,

mitochondrial morphology, the amount of mitochondrial DNA (mtDNA) expression, and the efficiency of adenosine-triphosphate (ATP) synthesis in GCs all influence oocyte developmental potential to some extent (91). Mitochondria from CGCs from mild EMS patients showed morphological edema, hazy mitochondria, and reduced mtDNA expression abundance (92). After surgery, the quantity of mtDNA expression in follicular GCs of females with severe EMS was enhanced compared to women without EMS (93). This could be related to a compensatory increase in mtDNA expression in GCs to compensate for the ovary's lack of mitochondrial energy metabolism in order to adapt to the hypoxia in the follicular development microenvironment. According to Hsu's research (94), CGCs in EMS patients produced less ATP, although mtDNA expression abundance remained unaffected. The respiratory chain's structural proteins are encoded by mtDNA. The loss of its nucleic acid sequence will hamper oxidative phosphorylation and decrease ATP generation. As a result, when the energy metabolism of GCs mitochondria is insufficient, oocyte quality suffers. EMS also decreases mitochondrial membrane potential in GCs (79). The stability of mitochondrial membrane potential promotes the preservation of normal cellular physiological function. Normal mitochondrial membrane potential is required for oxidative phosphorylation and the generation of ATP. Increased glucose intake and lactate generation are also signs of abnormal mitochondrial energy metabolism in GCs from EMS patients. This could be due to elevated Prohibitin 1(PHB1) expression in GCs of EMS patients. When PHB1 expression was reduced, GCs expression of enzymes involved in glucose metabolism, glucose consumption, and lactate generation decreased (95). Furthermore, Sirtuin 2 (SIRT2) inhibits phosphoenolpyruvate carboxykinase 1 degradation, and Phosphoenolpyruvate carboxykinase, cytosolic [GTP] is the rate-limiting enzyme in gluconeogenesis. SIRT2 expression is enhanced in granulosa cells of EMS patients, indicating that EMS impacts GCs metabolic pathways, which may be mediated by SIRT2 (96).

5 The relationship between EMS-induced GCs abnormalities and oocyte quality

Current research indicates that EMS-induced oxidative stress, inflammation, aberrant mitochondrial energy metabolism, inappropriate steroid production, and apoptosis in GCs can all impair the quality of oocytes to variable degrees. Although there have been few studies on the abnormal pathological states of GCs, it is known from the existing studies that the abnormal condition of GCs generated by EMS does not exist alone, but interacts with one another. A transcriptome analysis of CGCs revealed that the genes that differed between mature and immature CGCs were mostly involved in steroid metabolism, inflammation, apoptosis, cell cycle regulation, and extracellular matrix remodeling (97). This also implies that the aberrant status of GCs generated by EMS will have an effect on oocyte maturation. According to the current research, oxidative stress appears to be the root cause of various aberrant conditions. EMS-induced oxidative stress generates reactive oxygen species, and an increase in reactive oxygen species in the follicle leads to spindle instability, aberrant chromosomal formation, and impaired oocyte developmental capability (98). Furthermore, protein nitration is enhanced in follicular GCs of EMS patients, and protein nitration is a marker of peroxynitrite ions, which are reactive nitrogen free radicals (70). Peroxynitrite ions can change oocyte spindle shape and chromosomal organization in a dose-dependent way (99). Oxidative stress can damage GCs mitochondria, affecting mitochondrial energy metabolism and steroid hormone production. Mitochondrial dysfunction generates free radicals, which exacerbates oxidative stress (100). Furthermore, the lower mitochondrial membrane potential of GCs may cause nuclear and cytoplasmic maturation to be out of sync, eventually leading to embryo development failure (101). The failure of mitochondrial steroidogenesis in GCs can result in aberrant oocyte development, especially when estrogen levels in follicular fluid are low and progesterone levels are high, affecting proper meiosis and late cleavage of oocytes (80, 102, 103). Nevertheless, it is unknown whether the level of progesterone in follicular fluid of patients with EMS differs from that of normal individuals, and we do not yet know the mechanism by which progesterone influences oocyte quality. Oxidative stress can also cause GCs inflammation and apoptosis. GCs inflammation frequently interferes with oocyte meiotic capacity. Lipopolysaccharide-induced inflammation enhances IL-6 and IL-8 secretion in bovine GCs, resulting in meiotic block and failure of germinal vesicle rupture in oocytes (104). The IL-6 described above may interfere with estrogen synthesis in GCs, impacting oocyte development (88). Several investigations in embryo culture have demonstrated that IL-8, IL-12, and TNF-α in EMS patients' follicular fluid are inversely connected with oocyte maturity and embryo quality (105). Among these, TNF-α production by CGCs can increase senescence of mouse oocytes after ovulation, which may be the cause of oocyte quality decline (106). Although the mechanism of EMS-induced granulosa cell death is unknown, oxidative stress-induced apoptosis could be produced by endoplasmic reticulum stress. When EMS follicles were compared to normal women, GCs apoptosis was enhanced and endoplasmic reticulum stress was visible. GCs apoptosis was reduced after endoplasmic reticulum stress was relieved (73). MtDNA depletion causes apoptosis in granulosa cells (107). We noted previously that EMS patients' granulosa cells had abnormal mtDNA expression, but we don't know how EMS influences the abnormal expression of mtRNA. Figure 2 illustrates a potential process by which the granulosa cells' aberrant condition influences the quality of the oocyte.

6 Summary

The decrease in oocyte quality in EMS patients will make pregnancy hard for women. Even with assisted reproductive technology, the rate of pregnancy failure remains high. In conclusion, EMS primarily reduces oocyte quality by causing GCs apoptosis, inflammation, oxidative stress, steroidogenesis disorders, and abnormal mitochondrial energy metabolism, which also provides a therapeutic direction for improving assisted reproductive technology success rates in EMS patients. It is

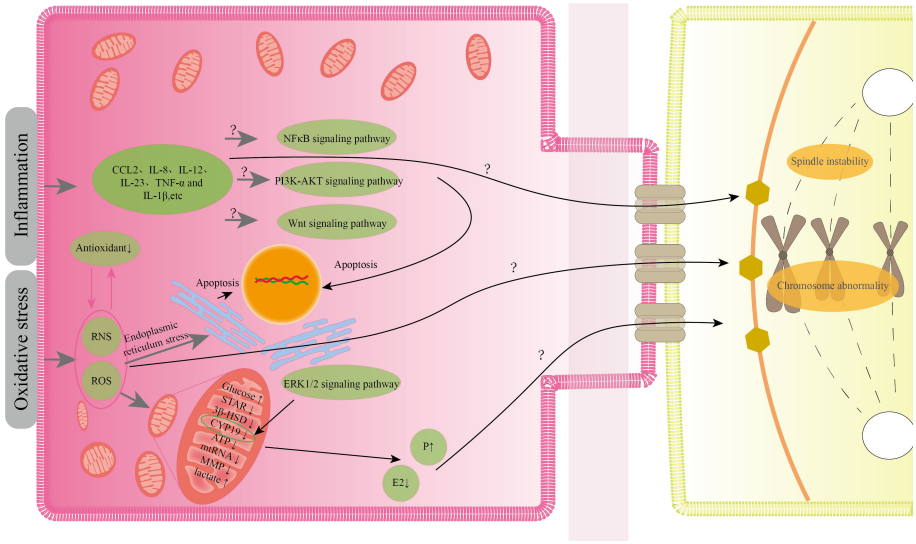


FIGURE 2
Possible ways by which the aberrant condition of granulosa cells impacts oocyte quality.

troubling that these aberrant states frequently affect each other, but the mechanism underlying the link between the multiple abnormal states of GCs generated by EMS is yet unknown. In addition, it is unclear how the aberrant status of GCs influences the oocyte by which mechanism or pathway. Although patients with EMS can achieve pregnancy using assisted reproductive technology, it is unknown whether the offspring's health would suffer as a result of poor oocyte quality. In terms of research technology, current study on the extraction of GCs from follicles leaves key questions unanswered. Because MGCs and CGCs serve different functions in follicles, most studies do not distinguish between the two when removing GCs from follicles. As a result of our summary, future research should concentrate on how to improve the abnormal condition of GCs induced by EMS and better understand the routes by which the abnormal state of GCs impacts oocyte quality. We can intervene earlier to achieve better GCs, enhance reproductive endocrinology, and so increase pregnancy rates and offspring health if we understand how EMS affects oocyte quality by altering GCs.

Author contributions

Writing—Original draft preparation: WF and ZY. Writing—review and editing, and supervision: ML and YZ. Draw diagram: WF. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by Scientific planning project of youth education in Shandong province Undergraduate Academic Project (23BSH251); The Natural Science Foundation of Shandong Province (ZR2021MH404); Ji 'nan Science and Technology Innovation Development Plan (202225006).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Horne AW, Missmer SA. Pathophysiology, diagnosis, and management of endometriosis. *BMJ* (2022) 379:e070750. doi: 10.1136/bmj-2022-070750

2. Saunders PTK, Horne AW. Endometriosis: Etiology, pathobiology, and therapeutic prospects. *Cell* (2021) 184(11):2807–24. doi: 10.1016/j.cell.2021.04.041

3. Edi R, Cheng T. Endometriosis: Evaluation and treatment. *Am Fam Physician* (2022) 106(4):397–404.

4. Muzii L, Galati G, Mattei G, Chinè A, Perniola G, Di Donato V, et al. Expectant, medical, and surgical management of ovarian endometriomas. *J Clin Med* (2023) 12 (5):1858. doi: 10.3390/jcm12051858

5. Bonavina G, Taylor HS. Endometriosis-associated infertility: From pathophysiology to tailored treatment. *Front Endocrinol (Lausanne)* (2022) 13:1020827. doi: 10.3389/fendo.2022.1020827

6. Qu H, Du Y, Yu Y, Wang M, Han T, Yan L. The effect of endometriosis on IVF/ICSI and perinatal outcome: A systematic review and meta-analysis. *J Gynecol Obstet Hum Reprod* (2022) 51(9):102446. doi: 10.1016/j.jogoh.2022.102446

7. Alshehre SM, Narice BF, Fenwick MA, Metwally M. The impact of endometrioma on in *vitro* fertilisation/intra-cytoplasmic injection IVF/ICSI reproductive outcomes: a systematic review and meta-analysis. *Arch Gynecol Obstet* (2021) 303(1):3–16. doi: 10.1007/s00404-020-05796-9

8. Kimber-Trojnar Ż, Pilszyk A, Niebrzydowska M, Pilszyk Z, Ruszała M, Leszczyńska-Gorzelak B. The potential of non-invasive biomarkers for early diagnosis of asymptomatic patients with endometriosis. *J Clin Med* (2021) 10 (13):2762. doi: 10.3390/jcm10132762

9. Eppig JJ. Reproduction: Oocytes call, granulosa cells connect. *Curr Biol* (2018) 28 (8):R354–6. doi: 10.1016/j.cub.2018.03.005

10. Wang X, Zhang M, Jiang L, Fang X, Zhang T. Exosomal AFAP1-AS1 binds to microRNA-15a-5p to promote the proliferation, migration, and invasion of ectopic endometrial stromal cells in endometriosis. *Reprod Biol Endocrinol* (2022) 20(1):77. doi: 10.1186/s12958-022-00942-1

11. Bulun SE, Yildiz S, Adli M, Chakravarti D, Parker JB, Milad M, et al. Endometriosis and adenomyosis: shared pathophysiology. *Fertil Steril* (2023) 119 (5):746–50. doi: 10.1016/j.fertnstert.2023.03.006

12. Khodarahmian M, Amidi F, Moini A, Kashani L, Salahi E, Danaii-Mehrabad S, et al. A randomized exploratory trial to assess the effects of resveratrol on VEGF and TNF-α 2 expression in endometriosis women. *J Reprod Immunol* (2021) 143:103248. doi: 10.1016/j.jri.2020.103248

13. Poirier D, Nyachieo A, ROmano A, Roy J, Maltais R, Chai D, et al. An irreversible inhibitor of 17β-hydroxysteroid dehydrogenase type 1 inhibits estradiol synthesis in human endometriosis lesions and induces regression of the non-human primate endometriosis. *J Steroid Biochem Mol Biol* (2022) 222:106136. doi: 10.1016/j.jsbmb.2022.106136

14. Minami T, Tsuzuki Y, Tanaka Y, Kitawaki J, Mori T. The Tpl2-MEK pathway plays a critical role in spheroid-cultured endometriotic stromal cells. *Am J Reprod Immunol* (2023) 89(5):e13689. doi: 10.1111/aji.13689

15. Han SJ, Jung SY, Wu SP, Hawkins SM, Park MJ, Kyo S, et al. Estrogen receptor β Modulates apoptosis complexes and the inflammasome to drive the pathogenesis of endometriosis. *Cell.* (2015) 163(4):960–74. doi: 10.1016/j.cell.2015.10.034

16. Juhasz-Böss I, Fischer C, Lattrich C, Skrzypczak M, Malik E, Ortmann O, et al. Endometrial expression of estrogen receptor β and its splice variants in patients with and without endometriosis. *Arch Gynecol Obstet* (2011) 284(4):885–91. doi: 10.1007/s00404-010-1768-7

17. Matsuzaki S, Pouly JL, Canis M. Dose-dependent pro- or anti-fibrotic responses of endometriotic stromal cells to interleukin-1β and tumor necrosis factor α. *Sci Rep* (2020) 10(1):9467. doi: 10.1038/s41598-020-66298-x

18. Zubrzycka A, Migdalska-Sęk M, Jędrzejczyk S, Brzeziańska-Lasota E. The expression of TGF-β1, SMAD3, ILK and miRNA-21 in the ectopic and eutopic endometrium of women with endometriosis. *Int J Mol Sci* (2023) 24(3):2453. doi: 10.3390/ijms24032453

19. Khan KN, Yamamoto K, Fujishita A, Muto H, Koshiba A, Kuroboshi H, et al. Differential levels of regulatory T cells and T-helper-17 cells in women with early and advanced endometriosis. *J Clin Endocrinol Metab* (2019) 104(10):4715–29. doi: 10.1210/jc.2019-00350

20. Check JH. What role does decreased ovarian reserve play in the aetiology of infertility related to endometriosis? *Hum Reprod* (2003) 18(3):653–4; author reply 654-5. doi: 10.1093/humrep/deg086

21. Dolmans MM, Martinez-Madrid B, Gadisseux E, Guiot Y, WY Y, Torre A, et al. Short-term transplantation of isolated human ovarian follicles and cortical tissue into nude mice. *Reproduction* (2007) 134(2):253–62. doi: 10.1530/REP-07-0131

22. David A, Van Langendonckt A, Gilliaux S, MM D, Donnez J, Amorim CA. Effect of cryopreservation and transplantation on the expression of kit ligand and anti-Mullerian hormone in human ovarian tissue. *Hum Reprod* (2012) 27(4):1088–95. doi: 10.1093/humrep/des013

23. Horton J, Sterrenburg M, Lane S, Maheshwari A, TC Li, Cheong Y. Reproductive, obstetric, and perinatal outcomes of women with adenomyosis and endometriosis: a systematic review and meta-analysis. *Hum Reprod Update* (2019) 25 (5):592–632. doi: 10.1093/humupd/dmz012

24. Zhou L, Wang L, Geng Q, Zhang H, Xu S, Diao L, et al. Endometriosis is associated with a lowered cumulative live birth rate: A retrospective matched cohort study including 3071 in *vitro* fertilization cycles. *J Reprod Immunol* (2022) 151:103631. doi: 10.1016/j.jri.2022.103631

25. Shebl O, Sifferlinger I, Habelsberger A, Oppelt P, RB M, Petek E, et al. Oocyte competence in in vitro fertilization and intracytoplasmic sperm injection patients suffering from endometriosis and its possible association with subsequent treatment outcome: a matched case-control study. *Acta Obstet Gynecol Scand* (2017) 96(6):736–44. doi: 10.1111/aogs.12941

26. Robin C, Uk A, Decanter C, Behal H, Collinet P, Rubod C, et al. Impact of endometriosis on oocyte morphology in IVF-ICSI: retrospective study of a cohort of more than 6000 mature oocytes. *Reprod Biol Endocrinol* (2021) 19(1):160. doi: 10.1186/s12958-021-00798-x

27. Kasapoglu I, Kuspinar G, Saribal S, Turk P, Avcı B, Uncu G. Detrimental effects of endometriosis on oocyte morphology in intracytoplasmic sperm injection cycles: a retrospective cohort study. *Gynecol Endocrinol* (2018) 34(3):206–11. doi: 10.1080/09513590.2017.1391203

28. Nada AM, El-Noury A, Al-Inany H, Bibars M, Taha T, Salama S, et al. Effect of laser-assisted zona thinning, during assisted reproduction, on pregnancy outcome in women with endometriosis: randomized controlled trial. *Arch Gynecol Obstet* (2018) 297(2):521–8. doi: 10.1007/s00404-017-4604-5

29. El-Hayek S, Yang Q, Abbassi L, FitzHarris G, Clarke HJ. MamMalian oocytes locally remodel follicular architecture to provide the foundation for germline-soma communication. *Curr Biol* (2018) 28(7):1124–1131.e3. doi: 10.1016/j.cub.2018.02.039

30. Anderson E, Wilkinson RF, Lee G, Meller S. A correlative microscopical analysis of differentiating ovarian follicles of mammals. *J Morphol* (1978) 156(3):339–66. doi: 10.1002/jmor.1051560303

31. Grieco D, Porcellini A, Avvedimento EV, Gottesman ME. Requirement for cAMP-PKA pathway activation by M phase-promoting factor in the transition from mitosis to interphase. *Science.* (1996) 271(5256):1718–23. doi: 10.1126/science.271.5256.1718

32. Clarke H. Control of mamMalian oocyte development by interactions with the maternal follicular environment. *Results Probl Cell Differ* (2017) 63:17–41. doi: 10.1007/978-3-319-60855-6_2

33. Kawamura K, Cheng Y, Kawamura N, Takae S, Okada A, Kawagoe Y, et al. Pre-ovulatory LH/hCG surge decreases C-type natriuretic peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes. *Hum Reprod* (2011) 26(11):3094–101. doi: 10.1093/humrep/der282

34. Okudaira Y, Wakai T, Funahashi H. Levels of cyclic-AMP and cyclic-GMP in porcine oocyte-cumulus complexes and cumulus-free oocytes derived from small and middle follicles during the first 24-hour period of in *vitro* maturation. *J Reprod Dev* (2017) 63(2):191–7. doi: 10.1262/jrd.2016-156

35. Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ. Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction* (2001) 121(5):647–53. doi: 10.1530/rep.0.1210647

36. Mattioli M, Barboni B. Signal transduction mechanism for LH in the cumulus-oocyte complex. *Mol Cell Endocrinol* (2000) 161(1-2):19–23. doi: 10.1016/s0303-7207(99)00218-x

37. Wang Y, Kong N, Li N, Hao X, Wei K, Xiang X, et al. Epidermal growth factor receptor signaling-dependent calcium elevation in cumulus cells is required for NPR2 inhibition and meiotic resumption in mouse oocytes. *Endocrinology* (2013) 154 (9):3401–9. doi: 10.1210/en.2013-1133

38. Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev* (2002) 61(3):414–24. doi: 10.1002/mrd.10102

39. Harris SE, Leese HJ, Gosden RG, Picton HM. Pyruvate and oxygen consumption throughout the growth and development of murine oocytes. *Mol Reprod Dev* (2009) 76 (3):231–8. doi: 10.1002/mrd.20945

40. Kaneko T, Iuchi Y, Takahashi M, Fujii J. Colocalization of polyol-metabolizing enzymes and immunological detection of fructated proteins in the female reproductive system of the rat. *Histochem Cell Biol* (2003) 119(4):309–15. doi: 10.1007/s00418-003-0516-5

41. Richani D, Constance K, Lien S, Agapiou D, Stocker WA, Hedger MP, et al. Cumulin and FSH cooperate to regulate inhibin B and activin B production by human granulosa-lutein cells in vitro. *Endocrinology* (2019) 160(4):853–62. doi: 10.1210/en.2018-01026

42. Liu H, Zhou D, Liu C, Zhuan Q, Luo Y, Mo X, et al. The Calcium-Sensing Receptor Is Involved in Follicle-Stimulating Hormone-Induced Cumulus Expansion in *in vitro* Cultured Porcine Cumulus-Oocyte Complexes. *Front Cell Dev Biol* (2021) 9:625036. doi: 10.3389/fcell.2021.625036

43. Mohan Jeena L, Kumar D, Rahangdale S, Pratap Singh A, Chandra Sarkhel B. Transcriptional profile of cumulus associated GJA1, PTX3, PRSS35, and SERPINE2 genes with oocytes and embryonic development in water buffalo. *Mol Biol Rep* (2022) 49(7):6285–93. doi: 10.1007/s11033-022-07435-9

44. Santiquet NW, Greene AF, Becker J, Barfield JP, Schoolcraft WB, Krisher RL. A pre-*in vitro* maturation medium containing cumulus oocyte complex ligand-receptor signaling molecules maintains meiotic arrest, supports the cumulus oocyte complex and improves oocyte developmental competence. *Mol Hum Reprod* (2017) 23(9):594–606. doi: 10.1093/molehr/gax032

45. Ploutarchou P, Melo P, Day AJ, Milner CM, Williams SA. Molecular analysis of the cumulus matrix: insights from mice with O-glycan-deficient oocytes. *Reproduction* (2015) 149(5):533–43. doi: 10.1530/REP-14-0503

46. Shimada M, Terada T. FSH and LH induce progesterone production and progesterone receptor synthesis in cumulus cells: a requirement for meiotic resumption in porcine oocytes. *Mol Hum Reprod* (2002) 8(7):612–8. doi: 10.1093/molehr/8.7.612

47. Abbassi L, El-Hayek S, Carvalho KF, Wang W, Yang Q, Granados-Aparici S, et al. Epidermal growth factor receptor signaling uncouples germ cells from the somatic follicular compartment at ovulation. *Nat Commun* (2021) 12(1):1438. doi: 10.1038/s41467-021-21644-z

48. Demirel LC, Cengiz B, Unlü C. Severe endometriosis and apoptotic granulosa cells. *Fertil Steril* (2001) 75(3):642. doi: 10.1016/s0015-0282(00)01771-4

49. Toya M, Saito H, Ohta N, Saito T, Kaneko T, Hiroi M. Moderate and severe endometriosis is associated with alterations in the cell cycle of granulosa cells in patients undergoing in *vitro* fertilization and embryo transfer. *Fertil Steril* (2000) 73 (2):344–50. doi: 10.1016/s0015-0282(99)00507-5

50. Sanchez AM, Somigliana E, Vercellini P, Pagliardini L, Candiani M, Vigano P. Endometriosis as a detrimental condition for granulosa cell steroidogenesis and development: From molecular alterations to clinical impact. *J Steroid Biochem Mol Biol* (2016) 155(Pt A):35–46. doi: 10.1016/j.jsbmb.2015.07.023

51. Nakahara K, Saito H, Saito T, Ito M, Ohta N, Takahashi T, et al. Ovarian fecundity in patients with endometriosis can be estimated by the incidence of apoptotic bodies. *Fertil Steril* (1998) 69(5):931–5. doi: 10.1016/s0015-0282(98)00038-7

52. Huang X, Yu Q. Bioinformatic analysis confirms differences in circular RNA expression profiles of cumulus cells between patients with ovarian and peritoneal endometriosis-associated infertility. *Front Endocrinol (Lausanne)* (2023) 14:1137235. doi: 10.3389/fendo.2023.1137235

53. Takeuchi A, Koga K, Satake E, Makabe T, Taguchi A, Miyashita M, et al. Endometriosis triggers excessive activation of primordial follicles via PI3K-PTEN-akt-foxo3 pathway. *J Clin Endocrinol Metab* (2019) 104(11):5547–54. doi: 10.1210/jc.2019-00281

54. Shi L, Wei X, Wu B, Yuan C, Li C, Dai Y, et al. Molecular Signatures Correlated With Poor IVF Outcomes: Insights From the mRNA and lncRNA Expression of Endometriotic Granulosa Cells. *Front Endocrinol (Lausanne)* (2022) 13:825934. doi: 10.3389/fendo.2022.825934

55. Veeman MT, Axelrod JD, Moon RT. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev Cell* (2003) 5(3):367–77. doi: 10.1016/s1534-5807(03)00266-1

56. Yland J, Carvalho LFP, Beste M, Bailey A, Thomas C, Abrão MS, et al. Endometrioma, the follicular fluid inflammatory network and its association with oocyte and embryo characteristics. *Reprod BioMed Online* (2020) 40(3):399–408. doi: 10.1016/j.rbmo.2019.12.005

57. Zhang H, Yang K, Chen F, Liu Q, Ni J, Cao W, et al. Role of the CCL2-CCR2 axis in cardiovascular disease: Pathogenesis and clinical implications. *Front Immunol* (2022) 13:975367. doi: 10.3389/fimmu.2022.975367

58. Vilotić A, Nacka-Aleksić M, Pirković A, Bojić-Trbojević Ž, Dekanski D, Jovanović Krivokuća M. IL-6 and IL-8: An overview of their roles in healthy and pathological pregnancies. *Int J Mol Sci* (2022) 23(23):14574. doi: 10.3390/ijms232314574

59. Zhang QF, Chen GY, Liu Y, Huang HJ, Song YF. Relationship between resistin and IL-23 levels in follicular fluid in infertile patients with endometriosis undergoing IVF-ET. *Adv Clin Exp Med* (2017) 26(9):1431–5. doi: 10.17219/acem/41149

60. Parham C, Chirica M, Timans J, Vaisberg E, Travis M, Cheung J, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. *J Immunol* (2002) 168(11):5699–708. doi: 10.4049/jimmunol.168.11.5699

61. Carlberg M, Nejaty J, Fröysa B, Guan Y, Söder O, Bergqvist A. Elevated expression of tumour necrosis factor alpha in cultured granulosa cells from women with endometriosis. *Hum Reprod* (2000) 15(6):1250–5. doi: 10.1093/humrep/15.6.1250

62. Li Y, Li R, Ouyang N, Dai K, Yuan P, Zheng L, et al. Investigating the impact of local inflammation on granulosa cells and follicular development in women with ovarian endometriosis. *Fertil Steril* (2019) 112(5):882–891.e1. doi: 10.1016/j.fertnstert.2019.07.007

63. Yamagata Y, Nakamura Y, Umayahara K, Harada A, Takayama H, Sugino N, et al. Changes in telomerase activity in experimentally induced atretic follicles of immature rats. *Endocr J* (2002) 49(6):589–95. doi: 10.1507/endocrj.49.589

64. Liang Y, Yang X, Lan Y, Lei L, Li Y, Wang S. Effect of Endometrioma cystectomy on cytokines of follicular fluid and IVF outcomes. *J Ovarian Res* (2019) 12(1):98. doi: 10.1186/s13048-019-0572-7

65. Rochette L, Lorin J, Zeller M, Guilland JC, Lorgis L, Cottin Y, et al. Nitric oxide synthase inhibition and oxidative stress in cardiovascular diseases: possible therapeutic targets? *Pharmacol Ther* (2013) 140(3):239–57. doi: 10.1016/j.pharmthera.2013.07.004

66. Lugrin J, Rosenblatt-Velin N, Parapanov R, Liaudet L. The role of oxidative stress during inflammatory processes. *Biol Chem* (2014) 395(2):203–30. doi: 10.1515/hsz-2013-0241

67. Al-Harbi NO, Nadeem A, Al-Harbi MM, Imam F, Al-Shabanah OA, Ahmad SF, et al. Oxidative airway inflammation leads to systemic and vascular oxidative stress in a murine model of allergic asthma. *Int Immunopharmacol* (2015) 26(1):237–45. doi: 10.1016/j.intimp.2015.03.032

68. Sun J, Jiao Z, Zhu W, Li X, Wang P, Wang J, et al. Astilbin attenuates cadmium-induced adipose tissue damage by inhibiting NF-κB pathways and regulating the expression of HSPs in chicken. *Biol Trace Elem Res* (2023) 201(5):2512–23. doi: 10.1007/s12011-022-03327-y

69. Matsuzaki S, Schubert B. Oxidative stress status in normal ovarian cortex surrounding ovarian endometriosis. *Fertil Steril* (2010) 93(7):2431–2. doi: 10.1016/j.fertnstert.2009.08.068

70. Da Broi MG, de Albuquerque FO, de Andrade AZ, Cardoso RL, Jordão Junior AA, Navarro PA. Increased concentration of 8-hydroxy-2'-deoxyguanosine in follicular fluid of infertile women with endometriosis. *Cell Tissue Res* (2016) 366(1):231–42. doi: 10.1007/s00441-016-2428-4

71. Goud PT, Goud AP, Joshi N, Puscheck E, Diamond MP, Abu-Soud HM. Dynamics of nitric oxide, altered follicular microenvironment, and oocyte quality in women with endometriosis. *Fertil Steril* (2014) 102(1):151–159.e5. doi: 10.1016/j.fertnstert.2014.03.053

72. Scutiero G, Iannone P, Bernardi G, Bonaccorsi G, Spadaro S, Volta CA, et al. Oxidative stress and endometriosis: A systematic review of the literature. *Oxid Med Cell Longev* (2017) 2017:7265238. doi: 10.1155/2017/7265238

73. Lin X, Dai Y, Tong X, Xu W, Huang Q, Jin X, et al. Excessive oxidative stress in cumulus granulosa cells induced cell senescence contributes to endometriosis-associated infertility. *Redox Biol* (2020) 30:101431. doi: 10.1016/j.redox.2020.101431

74. Kunitomi C, Harada M, Takahashi N, Azhary JMK, Kusamoto A, Nose E, et al. Activation of endoplasmic reticulum stress mediates oxidative stress-induced apoptosis of granulosa cells in ovaries affected by endometrioma. *Mol Hum Reprod* (2020) 26 (1):40–52. doi: 10.1093/molehr/gaz066

75. Ren Z, Huang J, Zhou C, Jia L, Li M, Liang X, et al. Transferrin and antioxidants partly prevented mouse oocyte oxidative damage induced by exposure of cumulus-oocyte complexes to endometrioma fluid. *J Ovarian Res* (2020) 13(1):139. doi: 10.1186/s13048-020-00738-0

76. Donabela FC, Meola J, Padovan CC, de Paz CC, Navarro PA. Higher SOD1 gene expression in cumulus cells from infertile women with moderate and severe endometriosis. *Reprod Sci* (2015) 22(11):1452–60. doi: 10.1177/1933719115585146

77. Lin X, Tong X, Zhang Y, Gu W, Huang Q, Zhang Y, et al. Decreased expression of EZH2 in granulosa cells contributes to endometriosis-associated infertility by targeting IL-1R2. *Endocrinology* (2022) 164(2):bqac210. doi: 10.1210/endocr/bqac210

78. González-Fernández R, Peña Ó, Hernández J, Martín-Vasallo P, Palumbo A, Ávila J. Patients with endometriosis and patients with poor ovarian reserve have abnormal follicle-stimulating hormone receptor signaling pathways. *Fertil Steril* (2011) 95(7):2373–8. doi: 10.1016/j.fertnstert.2011.03.030

79. Wang J, Shen XX, Huang XH, Zhao ZM. Follicular fluid levels of prostaglandin E2 and the effect of prostaglandin E2 on steroidogenesis in granulosa-lutein cells in women with moderate and severe endometriosis undergoing in *vitro* fertilization and embryo transfer. *Chin Med J (Engl)* (2012) 125(22):3985–90.

80. Sreerangaraja Urs DB, Wu WH, Komrskova K, Postlerova P, Lin YF, Tzeng CR, et al. Mitochondrial function in modulating human granulosa cell steroidogenesis and female fertility. *Int J Mol Sci* (2020) 21(10):3592. doi: 10.3390/ijms21103592

81. Wunder DM, Mueller MD, Birkhäuser MH, Bersinger NA. Steroids and protein markers in the follicular fluid as indicators of oocyte quality in patients with and without endometriosis. *J Assist Reprod Genet* (2005) 22(6):257–64. doi: 10.1007/s10815-005-5149-2

82. Garrido N, Navarro J, Remohí J, Simón C, Pellicer A. Follicular hormonal environment and embryo quality in women with endometriosis. *Hum Reprod Update* (2000) 6(1):67–74. doi: 10.1093/humupd/6.1.67

83. Pellicer A, Valbuena D, Bauset C, Albert C, Bonilla-Musoles F, Remohí J, et al. The follicular endocrine environment in stimulated cycles of women with endometriosis: steroid levels and embryo quality. *Fertil Steril* (1998) 69(6):1135–41. doi: 10.1016/s0015-0282(98)00085-5

84. Garrido N, Krüssel JS, Remohí J, Simón C, Pellicer A. Expression and function of 3beta hydroxisteroid dehydrogenase (3beta HSD) type II and corticosteroid binding globulin (CBG) in granulosa cells from ovaries of women with and without endometriosis. *J Assist Reprod Genet* (2002) 19(1):24–30. doi: 10.1023/a:1014058622697

85. Ding Y, Zhu Q, He Y, Lu Y, Wang Y, Qi J, et al. Induction of autophagy by Beclin-1 in granulosa cells contributes to follicular progesterone elevation in ovarian endometriosis. *Transl Res* (2021) 227:15–29. doi: 10.1016/j.trsl.2020.06.013

86. Lu X, Wu Y, Gao XH, Wang YW, Wang L, Sun XX. Effect of letrozole on estradiol production and P450 aromatase messenger RNA expression of cultured luteinized granulosa cells from women with and without endometriosis. *Fertil Steril* (2012) 98(1):131–5. doi: 10.1016/j.fertnstert.2012.03.055

87. Dokras A, Habana A, Giraldo J, Jones E. Secretion of inhibin B during ovarian stimulation is decreased in infertile women with endometriosis. *Fertil Steril* (2000) 74 (1):35–40. doi: 10.1016/s0015-0282(00)00568-9

88. Miyoshi T, Otsuka F, Inagaki K, Otani H, Takeda M, Suzuki J, et al. Differential regulation of steroidogenesis by bone morphogenetic proteins in granulosa cells: involvement of extracellularly regulated kinase signaling and oocyte actions in follicle-stimulating hormone-induced estrogen production. *Endocrinology* (2007) 148 (1):337–45. doi: 10.1210/en.2006-0966

89. Deura I, Harada T, Taniguchi F, Iwabe T, Izawa M, Terakawa N. Reduction of estrogen production by interleukin-6 in a human granulosa tumor cell line may have implications for endometriosis-associated infertility. *Fertil Steril* (2005) 83 Suppl 1:1086–92. doi: 10.1016/j.fertnstert.2004.12.014

90. Li Y, Liu YD, Chen SL, Chen X, Ye DS, Zhou XY, et al. Down-regulation of long non-coding RNA MALAT1 inhibits granulosa cell proliferation in endometriosis by up-regulating P21 via activation of the ERK/MAPK pathway. *Mol Hum Reprod* (2019) 25(1):17–29. doi: 10.1093/molehr/gay045

91. Cecchino GN, Seli E, Alves da Motta EL, García-Velasco JA. The role of mitochondrial activity in female fertility and assisted reproductive technologies: overview and current insights. *Reprod BioMed Online* (2018) 36(6):686–97. doi: 10.1016/j.rbmo.2018.02.007

92. Xu B, Guo N, Zhang XM, Shi W, Tong XH, Iqbal F, et al. Oocyte quality is decreased in women with minimal or mild endometriosis. *Sci Rep* (2015) 5:10779. doi: 10.1038/srep10779

93. Yamashita Y, Asano M, Morishima S, Fujino K, Terai Y, Ohmichi M. Mitochondrial gene expression in granulosa cells of severe endometriosis with in *vitro* fertilization and embryo transfer. *Fertil Steril* (2007) 88(6):1703–5. doi: 10.1016/j.fertnstert.2007.01.111

94. Liu Y, Han M, Li X, Wang H, Ma M, Zhang S, et al. Age-related changes in the mitochondria of human mural granulosa cells. *Hum Reprod* (2017) 32(12):2465–73. doi: 10.1093/humrep/dex309

95. Mao J, Zhang J, Cai L, Cui Y, Liu J, Mao Y. Elevated prohibitin 1 expression mitigates glucose metabolism defects in granulosa cells of infertile patients with endometriosis. *Mol Hum Reprod* (2022) 28(6):gaac018. doi: 10.1093/molehr/gaac018

96. González-Fernández R, Martín-Ramírez R, Rotoli D, Hernández J, Naftolin F, Martín-Vasallo P, et al. Granulosa-lutein cell sirtuin gene expression profiles differ between normal donors and infertile women. *Int J Mol Sci* (2019) 21(1):295. doi: 10.3390/ijms21010295

97. Wyse BA, Fuchs Weizman N, Kadish S, Balakier H, Sangaralingam M, Librach CL. Transcriptomics of cumulus cells - a window into oocyte maturation in humans. *J Ovarian Res* (2020) 13(1):93. doi: 10.1186/s13048-020-00696-7

98. Sasaki H, Hamatani T, Kamijo S, Iwai M, Kobanawa M, Ogawa S, et al. Impact of oxidative stress on age-associated decline in oocyte developmental competence. *Front Endocrinol (Lausanne)* (2019) 10:811. doi: 10.3389/fendo.2019.00811

99. Banerjee J, Shaeib F, Maitra D, Saed GM, Dai J, Diamond MP, et al. Peroxynitrite affects the cumulus cell defense of metaphase II mouse oocytes leading to disruption of the spindle structure *in vitro*. *Fertil Steril* (2013) 100(2):578–84.e1. doi: 10.1016/j.fertnstert.2013.04.030

100. Yang Q, Cong L, Wang Y, Luo X, Li H, Wang H, et al. Increasing ovarian NAD + levels improve mitochondrial functions and reverse ovarian aging. *Free Radic Biol Med* (2020) 156:1–10. doi: 10.1016/j.freeradbiomed.2020.05.003

101. Song ZQ, Li X, Wang YK, Du ZQ, Yang CX. DMBA acts on cumulus cells to desynchronize nuclear and cytoplasmic maturation of pig oocytes. *Sci Rep* (2017) 7 (1):1687. doi: 10.1038/s41598-017-01870-6

102. Smith DM, Tenney DY. Effects of steroids on mouse oocyte maturation in vitro. *J Reprod Fertil* (1980) 60(2):331–8. doi: 10.1530/jrf.0.0600331

103. Sakaguchi K, Huang W, Yang Y, Yanagawa Y, Nagano M. Relationship between in vitro growth of bovine oocytes and steroidogenesis of granulosa cells cultured in medium supplemented with bone morphogenetic protein-4 and follicle stimulating hormone. *Theriogenology* (2017) 97:113–23. doi: 10.1016/j.theriogenology.2017.04.030

104. Bromfield JJ, Sheldon IM. Lipopolysaccharide initiates inflammation in bovine granulosa cells via the TLR4 pathway and perturbs oocyte meiotic progression in vitro. *Endocrinology* (2011) 152(12):5029–40. doi: 10.1210/en.2011-1124

105. Corachán A, Pellicer N, Pellicer A, Ferrero H. Novel therapeutic targets to improve IVF outcomes in endometriosis patients: a review and future prospects. *Hum Reprod Update* (2021) 27(5):923–72. doi: 10.1093/humupd/dmab014

106. Kong QQ, Wang J, Xiao B, FH L, Zhu J, GY S, et al. Cumulus cell-released tumor necrosis factor (TNF)-α promotes post-ovulatory aging of mouse oocytes. *Aging (Albany NY)* (2018) 10(7):1745–57. doi: 10.18632/aging.101507

107. Au HK, Lin SH, Huang SY, Yeh TS, Tzeng CR, Hsieh RH. Deleted mitochondrial DNA in human luteinized granulosa cells. *Ann N Y Acad Sci* (2005) 1042:136–41. doi: 10.1196/annals.1338.014

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 18 August 2023
DOI 10.3389/fimmu.2023.1130738

OPEN ACCESS

EDITED BY
Iveta Yotova,
Medical University of Vienna, Austria

REVIEWED BY
Yogesh Singh,
University of Tübingen, Germany
Payel Roy,
La Jolla Institute for Immunology (LJI), United States
Hao Wang,
Shenzhen University, China

*CORRESPONDENCE
Jing Sun
sunjing61867@tongji.edu.cn

†These authors have contributed equally to this work

RECEIVED 30 December 2022
ACCEPTED 27 July 2023
PUBLISHED 18 August 2023

CITATION
Lv S-j, Sun J-n, Gan L and Sun J (2023) Identification of molecular subtypes and immune infiltration in endometriosis: a novel bioinformatics analysis and *In vitro* validation. *Front. Immunol.* 14:1130738. doi: 10.3389/fimmu.2023.1130738

Identification of molecular subtypes and immune infiltration in endometriosis: a novel bioinformatics analysis and *In vitro* validation

Si-ji Lv[1†], Jia-ni Sun[1†], Lei Gan[2] and Jing Sun[1*]

[1]Shanghai Key Laboratory of Maternal Fetal Medicine, Shanghai Institute of Maternal-Fetal Medicine and Gynecologic Oncology, Shanghai First Maternity and Infant Hospital, School of Medicine, Tongji University, Shanghai, China, [2]Department of Gynaecology and Obstetrics, Ningbo First Hospital, Ningbo, Zhejiang, China

Introduction: Endometriosis is a worldwide gynacological diseases, affecting in 6–10% of women of reproductive age. The aim of this study was to investigate the gene network and potential signatures of immune infiltration in endometriosis.

Methods: The expression profiles of GSE51981, GSE6364, and GSE7305 were obtained from the Gene Expression Omnibus (GEO) database. Core modules and central genes related to immune characteristics were identified using a weighted gene coexpression network analysis. Bioinformatics analysis was performed to identify central genes in immune infiltration. Protein-protein interaction (PPI) network was used to identify the hub genes. We then constructed subtypes of endometriosis samples and calculated their correlation with hub genes. qRTPCR and Western blotting were used to verify our findings.

Results: We identified 10 candidate hub genes (GZMB, PRF1, KIR2DL1, KIR2DL3, KIR3DL1, KIR2DL4, FGB, IGFBP1, RBP4, and PROK1) that were significantly correlated with immune infiltration. Our study established a detailed immune network and systematically elucidated the molecular mechanism underlying endometriosis from the aspect of immune infiltration.

Discussion: Our study provides comprehensive insights into the immunology involved in endometriosis and might contribute to the development of immunotherapy for endometriosis. Furthermore, our study sheds light on the underlying molecular mechanism of endometriosis and might help improve the diagnosis and treatment of this condition.

KEYWORDS

endometriosis, WGCNA, signature, immune infiltration, immune cell subset, molecular subtype

1 Introduction

Endometriosis is a chronic gynecological disorder characterized by the presence and infiltration of ectopic endometrial tissue (1, 2). It affects nearly 5% to 10% of women of reproductive age and could cause pelvic pain (50% to 80%) and infertility (up to 50%) (3–6). Other common symptoms include dysmenorrhea, dyspareunia, dysuria, dyschezia, and fatigue (2, 4). Although various biomarkers such as IL-2, anti-PEP, CA125, and miR-150-5p have been studied as clinical diagnostic tools, there is generally no single indicator that can directly diagnose endometriosis (7). Surgery remains the gold standard for diagnosis (8), but it has complications, and early lesions might be missed during operations (9). Therefore, a thorough understanding of the molecular mechanisms underlying endometriosis and exploration of a non-invasive diagnostic indicator with high specificity is essential.

Sampson's retrograde stigma theory is the most widely accepted theory on the pathogenesis of endometriosis (6). However, endometriosis is now recognized as a systemic disease that can spread through lymphatic and hematogenous metastases (10), not just limited to pelvic lesions (6). In addition, recent research suggests that endometriosis is closely related to inflammation and autoimmunity (11, 12).

Nowadays, the bioinformatics approach is widely used to reveal the molecular mechanisms associated with endometriosis. The underlying pathogenic factors are variable and could be related to the lesion microenvironment, individual differences, and environmental factors (4). As mentioned earlier, endometriosis is closely related to immunity, and some studies have revealed a part of the mechanisms. For instance, Wu et al. (13) reported that an increase in $CD8^+$ T cells and a decrease in $CD163^+$ macrophages might create a pro-inflammatory endometrial immune environment, leading to endometriosis. Zhong et al. (14) found that M2 macrophages significantly increase during the progression of endometriosis. In a previous study, it was discovered that PDLIM3, a specific biomarker in endometriosis, was correlated with multiple immune cells, such as M2 macrophages, activated NK cells, and $CD8^+$ T cells (15). However, many unknowns still exist regarding the immune mechanisms of endometriosis.

In our study, we downloaded three endometriosis datasets (GSE51981, GSE6364, and GSE7305) from the GEO database to evaluate immune cell infiltration and identify immune correlation with endometriosis for further analysis. We identified endometriosis subtypes, explored differentially expressed genes (DEGs), and constructed a co-expression network. Central genes were identified, and subsequently, hub genes were comprehensively analyzed, with a particular focus on their immunological characteristics. Our study established a detailed immune network and aimed to systematically clarify the underlying molecular mechanisms of endometriosis, particularly from the perspective of immune infiltration. The results of our study could help facilitate the development of immune therapeutic approaches for endometriosis.

2 Materials and methods

2.1 Data collection and preprocessing

To identify DEGs, the expression profiles of GSE51981, GSE6364, and GSE7305 were obtained from the GEO database using the GPL570[HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. We used the GEOquery (16) package of R software with the keyword "endometriosis". GSE5198 included 77 endometriosis samples, GSE6364 included 21 endometriosis samples, and GSE7305 included 10 endometriosis samples. We selected and combined the endometriosis samples from the three datasets, which underwent removal of batch effects, standardization and annotation of probes, and other data cleaning processes. We used the "removeBatchEffect" function to remove batch effects using the "limma" R package and employed "normalizeBetweenArrays" function to standardize the data. The overall analytical flow diagram is shown in Figure 1.

2.2 Evaluation and analysis of immune cell infiltration and correlation between immune cells

We used the CIBERSORT algorithm (17) to convert the normalized gene expression matrix into a matrix of 22 types of immune cells. We uploaded the gene expression matrix data to CIBERSORT and filtered out samples with $P < 0.05$ to obtain the immune cell infiltration matrix. A total of 108 samples were uploaded, and 32 samples were filtered out based on the criteria of $P < 0.05$. The Ggplot2 (18) package was used to draw bar charts showing the distribution of the 22 immune cell infiltrates in each sample. The Corrplot package was used to plot the correlation heatmap to visualize the correlations of these 22 immune cell infiltrates. The Ggplot2 package was used to visualize the expression of immune-related HLA family and KIR family genes.

2.3 Gene set variation analysis

GSVA (19) is a non-parametric, unsupervised analysis that calculates enrichment scores for specific sets of genes in each sample. GSVA contributes to the construction of pathway-central models of biology and meets the need for bioinformatic methods for RNA-seq data (20). In our study, we used the expression profile data of endometriosis patients based on the c2.cp.v7.4.symbols.gmt dataset to analyze the corresponding biological characteristics and observe changes in immune-related pathways to explore the underlying mechanism of pathogenesis in endometriosis. The c2.cp.v7.4.gm geneset is a collection of gene sets in the Molecular Signatures Database (MSigDB) version 7.4. It contains co-expression-based gene sets that capture potential functional relationships among genes across diverse tissues or cell types (https://docs.gsea-msigdb.org/#MSigDB/Release_Notes/MSigDB_7.4/#_top).

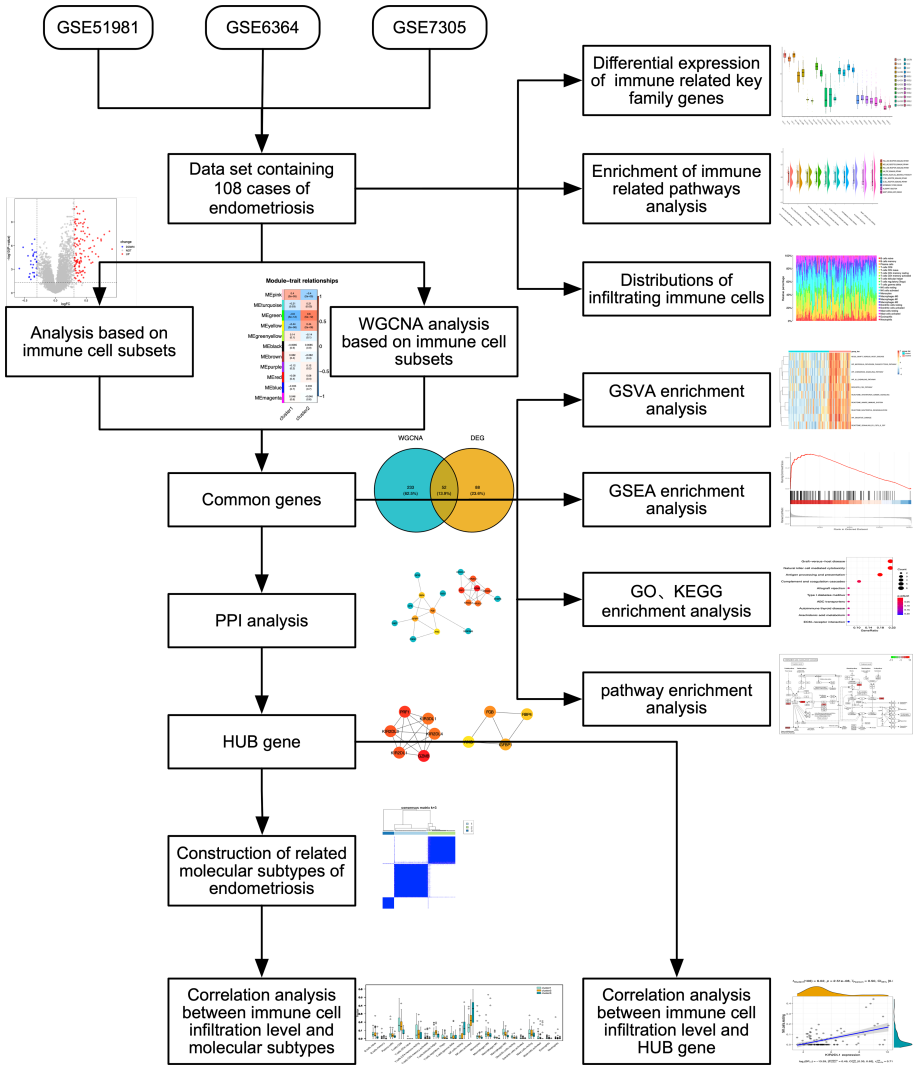


FIGURE 1
Flow diagram of methodologies applied to explore the biological characteristics of endometriosis.

2.4 Construction and analysis of related molecular subtypes

Consensus clustering involves the identification of molecularly related subtypes using the Consensus Cluster Plus algorithm (21). The consensus matrix is determined by consensus clustering to classify the samples, with the k value of the cluster number set between 2 and 9. When the cumulative distribution function index reaches the approximate maximum value, the optimal K value is determined. The classification is then validated by principal component analysis (PCA) of mRNA expression profiles.

The construction of immune cell-related subtypes was based on the consensus clustering of immune cell infiltration data. The results were used to define Cluster 1 and Cluster 2. DEGs were identified by limma (22) with the criteria of P-value < 0.05 and |log2FC| > 0.3. We used the ggplot2 package to draw a volcano plot and a heatmap of the DEGs to visualize their expression.

2.5 Weighted gene co-expression network based on immune cell subsets

We used WGCNA based on immune cell subsets (23) to process data. We used the c2.cp.v7.4.symbols.gmt dataset, which includes immune-related pathways and genes selected from the dataset obtained through GSVA. First, the soft threshold value of network construction was selected, and the adjacency matrix was a continuous value between 0 and 1, ensuring that the constructed network followed a power-law distribution and was closer to the real biological network state. Second, a scale-free network was constructed using the function of block modules, and the co-expression modules were identified by block partitioning analysis, grouping genes with similar expression patterns. All modules were summarized by modular characteristic genes, which were the most important major components of each module and were defined as synthetic genes representing the expression profile of all genes in a given module.

2.6 Functional enrichment analysis

The ClusterProfiler package (24) was used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the DEGs, and the common genes were analyzed using WGCNA. The critical value of false discovery rate < 0.05 was considered statistically significant. The GO and KEGG results were visualized using the pathview package (25), and the first two KEGG enrichment results were evaluated.

To investigate the differences in biological processes among different subgroups, we used gene set enrichment analysis (GSEA) based on the gene expression profile dataset of patients with endometriosis. The C2.cp.v7.4.symbols.gmt gene set was downloaded from MSIGDB for GSEA, and a P-value < 0.05 was considered statistically significant.

2.7 PPI network construction

We used the STRING database (26) (http://string-db.org, version 11.09) online tool to obtain PPI data. Candidate targets were input into STRING, and genes with scores greater than 0.3 were selected to construct a network model using Cytoscape (V3.7.2) (27). Cytohubba plug-in (28) was used to screen the top 10 hub genes based on the score. Pearson correlation analysis was conducted between the hub genes and immune cells, and both cor > 0.55 and > 0.7 were tried. An interaction score of 0.55 and P < 0.05 was finally set as the significance criteria for further analysis. The correlation between hub genes and immune checkpoint genes was assessed as previously using corrgram (29).

2.8 Construction of miRNA-mRNA interaction network

The MultiMiR (30) package was used to construct the interaction between mRNA and miRNA. We obtained data from the miRTarBase database (http://mirtarbase.mbc.nctu.edu.tw/php/index.php) and TarBase V.8 database (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8%2Findex). The "Functional MTI" and "positive" results were selected, and Cytoscape (V3.7.2) was used to construct the miRNA-mRNA network.

2.9 Quantitative real-time PCR

Total RNA was isolated from 6 ovarian endometriosis tissue samples and 9 eutopic endometrial tissue samples from endometriosis-free women (normal control, NC) using Trizol (RNAiso Plus; Takara, Japan) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara) according to the manufacturer's protocol. Amplification was performed on a quantitative real-time (qRT)-PCR device (QuantStudio5; Thermo Fisher Scientific, Waltham, MA, USA) using TB Green® Premix EX TaqTM II (Takara) and gene-specific primers (Sangon, Shanghai, China). β-Actin was used as the internal control. The relative expression levels of the genes were calculated using the $2^{-\Delta\Delta CT}$ method.

2.10 Protein extraction and western blotting

Ovarian endometriosis tissues and normal eutopic endometrium tissues were lysed in RIPA buffer containing protease and phosphatase inhibitor cocktail. After centrifugation at 12,000 rpm and 4°C for 15 min, the protein concentration in the supernatant was determined using a BCA protein assay. Equal amounts of total protein (30 μg) were loaded and separated on 12.5% SDS-PAGE and then transferred to PVDF membranes. After blocking with protein-free rapid blocking buffer for 0.5 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C. Rabbit anti-RBP4 antibody (1:5000; Abcam) and rabbit anti-β-actin (1:1000, cell signaling technology) were used as primary antibodies, with β-actin serving as the internal loading control. Then, the membranes were then incubated with secondary antibodies (1:3000) for 1 h at room temperature. Excess secondary antibody was removed by three washes in TBST. The targeted protein bands were visualized and imaged using an ECL Western blotting kit (NCM Biotech, China).

2.11 Statistical analysis

All data processing and analysis were conducted using R software (version 4.1.0). The statistical significance of the normally distributed continuous variables was estimated using the independent Student t test, while the differences between non-normally distributed continuous variables were analyzed using the Mann-Whitney U test (Wilcoxon rank-sum test). The Chi-square test or Fisher's exact test was used to compare and analyze the statistical significance between categorical variables in two groups. All statistical P values were bilateral, and P < 0.05 was considered statistically significant.

3 Results

3.1 Evaluation of immune cells infiltration and immune correlation analysis in endometriosis

To evaluate the extent of immune infiltration in patients with endometriosis, we used the CIBERSORT algorithm to analyze the proportions of the immune subset in endometriosis. We constructed immune cell maps (Figure 2A) and correlation maps of these 22 types of immune cells (Figure 2B) in endometriosis samples. The 22 types of immune cells are listed in Supplementary Table 1. We used GSVA to calculate the enrichment scores for all genes in each immune cell type. We observed changes in specific

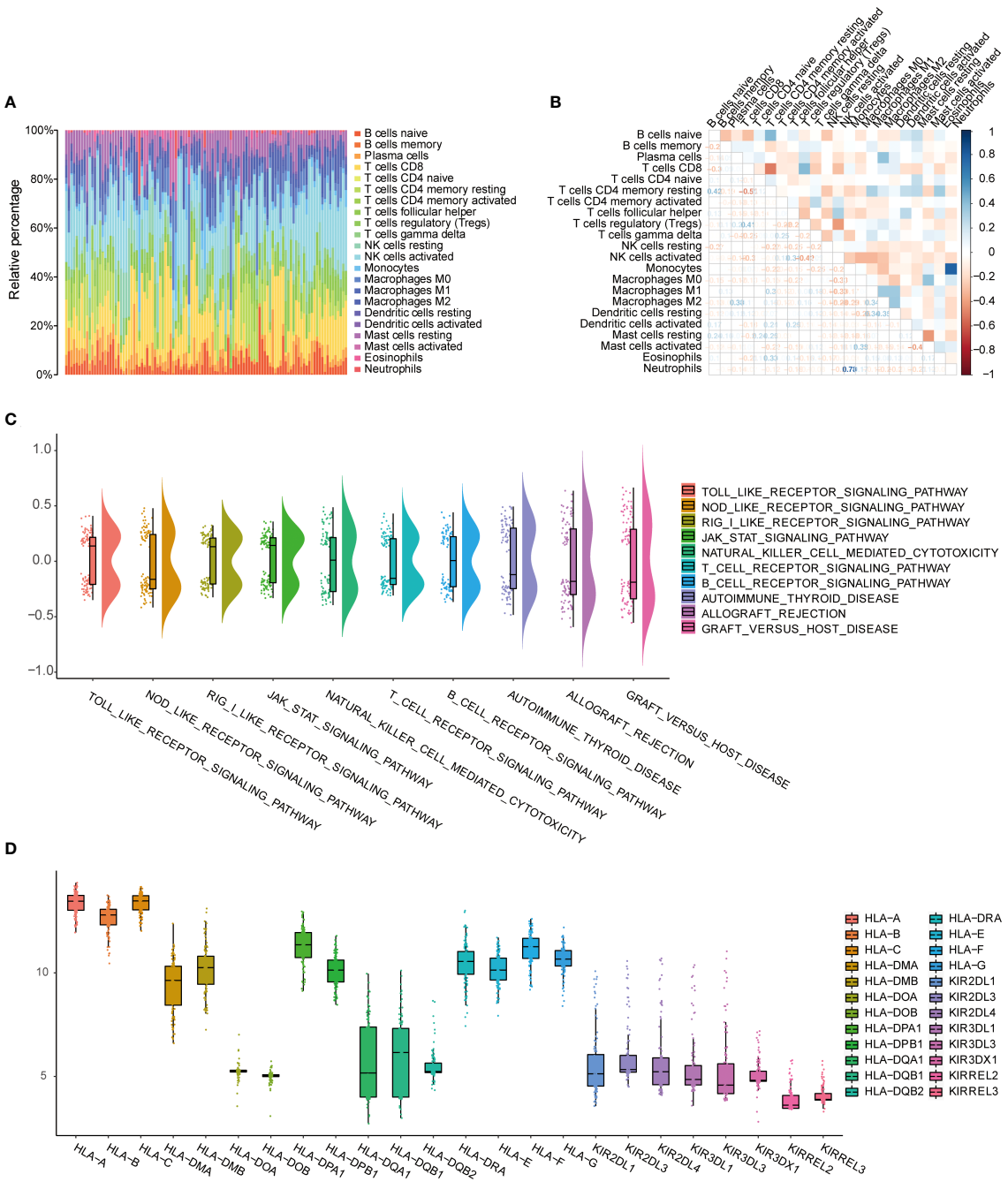


FIGURE 2
Evaluation of immune cell infiltration and immune correlation analysis. **(A)** Barplot shows the proportion of 22 types of immune cells in endometriosis samples. The column of the graph is sample. **(B)** Correlation heat map of 22 immune cell infiltrates; Blue represents positive, red represents negative. The depth of the color indicates the strength of the correlation. **(C)** Changes in enrichment of 10 immune-related pathways. **(D)** Expression differences of HLA and KIR family genes.

gene sets associated with 10 immune-related pathways in the c2.cp.v7.4.symbols.gmt dataset, including Toll-like receptor signaling pathway, Nod-like receptor signaling pathway, Rigi-like receptor signaling pathway, Jak-stat signaling pathway, NK cell-mediated cytotoxicity, T cell receptor signaling pathway, B cell receptor signaling pathway, autoimmune thyroid disease, allograft rejection, and graft versus host disease the gene sets and (Figure 2C). The HLA and KIR family genes were immune-related gene families, and the expression of each gene in the family showed different manifestations. HLA-A, HLA-B, and HLA-C showed relatively high expression, while the expression of the KIR family was relatively low (Figure 2D).

3.2 Identification of two endometriosis subtypes and exploration of DEGs based on immune characteristics

To determine the biological differences between different immune subtypes in endometriosis, we classified the endometriosis samples into cluster 1 and cluster 2 based on the consensus clustering of immune cell infiltration data. The two subtypes were clearly distinguished (Figure 3A). Differential gene expression was identified with limma (P-value < 0.05 and |log2FC| > 0.3). A total of 140 DEGs were obtained, including 29 low-expression genes and 111 high-expression genes (Supplementary Table 7). The volcano plot indicated a higher number of genes with upregulated expression compared to genes with downregulated expression (Figure 3B). In the heat map, the genes in cluster 1 exhibited an overall downregulated trend, while the genes in cluster 2 showed an overall upregulated trend (Figure 3C). GSEA and GSVA were applied for all the DEGs between cluster 1 and cluster 2, and the results were shown in Supplementary Tables 2, 3, respectively. The results of GSEA were visualized in Supplementary Figure 1A, indicating that immunoregulatory interactions and the complement system play crucial roles in the pathology of endometriosis. The results of GSVA (Supplementary Figure 1C) revealed that the B cell receptor signaling pathway and CD8 TCR downstream pathway are important pathways involved in endometriosis. These findings indicate that the immunology system plays an essential role in the pathologies of endometriosis.

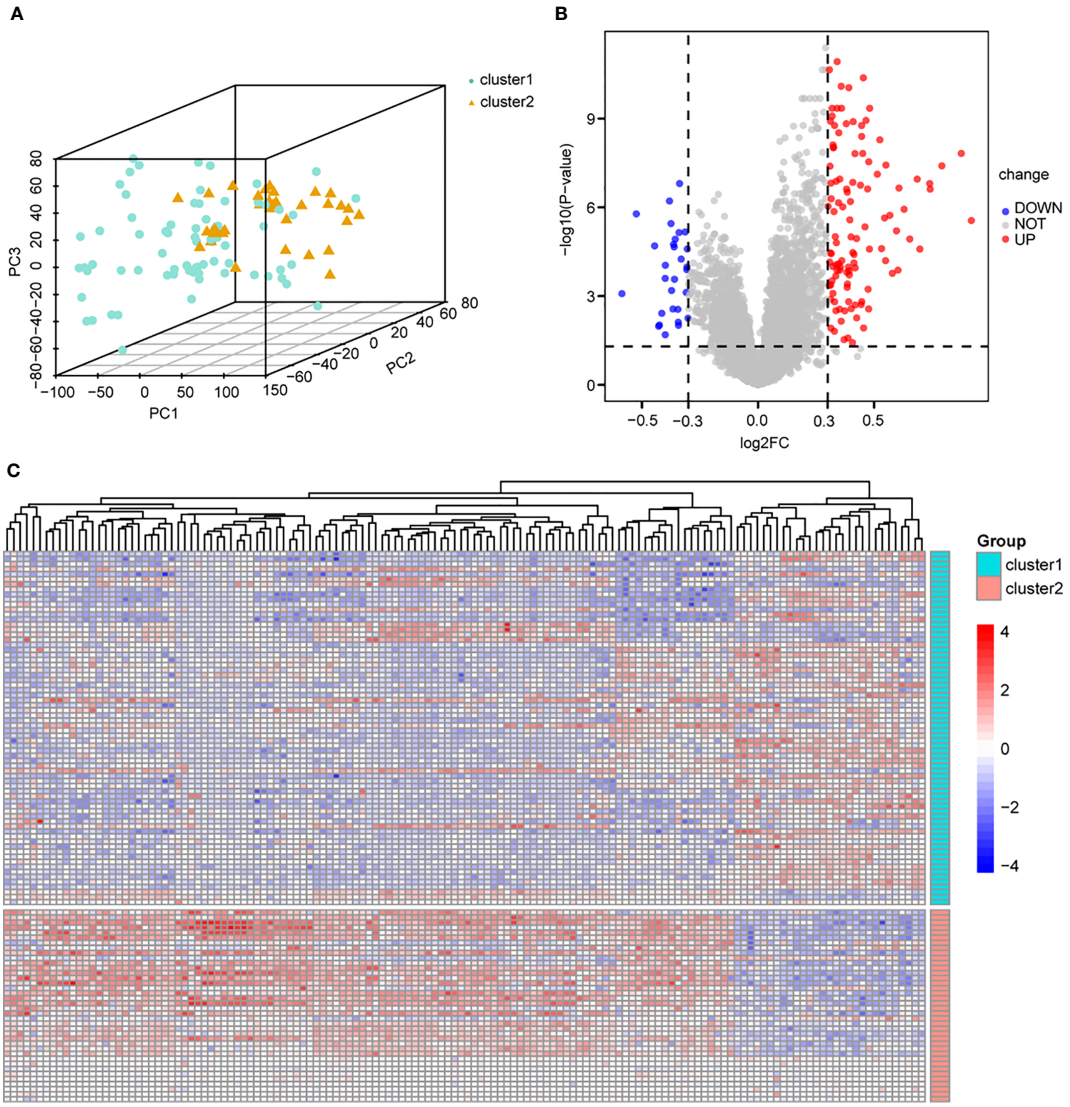


FIGURE 3
Differential analysis based on immune cell subtypes. **(A)** PCA analysis based on immune cell subtypes, cluster1 is green, cluster 2 is yellow. **(B)** Volcano map of differential analysis based on immune cell subtype, blue dots represent low expression and red dots represent high expression. **(C)** Heat map based on differential analysis of immune cell subsets, cluster1 is green, cluster 2 is red; blue dots represent low expression and red dots represent high expression. PCA, principal component analysis.

3.3 Co-expression network construction and central genes identification

To explore the central genes involved in immune infiltration in endometriosis, we constructed a gene co-expression network using the WGCNA algorithm. The gene expression profile of the endometriosis immune subtype was evaluated using cluster analysis (Figure 4A). To ensure that the network was scale-free, we selected a soft threshold of $\beta = 2$ (Figure 4B). We then transformed the representation matrix into an adjacency matrix and a topological matrix and used the average linkage hierarchical clustering method to cluster the genes. We set a minimum number of 50 genes in each gene network module, based on the standard of the hybrid dynamic clipping tree. We used the dynamic shearing method to determine the gene module and calculated the characteristic gene values for each module.

We then conducted Cluster analysis on the modules and merged modules that were close to each other using the following parameters: height = 0.25, deep split = 4, and minimum module = 50, resulting in a total of 11 modules (Figure 4C). The module with the highest correlation with immune characteristics was the green module (r = 0.6, P = 5e-12; Figure 4D). We identified 285 genes in the green module. We then compared these genes to the 140 DEGs based on immune characteristics and identified 52 common genes (Figure 4E).

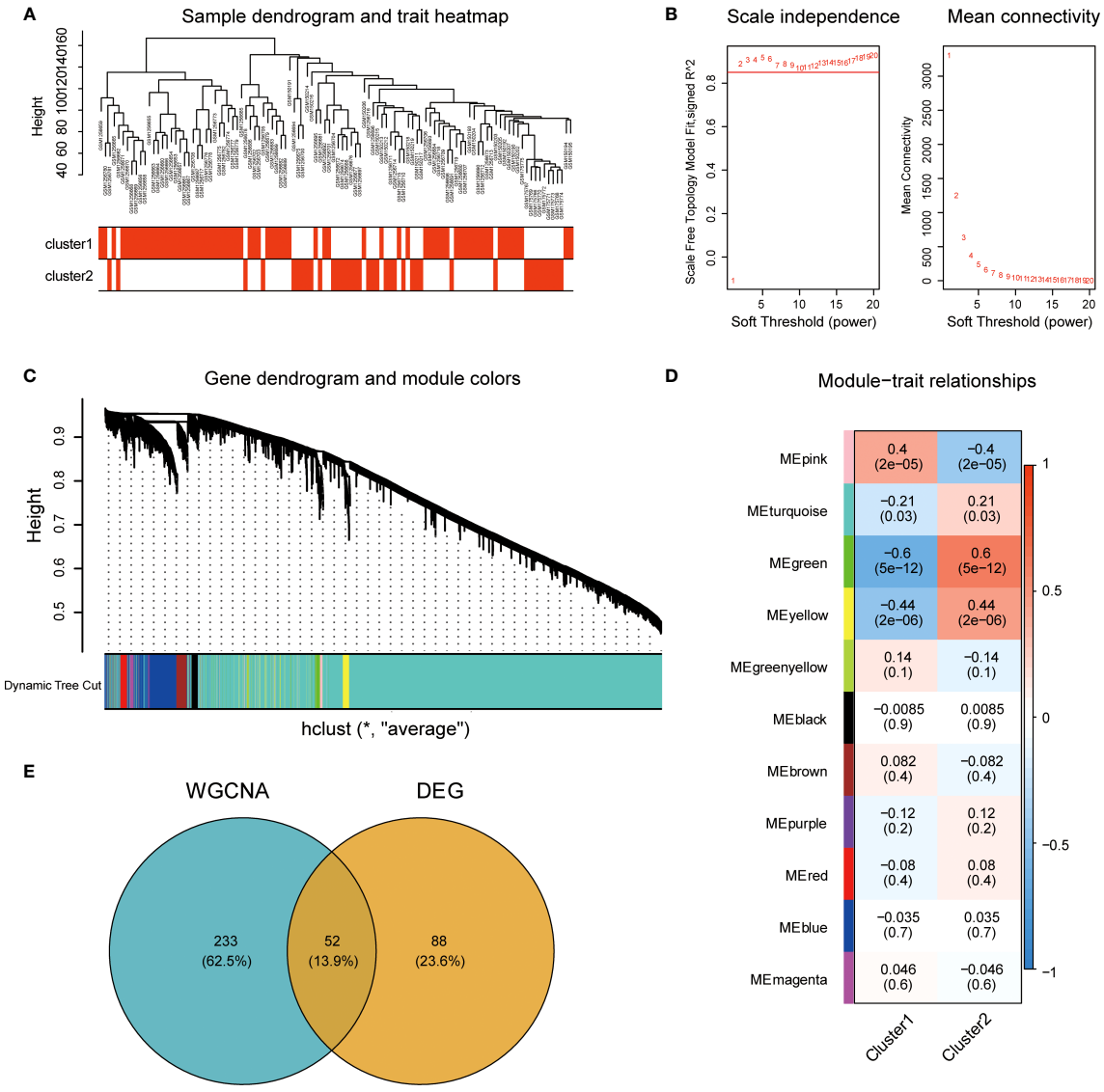


FIGURE 4
Construction and module analysis of the weighted co-expression network. **(A)** Sample clustering tree based on Euclidean distance. **(B)** Network topology analysis under various soft threshold power settings. Left: The X-axis represents the soft threshold power, and the Y-axis represents the fitting index of scale-free topological model. Right: The X-axis represents the soft threshold power, and the Y-axis reflects the average connectivity (degree). **(C)** Clustering tree of genes with different similarities based on topological overlap, and assigned module colors. **(D)** Association with Module-trait. Each row corresponds to a module, and each column corresponds to a feature. Each cell contains the corresponding correlation and P values. This table is color-coded according to the correlation of the color legends. **(E)** The intersection Venn diagram of core modules and central genes of WGCNA. WGCNA, weighted gene co-expression network based on immune cell subsets.

3.4 GO and KEGG enrichment analysis of cerntral genes in endometriosis

To further investigate the functions of the 52 common genes, we performed GO and KEGG analyses. We used a cutoff p value of 0.5 for these analyses. GO analysis showed that the common genes were mainly related to leukocyte-mediated cytotoxicity, negative regulation of growth, NK cell-mediated cytotoxicity, NK cell-mediated immunity, and positive regulation of protein transport (Figures 5A, C; Supplementary Table 4). KEGG analysis showed that the pathway was mainly related to graft-versus-host disease, NK cell-mediated cytotoxicity, antigen processing and presentation, complement and coagulation cascades, allograft rejection, and NK cells in both resting and activated states (Figures 5B, D; Supplementary Table 5). Pathway enrichment analysis also showed that the common genes were significantly expressed in the complement and coagulation cascade (Figure 5E) and NK cell-mediated cytotoxicity (Figure 5F) pathways.

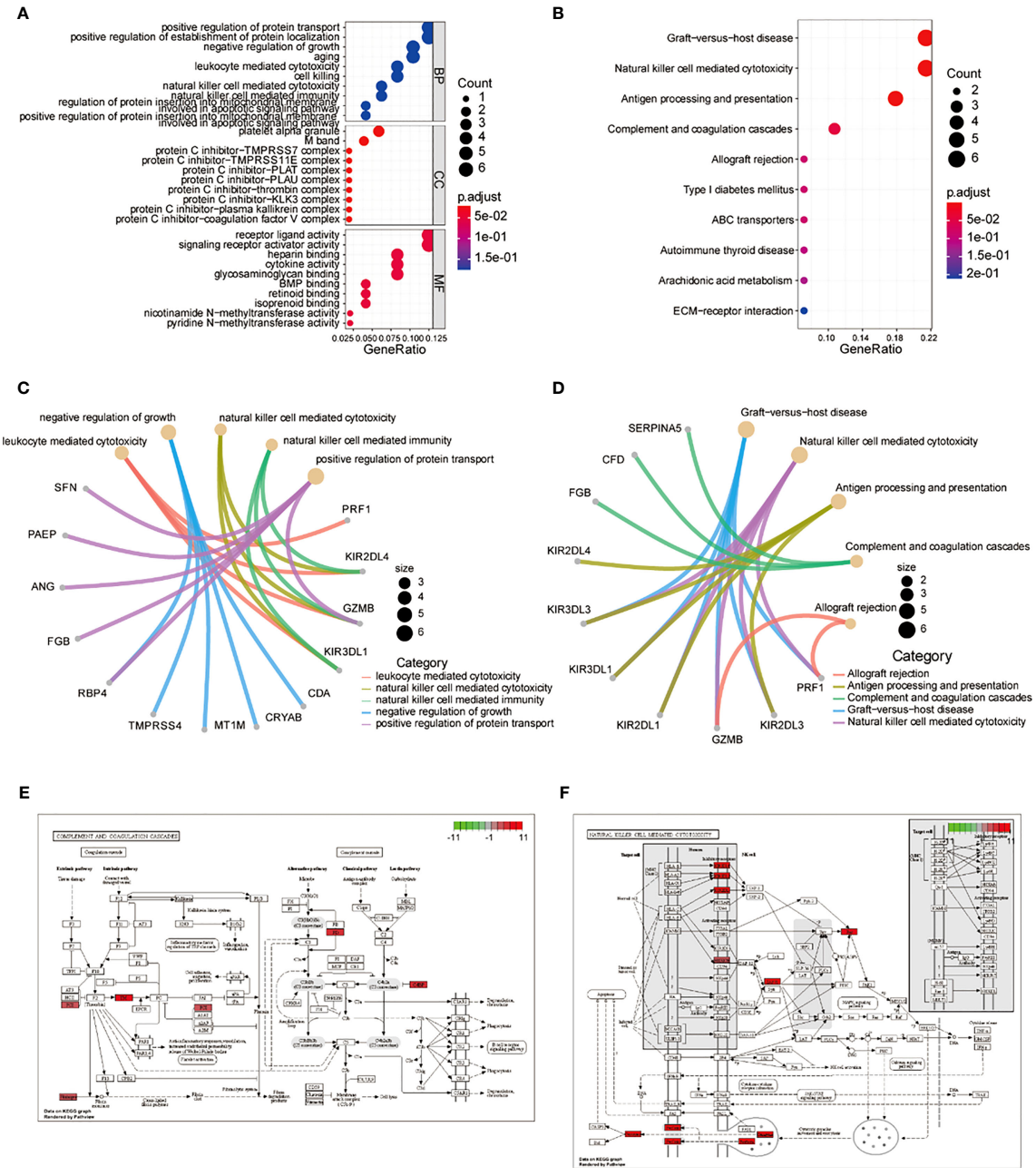


FIGURE 5
Central genes in GO, KEGG, and PATHWAY enrichment analysis. **(A)** Dot plot of central genes GO analysis. **(B)** Dot plot of central genes KEGG analysis. **(C)** Loop diagram of central genes GO analysis. **(D)** Loop diagram of central genes KEGG analysis. **(E)** Enrichment diagram of central genes in the complement and coagulation cascade pathways. **(F)** Enrichment diagram of central genes in the natural killer cell-mediated cytotoxicity pathway. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

3.5 Pathway enrichment of central genes in endometriosis

To address potential biases in the enrichment analysis of intersection genes, we performed GSEA on all 140 DEGs related to immune subtypes in the endometriosis expression profile. We used the C2.cp.v7.4.Symbols.gmt reference gene set for this analysis. The results showed that DEGs were significantly enriched in the chemokine signaling pathway, cytokine cytokine receptor interaction, NK cell mediated cytotoxicity, cell cycle checkpoints, cell cycle mitotic, homology directed repair, and reactome mitotic prometaphase (Figures 6A–G). We also performed GSVA using the C2.cp.v7.4.symbols.gmt reference gene set to explore the potential mechanism underlying the pathogenesis of endometriosis. We observed significant differences in 10 gene sets based on immune subtypes, including innate immune system, biocarta csk pathway, graft versus host disease, WP oxidative damage, neutrophil degranulation, signaling by CSF3 G CSF, WP microglia pathogen phagocytosis pathway, WP chemokine signaling pathway, WP IL3 signaling pathway, and interferon-gamma signaling. Most of these DEG sets were immune-related pathways (Figure 6H).

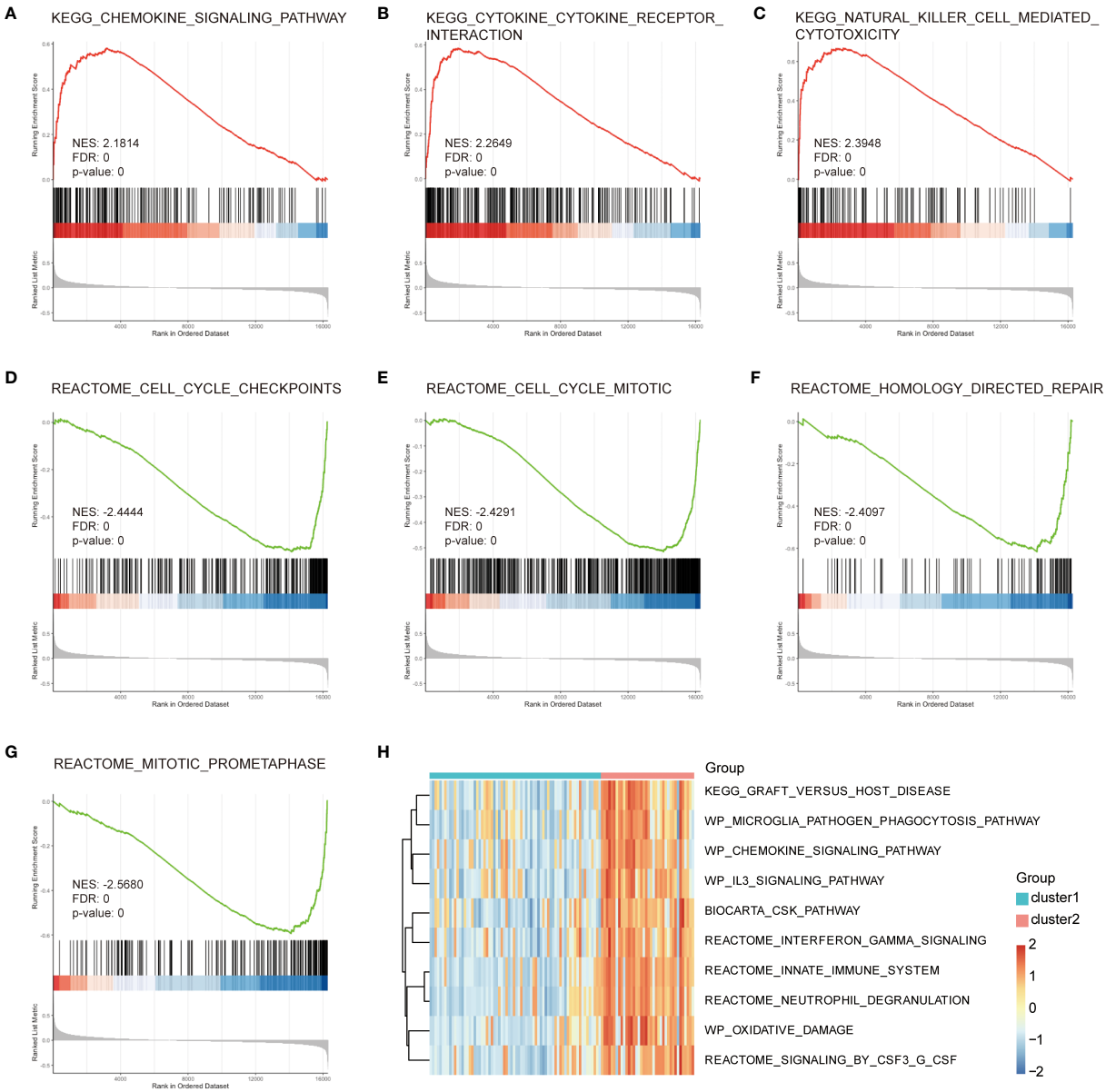


FIGURE 6
GSEA and GSVA enrichment analysis of central genes. **(A)** Cytokine–cytokine receptor interaction. **(B)** Natural killer cell-mediated cytotoxicity. **(C)** Cell cycle checkpoints. **(D)** Cell cycle mitosis. **(E)** Homology-directed repair. **(F)** Mitotic prometaphase. **(G, H)** GSVA analysis, green represents Cluster1, red represents Cluster2, small blue squares represent low expression, and small red squares represent high expression. GSEA, gene set enrichment analysis; GSVA, gene set variation analysis; BP, Biological Process; CC, Cellular Component; MF, Molecular Function.

3.6 Construction of PPI network

To explore the protein functions of the 52 candidate genes (Supplementary Table 6), we constructed a PPI network using the STRING database and Cytoscape software. With a confidence score of 0.55, we identified 45 genes that showed close interactions with each other (Supplementary Figure 2A), while only 8 genes remained with a cut-off of > 0.7 (Supplementary Figure 3). We utilized an interaction score of 0.55 to identify the hub genes. Using the cytoHubba plugin, we calculated the value of the PPI network and selected the top 10 hub genes (Supplementary Figure 2B) and their extensions: GZMB, PRF1, KIR2DL1, KIR2DL3, KIR3DL1, KIR2DL4, FGB, IGFBP1, RBP4, and PROK1 (Supplementary Figure 2C). To further explore the upstream regulatory relationships, we predicted miRNA interactions with the hub gene by selecting the results of "Functional MTI" and "positive" evidence, based on the multiMiR and TarBase v.8 databases. We found that 8 mRNAs in the hub genes interacted with 11 miRNAs (Supplementary Figure 2D).

3.7 Identification of three endometriosis subtypes through unsupervised clustering based on hub genes

To investigate the biological differences among different subtypes based on the characteristics of endometriosis, we used the ConsensusClusterPlus software package to construct subtypes based on the expression profile of the 10 hub genes. When k = 3, the classification was reliable and stable (Figures 7A–C). The samples were divided into cluster 1, cluster 2, and cluster 3. PCA confirmed that cluster 1, cluster 2, and cluster 3 showed significant differences (Figure 7D). We also analyzed the correlation of immune cell fractions by disease subtypes and found that the three components showed different expressions in different immune cells. NK.cells.activated, T.cells.CD8, and NK.cells.resting showed a large difference in expression (Supplementary Figure 4G). For the hub genes, we calculated the correlation between the hub gene expression and immune cell fraction (Pearson's coefficient). Most hub genes were significantly correlated with immune markers. We

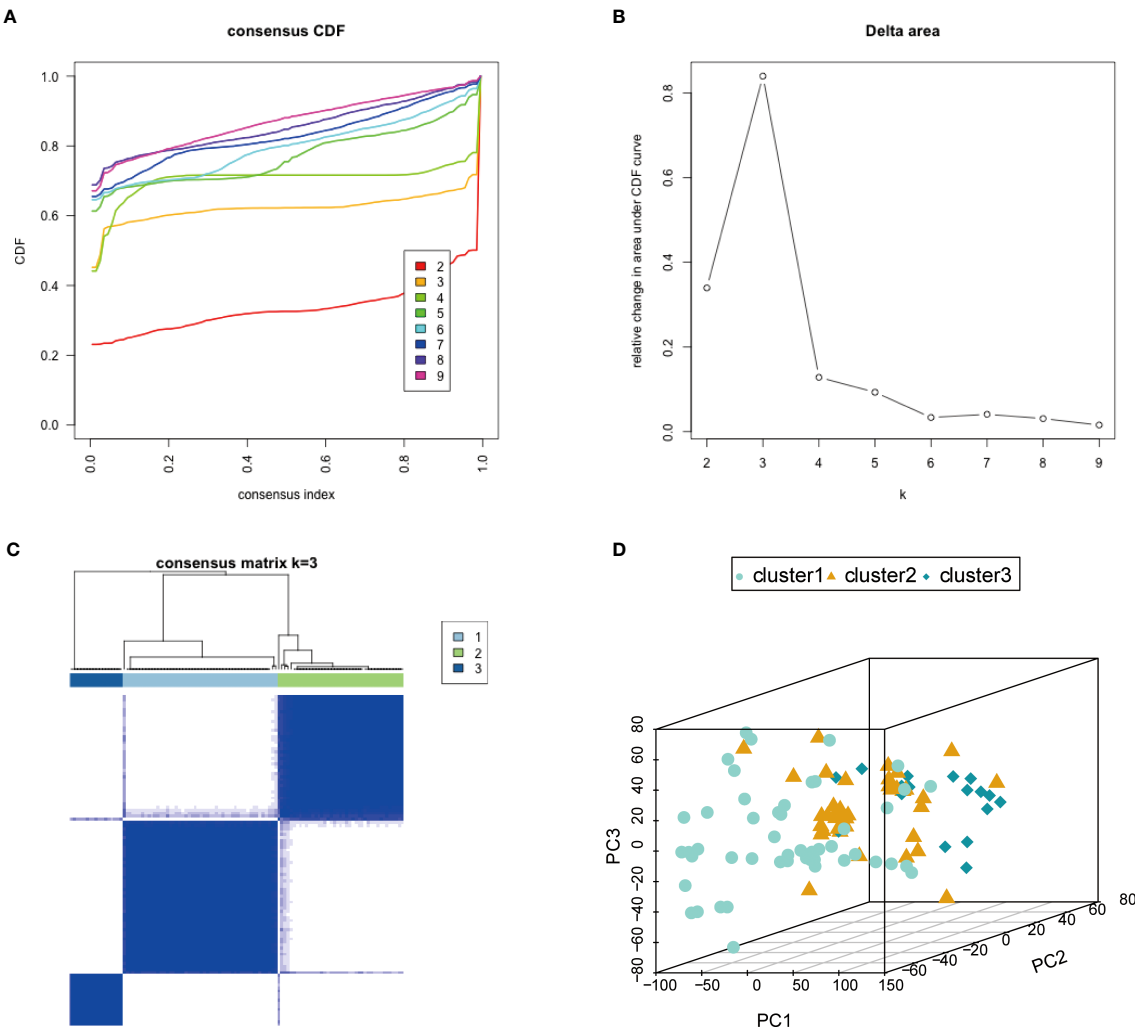


FIGURE 7
Characteristics based on endometriosis-related molecular typing. **(A)** Consensus CDF. **(B)** Delta region. **(C)** Consistent matrix at k = 3. The rows and columns of the matrix represent samples. **(D)** The PCA diagram verifies the stability and reliability of classification. PCA, principal component analysis.

observed a significant positive correlation between GZMB and NK.cells.activated (P = 1.23e-11, R = 0.59) (Supplementary Figure 4A), KIR2DL1 and NK.cells.resting (P = 2.51e-8, R = 0.50) (Supplementary Figure 4B), KIR2DL3 and NK.cells.resting (P = 1.55e-11, R = 0.59) (Supplementary Figure 4C), KIR2DL4 and NK.cells.resting (P = 4.26e-12, R = 0.60) (Supplementary Figure 4D), PRF1 and NK.cells.activated (P = 3.54e-10, R = 0.56) (Supplementary Figure 4E), and PRF1 and NK.cells.resting (P = 1.15e-11, R = 0.59) (Supplementary Figure 4F). Further analysis of the correlation between hub genes and immune checkpoint genes revealed that hub genes were strongly positively correlated with CD40, IDO1, LAG3, TNF, and TNFRSF18 immune checkpoint genes (Supplementary Figure 4H). These results showed that the expression of hub genes was significantly positively correlated with immune characteristics.

3.8 Expression validation of hub genes

To validate the transcriptional and protein expression of the hub genes, we used qRT-PCR and Western blot analyses to detect their expression in both ovarian endometriosis tissue and normal eutopic endometrial tissue from the same patients (Figure 8). We found that, except for FGB, KIR2DL1, and KIR2DL3, all other hub genes showed significantly different expression levels in the endometriosis and normal control groups (Figure 8A). Specifically, the expression of KIR2DL4 was remarkably downregulated, while the expressions of PROK1, IGFBP1, RBP4, G2MB, KIR3DL1, and PRF1 were significantly upregulated in ovarian endometriosis. Moreover, the protein expression of RBP4 was remarkably higher in ovarian endometriosis than in normal control (Figure 8B).

4 Discussion

Endometriosis is a common disease that affects up to 10% of women. It is characterized by pain and infertility (31). Up to 90% of cases of chronic pelvic pain in women of reproductive age are associated with endometriosis (32). Endometriosis is also a risk factor for ovarian cancer (33). However, endometriosis is not curable, and its underlying etiology remains unclear. In a previous study, we identified PDLIM3 as a specific biomarker for endometriosis that was associated with multiple immune cells (15). However, that analysis focused on the relationship between a single gene and the immune cell environment.

In the present study, we conducted a more comprehensive analysis of the immune cell environment and explored immune-

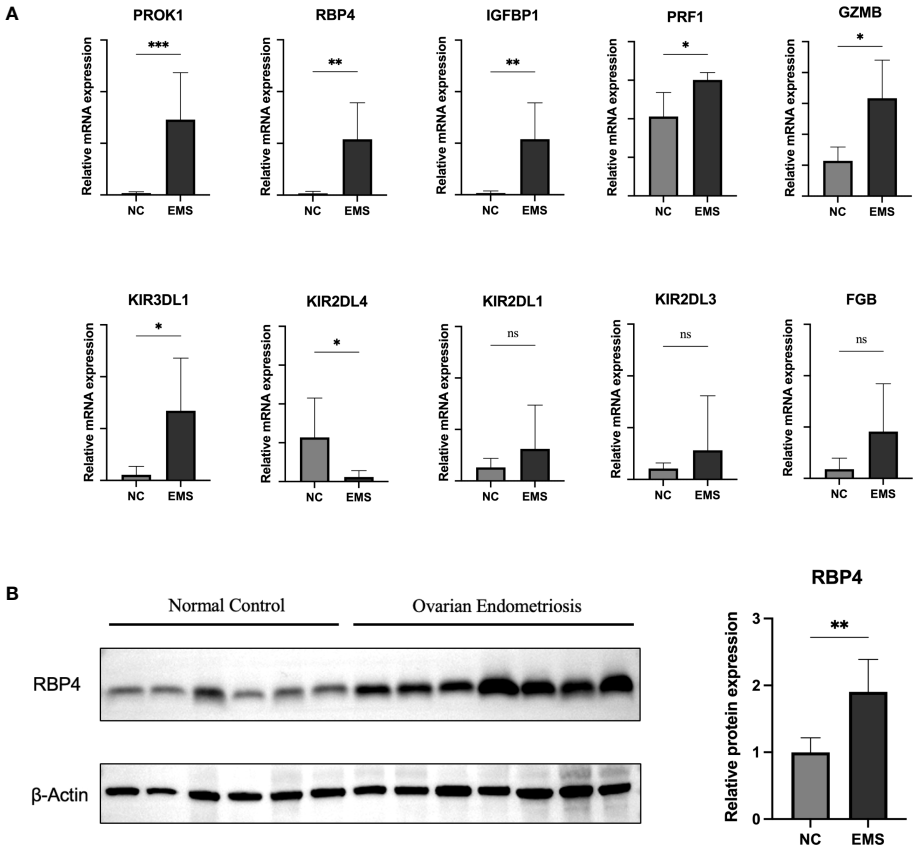


FIGURE 8
Expression validation of hub genes. **(A)** qRT-PCR was performed to determine mRNA expression of hub genes in normal eutopic endometrial tissue and ovarian endometriosis tissue. **(B)** Western blotting was performed to determine the protein expression of RBP4 in normal eutopic endometrial tissue and ovarian endometriosis tissue. EMS, endometriosis. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01; ns, non-significant.

associated genes in endometriosis. We merged three mRNA microarray datasets (GSE51981, GSE6364, GSE7305) into one dataset with 108 samples of endometriosis and conducted several analyses. Our findings indicated that hub genes, enriched modules, and pathways have genetic effects on endometriosis. Moreover, the predicted genes in the merged dataset might interact with each other and coregulate endometriosis.

HLA and KIR are immune-related gene families. KIRs are expressed by NK cells and can recognize class I HLA on target cells. HLA-C is a ligand of KIRs and is an essential regulator of NK cell activity (34, 35). Modulation of the HLA-KIR axis provides new prospects for cancer treatment (36) and immunotherapy of other diseases such as COPD (37). A previous study revealed that the expression of HLA-C*03:03*01 was increased in endometriosis. Aberrant expression of KIR2DL1 impaired NK cell cytotoxicity in endometriosis (38). KIR2DS5-positive women with endometriosis had a lesser chance of peritoneal disease, and KIR2DS5 might be a protective factor for endometriosis (34). In this study, we constructed 22 immune profiles from endometriosis samples. GSVA was used to calculate enrichment scores for specific gene sets in each sample, and variations of 10 immune pathways, including Toll-like receptor signaling pathway, Nod-like receptor signaling pathway, and Rig I-like receptor signaling pathway, were observed. HLA-A, HLA-B, and HLA-C expression levels were relatively high, and KIR expression was relatively low, which is consistent with previous reports (34, 35).

To investigate the biological functions of the differentially expressed genes, we analyzed 52 common genes that were common to both immunogrouping and WGCNA analyses and found several functions were associated with endometriosis, including leukocyte-mediated cytotoxicity, negative regulation of growth, NK cell-mediated cytotoxicity, NK cell-mediated immunity, and positive regulation of protein transport. Further analysis using GO and KEGG pathways showed that the complement and coagulation cascade pathways and NK cell-mediated cytotoxicity pathways were significantly associated with endometriosis. Consistent with our findings, the abnormal immune function of NK cells is closely related to endometriosis (39). In particular, impaired NK cell cytotoxic activity has been linked to the development of endometriosis (40, 41). To validate our results, we performed GSEA and GSVA analysis on differential gene sets related to immune subtypes. Our analysis showed common enrichment in NK cell-mediated cytotoxicity and graft versus host disease, and most differential gene sets were immune-related pathways. These results further support the important role of NK cells in the development of endometriosis.

The immune system plays a crucial role in the pathogenesis of endometriosis, with immune cells infiltrating the ectopic endometrial lesions. Immune infiltration points in endometriosis include the involvement of various immune cell types, such as macrophages, neutrophils, NK cells, and $CD8^+$ T cells. Macrophages, found in increased numbers in the peritoneal fluid and at the site of the lesions, contribute to the development and maintenance of endometriotic lesions by producing growth factors, angiogenic factors, and pro-inflammatory cytokines (42–44). Previous study suggests that M2 macrophages may play a role in the development and recurrence of endometriosis (45). Additionally, microvesicles secreted by M1 macrophages in endometriosis can induce polarization of M2 macrophages towards an M1 phenotype, potentially inhibiting the development of endometriosis (46). These studies indicate the presence of both M1 and M2 macrophages in endometriotic lesions and suggest that repolarizing M2 macrophages towards an M1 phenotype could be a potential therapeutic strategy. However, further research and clinical studies are needed to fully understand the role of macrophage subtypes and their potential for the treatment of endometriosis. Similarly, neutrophils are present in the peritoneal fluid of women with endometriosis and promote angiogenesis and tissue remodeling in the formation of endometriotic lesions (47, 48). Moreover, NK cells in peripheral blood might help to destroy retrograde endometrial cells, thus preventing endometriosis development. The reduction in NK cell cytotoxic activity might result in endometriosis (49). The alterations in the immunological parameters of NK cells not only included lower NK cell cytotoxicity but also involved a shift in the balance between type 1 and type 2 NK cells, as well as changes in the percentages of inhibitory and activating NK cell receptors (39). Thus, abnormalities in the function of NK cells, such as reduced cytotoxicity, altered activity and phenotype, and imbalances in receptor expression, might contribute to the development and pathology of endometriosis. In our study, the NK cell-mediated cytotoxicity pathway is implicated in several analyses, aligning with previous findings. CD8 T cells also play a role in the development and progression of endometriosis. An increased number of CD8 T cells has been observed in the peritoneal fluid of women with endometriosis compared to those without the disease, suggesting a possible role of CD8 T cells in the local immune response (50). CD8 T cells in the peritoneal fluid of endometriosis patients exhibited a reduced cytotoxic capacity, potentially contributing to impaired immune surveillance and facilitating the establishment of endometrial implants in the peritoneal cavity (51).

To further elucidate the pathogenesis of endometriosis, we performed PPI analyses to identify the endometriosis-associated hub genes. We selected 10 DEGs as hub genes, namely, GZMB, PRF1, KIR2DL1, KIR2DL3, KIR3DL1, KIR2DL4, FGB, IGFBP1, RBP4, and PROK1. Among these, KIR2DL1, KIR2DL3, KIR3DL1, and KIR2DL4 belong to NK cell inhibitory receptors (39). Their functions are mentioned above. GZMB is a component of cytolytic granules within NK cells (52) and was reported to be correlated with cervical cancer (53). RBP4 belongs to the lipocalin family and is the major transport protein of the hydrophobic molecule retinol, which is known as vitamin A (54). A recent study showed that RBP4 is involved in the pathological process of endometriosis and could promote the activity, proliferation, and invasion of ectopic cells (55). We validated that RBP4 was overexpressed in endometriosis patients. In addition, there are no reports about RBP4 and other uterine pathologies, which indicates that this signature is unique to endometriosis. PROK1 is a secreted peptide that belongs to the prokineticin family and performs a wide range of functions in angiogenesis, modulation of inflammatory responses, and regulation of hematopoiesis (56). Tiberi et al. discovered that the expression of PROK1 in normal female tissues was much higher

than that in women with endometriosis, suggesting that women with endometriosis might show abnormal vascular function (57).

In our study, all the hub genes were found to be highly correlated with endometriosis. For example, GZMB (Granzyme B) belongs to the granzyme family of serine proteases that are found in the cytotoxic granules of cytotoxic T lymphocytes and NK cells (58). Granzymes play a critical role in the immune system by inducing apoptosis in target cells, such as virally infected cells or tumor cells (59). GZMB is a therapeutic target for endometriosis, based on its role in immune response, tissue remodeling, and angiogenesis. Targeting GZMB might alleviate endometriosis-associated pain and inflammation while preserving fertility (60). Apart from their association with endometriosis, some of the hub genes have also been linked to other uterine pathologies. For instance, the KIR family (61), IGFBP1 (62), and PROK1 (63) have been correlated with recurrent implantation failure, while GZMB (64) and FGB (65) have been implicated in cervical carcinoma. However, there is no report about the relationship between PRF1 and RBP4 and other uterine pathologies, which suggests that these two might be unique signatures of endometriosis.

To further explore upstream regulatory relationships, multiMiR database, and TarBase V. 8 database were used to predict miRNAs interacting with hub genes. Eight mRNAs in hub genes were found to interact with 11 miRNAs, namely, miR-29c-3p, miR-542-3p, miR-27a-3p, miR-31-5p, miR-16-5p, miR-29b-3p, miR-124-3p, miR-21-5p, miR-409-3p, miR-107, and miR-452-5p. MiRNAs could inhibit the translation of their target genes by binding to their messenger RNA 3′-untranslated region (66). This posttranscriptional regulation occurs during physiopathologic processes, including endometriosis (67). Changes in the function of mir-542-3p and its target gene IGFBP1 have been reported to alter the decidualization of endometriosis stromal cells, affecting the metastasis and invasion of ectopic endometriosis cells (68, 69). Braza-Boïls et al. indicated that many anomalously expressed miRNAs were identified in peritoneal fluid, including miR-16-5p. These miRNAs affected the expression of VEGF-A and had an important influence on the occurrence and development of endometriosis (70). Overall, these results are similar to those from our mining data.

Our study has some limitations. Firstly, as the pathogenesis of endometriosis is multidimensional, discussing it only from the aspect of immune infiltration might not be comprehensive enough. Secondly, all study data were obtained from publicly available databases, and additional clinical characteristics of endometriosis patients should be included in subgroup analysis. Thirdly, although we validated the expression of hub genes through qRT-PCR and western blot, further multicenter and prospective studies are required to evaluate the possible applications of molecular signatures. Additionally, more *in vivo* and *in vitro* experiments are needed to elucidate the molecular mechanisms of hub genes for clinical applications. Furthermore, while we have analyzed gene expression data for HLA and KIR genes, we acknowledge that further investigation in specific cell types and disease contexts through sequencing or genotyping approaches is necessary to better understand the heterogeneity of these gene families. Lastly, scRNA-seq analysis may provide more valuable insights into immune cell heterogeneity compared to bulk RNA-seq, and we plan to conduct scRNA-seq analysis in future studies.

This study presents several novel aspects compared to previous similar research. Firstly, we incorporate a novel approach by considering the immune phenotype of patients with endometriosis. By analyzing the immune cell composition and immune-related pathways in endometriosis, we provide valuable insights into immune dysregulation associated with the disease. Secondly, we utilize diverse datasets to comprehensively analyze the immune landscape, revealing new relationships between immune cell subgroups, gene expression patterns, and immune pathways in endometriosis. Advanced analysis methods, such as CIBERSORT, GSVA, and WGCNA, further deepened the exploration, identifying key genes associated with immune subtypes. Notably, the study uncovers two previously unknown genes, KIR2DL3 and FGB, in endometriosis, contributing to the understanding of potential genetic factors in the disease.

In conclusion, this study provides comprehensive and reliable evidence for the pathogenesis of endometriosis from the perspective of immune infiltration. The results showed that most of the DEGs are involved in immune-related pathways. Ten hub genes, which show significant correlation with immune markers, might be potential diagnostic and therapeutic targets for endometriosis.

Our study provided a reliable and deep analysis of the mechanism underlying endometriosis from the perspective of immune infiltration. A total of 10 hub genes were identified and were validated by qRT-PCR and western blotting. The majority of hub genes showed significantly different expression between endometriosis patients and normal controls. Of these genes, PROK1 (Prokineticin 1) has been suggested to be associated with endometriosis due to its role in promoting angiogenesis (71). Altered expression of IGFBP1 (Insulin-like growth factor-binding protein 1) has been found in the endometrium of women with endometriosis, suggesting a potential association (72). KIR2DL4 and KIR3DL1 are members of the killer cell immunoglobulin-like receptor family and might be associated with the pathogenesis of endometriosis through their involvement in the regulation of NK cell activity (35). No direct link has been established between RBP4, G2MB, or PRF1 and endometriosis, necessitating further investigation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of Shanghai First Maternity and Infant Hospital. The studies were conducted in accordance with the local legislation and

institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

JS: Conceptualization, Supervision. S-JL: Project administration, Formal analysis, Investigation, Methodology, Writing -original draft J-NS: Software, Investigation, Visualization, Writing -review & editing, Validation. LG: Data curation, Resources. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Science and Technology Commission of Shanghai Municipality (grant number 22Y11906100) and the Shanghai Municipality: Shanghai Outstanding Academic Leaders Plan (2019).

Acknowledgments

All the authors are thankful to Shanghai First Maternity and Infant Hospital and Tongji University for successful completion of this research.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1130738/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Pathways enrichment of DEGs in endometriosis. **(A)** GSEA results in the endometriosis group. **(B)** GSEA results in the control group. **(C)** Heatmap of the enriched pathways. DEGs, differentially expressed genes; GSEA, gene set enrichment analysis.

SUPPLEMENTARY FIGURE 2
PPI network and miRNA-mRNA network analysis. **(A)** PPI network of 45 common genes constructed by the STRING database. The higher the degree value of the genes, the darker the color and the larger the diameter. **(B)** Top 10 hub genes and their extensions calculated by cytoHubba. Extension genes were green. The higher enrichment scores were indicated by darker colors. **(C)** The top 10 hub genes calculated by cytoHubba. The higher enrichment scores were indicated by darker colors. **(D)** miRNA-mRNA interaction network. Green circles represent mRNA and brown triangles represent miRNAs. PPI, Protein-Protein Interaction.

SUPPLEMENTARY FIGURE 3
PPI network with an interaction score of 0.7. PPI: Protein-Protein Interaction.

SUPPLEMENTARY FIGURE 4
Correlation analysis of key genes, immune cell infiltration level, and molecular subtypes. **(A)** Correlation point plot between GZMB and NK.cells.activated (P = 1.23e-11, R = 0.59). **(B)** Correlation point plot between KIR2DL1 and NK.cells.resting (P = 2.51e-8, R = 0.50). **(C)** Correlation point plot between KIR2DL3 and NK.cells.resting (P = 1.55e-11, R = 0.59). **(D)** Correlation point plot between KIR2DL4 and NK.cells.resting (P = 4.26e-12, R = 0.60). **(E)** Correlation point plot between PRF1 and NK.cells.activated (P = 3.54e-10, R = 0.56). **(F)** Correlation point plot between PRF1 and NK.cells.resting (P = 1.15e-11, R = 0.59). **(G)** Correlation analysis of 22 immune cell fractions in 3 disease subtypes. **(H)** Correlation between hub genes and immune checkpoint genes.

SUPPLEMENTARY TABLE 1
The list of 22 immune cells.

SUPPLEMENTARY TABLE 2
The GSEA analysis results.

SUPPLEMENTARY TABLE 3
The GSVA analysis results.

SUPPLEMENTARY TABLE 4
GO analysis of central genes.

SUPPLEMENTARY TABLE 5
KEGG analysis of central genes.

SUPPLEMENTARY TABLE 6
The list of candidate targets for STRING analysis.

References

1. Noh EJ, Kim DJ, Lee JY, Park JH, Kim JS, Han JW, et al. Ureaplasma urealyticum infection contributes to the development of pelvic endometriosis through toll-like receptor 2. *Front Immunol* (2019) 10:2373. doi: 10.3389/fimmu.2019.02373

2. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *N Engl J Med* (2020) 382 (13):1244–56. doi: 10.1056/NEJMra1810764

3. Macer ML, Taylor HS. Endometriosis and infertility: a review of the pathogenesis and treatment of endometriosis-associated infertility. *Obstet Gynecol Clin North Am* (2012) 39(4):535–49. doi: 10.1016/j.ogc.2012.10.002

4. Zondervan KT, Becker CM, Koga K, Missmer SA, Taylor RN, Vigano P. Endometriosis. *Nat Rev Dis Primers* (2018) 4(1):9. doi: 10.1038/s41572-018-0008-5

5. Lin X, Dai Y, Tong X, Xu W, Huang Q, Jin X, et al. Excessive oxidative stress in cumulus granulosa cells induced cell senescence contributes to endometriosis-associated infertility. *Redox Biol* (2020) 30:101431. doi: 10.1016/j.redox.2020.101431

6. Taylor HS, Kotlyar AM, Flores VA. Endometriosis is a chronic systemic disease: clinical challenges and novel innovations. *Lancet* (2021) 397(10276):839–52. doi: 10.1016/S0140-6736(21)00389-5

7. Kiesel L, Sourouni M. Diagnosis of endometriosis in the 21st century. *Climacteric* (2019) 22(3):296–302. doi: 10.1080/13697137.2019.1578743

8. Falcone T, Flyckt R. Clinical management of endometriosis. *Obstet Gynecol* (2018) 131(3):557–71. doi: 10.1097/AOG.0000000000002469

9. Ianieri MM, Mautone D, Ceccaroni M. Recurrence in deep infiltrating endometriosis: A systematic review of the literature. *J Minim Invasive Gynecol* (2018) 25(5):786–93. doi: 10.1016/j.jmig.2017.12.025

10. Ichimiya M, Hirota T, Muto M. Intralymphatic embolic cells with cutaneous endometriosis in the umbilicus. *J Dermatol* (1998) 25(5):333–6. doi: 10.1111/j.1346-8138.1998.tb02407.x

11. Othman Eel D, Hornung D, Salem HT, Khalifa EA, El-Metwally TH, Al-Hendy A. Serum cytokines as biomarkers for nonsurgical prediction of endometriosis. *Eur J Obstet Gynecol Reprod Biol* (2008) 137(2):240–6. doi: 10.1016/j.ejogrb.2007.05.001

12. Shigesi N, Kvaskoff M, Kirtley S, Feng Q, Fang H, Knight JC, et al. The association between endometriosis and autoimmune diseases: a systematic review and meta-analysis. *Hum Reprod Update* (2019) 25(4):486–503. doi: 10.1093/humupd/dmz014

13. Wu XG, Chen JJ, Zhou HL, Wu Y, Lin F, Shi J, et al. Identification and validation of the signatures of infiltrating immune cells in the eutopic endometrium endometria of women with endometriosis. *Front Immunol* (2021) 12:671201. doi: 10.3389/fimmu.2021.671201

14. Zhong Q, Yang F, Chen X, Li J, Zhong C, Chen S. Patterns of immune infiltration in endometriosis and their relationship to r-AFS stages. *Front Genet* (2021) 12:631715. doi: 10.3389/fgene.2021.631715

15. Gan L, Sun J, Sun J. Bioinformatical analysis identifies PDLIM3 as a potential biomarker associated with immune infiltration in patients with endometriosis. *PeerJ* (2022) 10:e13218. doi: 10.7717/peerj.13218

16. Davis S, Meltzer PS. GEOquery: a bridge between the Gene Expression Omnibus (GEO) and BioConductor. *Bioinformatics* (2007) 23(14):1846–7. doi: 10.1093/bioinformatics/btm254

17. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods* (2015) 12 (5):453–7. doi: 10.1038/nmeth.3337

18. Wickham H. ggplot2 : Elegant Graphics for Data Analysis. In: *Use R!*. Cham: Springer International Publishing : Imprint: Springer (2016).

19. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* (2005) 102(43):15545–50. doi: 10.1073/pnas.0506580102

20. Hanzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinf* (2013) 14:7. doi: 10.1186/1471-2105-14-7

21. Wilkerson MD, Hayes DN. ConsensusClusterPlus: a class discovery tool with confidence assessments and item tracking. *Bioinformatics* (2010) 26(12):1572–3. doi: 10.1093/bioinformatics/btq170

22. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* (2015) 43(7):e47. doi: 10.1093/nar/gkv007

23. Liang JW, Fang ZY, Huang Y, Liuyang ZY, Zhang XL, Wang JL, et al. Application of weighted gene co-expression network analysis to explore the key genes in alzheimer's disease. *J Alzheimers Dis* (2018) 65(4):1353–64. doi: 10.3233/JAD-180400

24. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* (2012) 16(5):284–7. doi: 10.1089/omi.2011.0118

25. Luo W, Brouwer C. Pathview: an R/Bioconductor package for pathway-based data integration and visualization. *Bioinformatics* (2013) 29(14):1830–1. doi: 10.1093/bioinformatics/btt285

26. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res* (2019) 47(D1):D607–13. doi: 10.1093/nar/gky1131

27. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* (2003) 13(11):2498–504. doi: 10.1101/gr.1239303

28. Chin CH, Chen SH, Wu HH, Ho CW, Ko MT, Lin CY. cytoHubba: identifying hub objects and sub-networks from complex interactome. *BMC Syst Biol* (2014) 8 Suppl 4:S11. doi: 10.1186/1752-0509-8-S4-S11

29. Friendly M. Corrgrams: Exploratory displays for correlation matrices. *Am Statistician* (2002) 56(4):316–24. doi: 10.1198/000313002533

30. Ru Y, Kechris KJ, Tabakoff B, Hoffman P, Radcliffe RA, Bowler R, et al. The multiMiR R package and database: integration of microRNA-target interactions along with their disease and drug associations. *Nucleic Acids Res* (2014) 42(17):e133. doi: 10.1093/nar/gku631

31. Lalami I, Abo C, Borghese B, Chapron C, Vaiman D. Genomics of endometriosis: from genome wide association studies to exome sequencing. *Int J Mol Sci* (2021) 22(14):7297. doi: 10.3390/ijms22147297

32. McAllister SL, Sinharoy P, Vasu M, Gross ER. Aberrant reactive aldehyde detoxification by aldehyde dehydrogenase-2 influences endometriosis development and pain-associated behaviors. *Pain* (2021) 162(1):71–83. doi: 10.1097/j.pain.0000000000001949

33. Lin SC, Lee HC, Hsu CT, Huang YH, Li WN, Hsu PL, et al. Targeting anthrax toxin receptor 2 ameliorates endometriosis progression. *Theranostics* (2019) 9(3):620–32. doi: 10.7150/thno.30655

34. Nowak I, Ploski R, Barcz E, Dziunycz P, Kaminski P, Kostrzewa G, et al. KIR2DS5 in the presence of HLA-C C2 protects against endometriosis. *Immunogenetics* (2015) 67(4):203–9. doi: 10.1007/s00251-015-0828-3

35. Chou YC, Chen CH, Chen MJ, Chang CW, Chen PH, Yu MH, et al. Killer cell immunoglobulin-like receptors (KIR) and human leukocyte antigen-C (HLA-C) allorecognition patterns in women with endometriosis. *Sci Rep* (2020) 10(1):4897. doi: 10.1038/s41598-020-61702-y

36. Pende D, Falco M, Vitale M, Cantoni C, Vitale C, Munari E, et al. Killer ig-like receptors (KIRs): Their role in NK cell modulation and developments leading to their clinical exploitation. *Front Immunol* (2019) 10:1179. doi: 10.3389/fimmu.2019.01179

37. Mkorombindo T, Tran-Nguyen T, Yuan K, Zhang Y, Xue J, Criner GJ, et al. HLA-C and KIR permutations influence chronic obstructive pulmonary disease risk. *JCI Insight* (2021) 6(19):e150187. doi: 10.1172/jci.insight.150187

38. Du Y, Liu X, Guo SW. Platelets impair natural killer cell reactivity and function in endometriosis through multiple mechanisms. *Hum Reprod* (2017) 32(4):794–810. doi: 10.1093/humrep/dex014

39. Fukui A, Mai C, Saeki S, Yamamoto M, Takeyama R, Kato T, et al. Pelvic endometriosis and natural killer cell immunity. *Am J Reprod Immunol* (2021) 85(4): e13342. doi: 10.1111/aji.13342

40. Maeda N, Izumiya C, Yamamoto Y, Oguri H, Kusume T, Fukaya T. Increased killer inhibitory receptor KIR2DL1 expression among natural killer cells in women with pelvic endometriosis. *Fertil Steril* (2002) 77(2):297–302. doi: 10.1016/s0015-0282(01)02964-8

41. Mei J, Zhou WJ, Zhu XY, Lu H, Wu K, Yang HL, et al. Suppression of autophagy and HCK signaling promotes PTGS2(high) FCGR3(-) NK cell differentiation triggered by ectopic endometrial stromal cells. *Autophagy* (2018) 14(8):1376–97. doi: 10.1080/15548627.2018.1476809

42. Bacci M, Capobianco A, Monno A, Cottone L, Di Puppo F, Camisa B, et al. Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. *Am J Pathol* (2009) 175(2):547–56. doi: 10.2353/ajpath.2009.081011

43. Capobianco A, Rovere-Querini P. Endometriosis, a disease of the macrophage. *Front Immunol* (2013) 28:9. doi: 10.3389/fimmu.2013.00009

44. Chen S, Liu Y, Zhong Z, Wei C, Liu Y, Zhu X. Peritoneal immune microenvironment of endometriosis: Role and therapeutic perspectives. *Front Immunol* (2023) 14:1134663. doi: 10.3389/fimmu.2023.1134663

45. Sun SG, Guo JJ, Qu XY, Tang XY, Lin YY, Hua KQ, et al. The extracellular vesicular pseudogene LGMNP1 induces M2-like macrophage polarization by upregulating LGMN and serves as a novel promising predictive biomarker for ovarian endometriosis recurrence. *Hum Reprod* (2022) 37(3):447–65. doi: 10.1093/humrep/deab266

46. Li QJ, Yuan M, Jiao X, Huang YF, Li J, Li D, et al. M1 macrophage-derived nanovesicles repolarize M2 macrophages for inhibiting the development of endometriosis. *Front Immunol* (2021) 12:707784. doi: 10.3389/fimmu.2021.707784

47. Gmyrek GB, Sozanski R, Jerzak M, Chrobak A, Wickiewicz D, Skupnik A, et al. Evaluation of monocyte chemotactic protein-1 levels in peripheral blood of infertile women with endometriosis. *Eur J Obstet Gynecol Reprod Biol* (2005) 122(2):199–205. doi: 10.1016/j.ejogrb.2005.03.019

48. Santulli P, Lamau MC, Marcellin L, Gayet V, Marzouk P, Borghese B, et al. Endometriosis-related infertility: Ovarian endometrioma per se is not associated with presentation for infertility. *Hum Reprod* (2016) 8:1765–75. doi: 10.1093/humrep/dew093

49. Osuga Y, Koga K, Hirota Y, Hirata T, Yoshino O, Taketani Y. Lymphocytes in endometriosis. *Am J Reprod Immunol* (2011) 65(1):1–10. doi: 10.1111/j.1600-0897.2010.00887.x

50. Berkkanoglu M, Arici A. Immunology and endometriosis. *Am J Reprod Immunol* (2003) 50(1):48–59. doi: 10.1034/j.1600-0897

51. Podgaec S, Abrao MS, Dias JAi Jr., Rizzo LV, de Oliveira RM, Baracat EC. Endometriosis: An inflammatory disease with a th2 immune response component. *Hum Reprod* (2007) 22(5):1373–9. doi: 10.1093/humrep/del516

52. Oboshi W, Watanabe T, Hayashi K, Nakamura T, Yukimasa N. QPY/RAH haplotypes of the GZMB gene are associated with natural killer cell cytotoxicity. *Immunogenetics* (2018) 70(1):29–36. doi: 10.1007/s00251-017-1014-6

53. Zhou C, Li C, Zheng Y, Liu X. Identification of pyroptosis-related signature for cervical cancer predicting prognosis. *Aging (Albany NY)* (2021) 13(22):24795–814. doi: 10.18632/aging.203716

54. Steinhoff JS, Lass A, Schupp M. Biological functions of RBP4 and its relevance for human diseases. *Front Physiol* (2021) 12:659977. doi: 10.3389/fphys.2021.659977

55. Lee JC, Kim SH, Oh YS, Kim JH, Lee SR, Chae HD. Increased expression of retinol-binding protein 4 in ovarian endometrioma and its possible role in the pathogenesis of endometriosis. *Int J Mol Sci* (2021) 22(11):5827. doi: 10.3390/ijms22115827

56. Su MT, Huang JY, Tsai HL, Chen YC, Kuo PL. A common variant of PROK1 (V67I) acts as a genetic modifier in early human pregnancy through down-regulation of gene expression. *Int J Mol Sci* (2016) 17(2):162. doi: 10.3390/ijms17020162

57. Tiberi F, Tropea A, Apa R, ROmani F, Lanzone A, Marana R. Prokineticin 1 mRNA expression in the endometrium of healthy women and in the eutopic endometrium of women with endometriosis. *Fertil Steril* (2010) 93(7):2145–9. doi: 10.1016/j.fertnstert.2009.01.105

58. Afonina IS, Tynan GA, Logue SE, Cullen SP, Bots M, Luthi AU, et al. Granzyme B-dependent proteolysis acts as a switch to enhance the proinflammatory activity of il-1alpha. *Mol Cell* (2011) 44(2):265–78. doi: 10.1016/j.molcel.2011.07.037

59. Trapani JA. Granzymes: A family of lymphocyte granule serine proteases. *Genome Biol* (2001) 2(12):REVIEWS3014. doi: 10.1186/gb-2001-2-12-reviews3014

60. Aubert A, Lane M, Jung K, Granville DJ. Granzyme B as a therapeutic target: An update in 2022. *Expert Opin Ther Targets* (2022) 26(11):979–93. doi: 10.1080/14728222.2022.2161890

61. Nowak I, Wilczynska K, Wilczynski JR, Malinowski A, Radwan P, Radwan M, et al. KIR, LILRB and their ligands' Genes as potential biomarkers in recurrent implantation failure. *Arch Immunol Ther Exp (Warsz)* (2017) 65(5):391–9. doi: 10.1007/s00005-017-0474-6

62. Fu YX, Yang HM, OuYang XE, Hu R, Hu T, Wang FM. Assessment of anti-mullerian hormone and anti-mullerian hormone type II receptor variants in women with repeated implantation failures. *Reprod Sci* (2021) 28(2):406–15. doi: 10.1007/s43032-020-00303-6

63. Karaer A, Tuncay G, Uysal O, Semerci Sevimli T, Sahin N, Karabulut U, et al. The role of prokineticins in recurrent implantation failure. *J Gynecol Obstet Hum Reprod* (2020) 49(9):101835. doi: 10.1016/j.jogoh.2020.101835

64. Guzman VB, Silva ID, Brenna SM, Carvalho CR, Ribalta JC, Gerbase-Delima M. High levels of granzyme B expression in invasive cervical carcinoma correlates to poor response to treatment. *Cancer Invest* (2008) 26(5):499–503. doi: 10.1080/07357900701805678

65. Lee SY, Lee KP, Lim JW. Identification and biosynthesis of fibrinogen in human uterine cervix carcinoma cells. *Thromb Haemost* (1996) 75(3):466–70. doi: 10.1055/s-0038-1650298

66. Mari-Alexandre J, Sanchez-Izquierdo D, Gilabert-Estelles J, Barcelo-Molina M, Braza-Boils A, Sandoval J. miRNAs regulation and its role as biomarkers in endometriosis. *Int J Mol Sci* (2016) 17(1):93. doi: 10.3390/ijms17010093

67. Mari-Alexandre J, Barcelo-Molina M, Belmonte-Lopez E, Garcia-Oms J, Estelles A, Braza-Boils A, et al. Micro-RNA profile and proteins in peritoneal fluid from women with endometriosis: their relationship with sterility. *Fertil Steril* (2018) 109(4):675–684 e2. doi: 10.1016/j.fertnstert.2017.11.036

68. Sultana S, Kajihara T, Mizuno Y, Sato T, Oguro T, Kimura M, et al. Overexpression of microRNA-542-3p attenuates the differentiating capacity of endometriotic stromal cells. *Reprod Med Biol* (2017) 16(2):170–8. doi: 10.1002/rmb2.12028

69. Cai H, Zhu XX, Li ZF, Zhu YP, Lang JH. MicroRNA dysregulation and steroid hormone receptor expression in uterine tissues of rats with endometriosis during the implantation window. *Chin Med J (Engl)* (2018) 131(18):2193–204. doi: 10.4103/0366-6999.240808

70. Braza-Boils A, Salloum-Asfar S, Mari-Alexandre J, Arroyo AB, Gonzalez-Conejero R, Barcelo-Molina M, et al. Peritoneal fluid modifies the microRNA expression profile in endometrial and endometriotic cells from women with endometriosis. *Hum Reprod* (2015) 30(10):2292–302. doi: 10.1093/humrep/dev204

71. Ujvari D, Jakson I, Oldmark C, Attarha S, Alkasalias T, Salamon D, et al. Prokineticin 1 is up-regulated by insulin in decidualizing human endometrial stromal cells. *J Cell Mol Med* (2018) 22(1):163–72. doi: 10.1111/jcmm.13305

72. Naqvi H, Mamillapalli R, Krikun G, Taylor HS. Endometriosis located proximal to or remote from the uterus differentially affects uterine gene expression. *Reprod Sci* (2016) 23(2):186–91. doi: 10.1177/1933719115613449

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 07 November 2023
DOI 10.3389/fendo.2023.1156169

OPEN ACCESS

EDITED BY
Achmad Kemal Harzif,
University of Indonesia, Indonesia

REVIEWED BY
Lu Lu,
Guangzhou University of Chinese Medicine, China
Igor Govorov,
Almazov National Medical Research Centre, Russia

*CORRESPONDENCE
Huaijun Zhou
zhouhj2007@126.com
Rong Li
glyylr@163.com

RECEIVED 01 February 2023
ACCEPTED 23 October 2023
PUBLISHED 07 November 2023

CITATION
Miao M, Zhu Y, Wang L, Miao Y, Li R and Zhou H (2023) A nomogram for predicting recurrence in endometrial cancer patients: a population-based analysis.
Front. Endocrinol. 14:1156169.
doi: 10.3389/fendo.2023.1156169

A nomogram for predicting recurrence in endometrial cancer patients: a population-based analysis

Mengdan Miao[1,2], Yanping Zhu[1], Lulu Wang[1], Yifei Miao[2], Rong Li[1]* and Huaijun Zhou[1,2]*

[1]Affiliated Drum Tower Hospital, Medical School, Nanjing University, Nanjing, China, [2]Department of Gynecology, Nanjing Drum Tower Hospital Clinical College of Nanjing Medical University, Nanjing, China

Objective: Endometrial cancer recurrence is one of the main factors leading to increased mortality, and there is a lack of predictive models. Our study aimed to establish a nomogram predictive model to predict recurrence in endometrial cancer patients.

Method: Screen 517 endometrial cancer patients who came to Nanjing Drum Tower Hospital from 2008 to 2018. All these data are listed as the training group, and then 70% and 60% are randomly divided into verification groups 1 and 2. Univariate, Multivariate logistic regression, stepwise regression were used to select variables for nomogram. Nomogram identification and calibration were evaluated by concordance index (c-index), area under receiver operating characteristic curve (AUC) over time and calibration plot Function. By decision curve analysis (DCA), net reclassification index (NRI), integrated discrimination improvement (IDI), we compared and quantified the net benefit of nomogram and ESMO-ESGO-ESTRO model-based prediction of tumor recurrence.

Results: A nomogram predictive model of endometrial cancer recurrence was established with the eight variables screened. The c-index (for the training cohort and for the validation cohort) and the time-dependent AUC showed good discriminative power of the nomogram. Calibration plots showed good agreement between nomogram predictions and actual observations in both the training and validation sets.

Conclusions: We developed and validated a predictive model of endometrial cancer recurrence to assist clinicians in assessing recurrence in endometrial cancer patients.

KEYWORDS

endometrial cancer, recurrence, risk factor, nomogram, predictive model

Introduction

As an epithelial cancer, endometrial cancer (EC) forms in the endometrium and is prevalent in perimenopausal and menopausal women. Current research indicates that EC is the most prevalent gynecological cancer in affluent nations (1–3). Moreover, its incidence rose from 2013 to 2017 (4, 5). According to the most current worldwide cancer statistics published in 2020 by the IARC of WHO, EC is second among prevalent genital tract malignancy after cervical cancer (6). In recent years, due to lifestyle changes and the popularity of hormone replacement therapy, the incidence of EC has gradually increased and gradually become younger (7).The progression of EC is relatively slow, and it is often detected at an early stage (8), so the prognosis is relatively good (9). However, there are still about 20% of patients with recurrence and metastasis (10), resulting in increased mortality (11, 12).

Age, grade and type of histology, myometrial invasion, and lymphovascular space invasion (LVSI) represent the risk factors for conventional endometrial cancer recurrence (12) and based on these risk variables, a set of recurrence patterns have been created. Some instances are EC recurrence patterns based on the European Society for Medical Oncology (ESMO), the European Society for Radiotherapy & Oncology (ESTRO), and the European Society of Gynaecological Oncology (ESGO) (ESMO-ESGO-ESTRO) consensus conference (8, 13, 14). However, these methods' limitations are their relatively low accuracy and inferior capacity for the prediction of recurrence risk among individuals. Thus, tailoring a model for predicting EC patients is necessary.

The nomogram tumor prediction model has been extensively utilized recently (15–17). For instance, the nomogram prediction model was used to construct treatment and monitoring regimens for patients in stages IIIB and IIIC in melanoma (18) and hepatocellular carcinoma to predict the recurrence of patients after laparoscopic liver resection (19). The nomogram tumor prediction model meets the requirements of an ensemble model and plays a role in promoting personalized medicine, valuable to physicians for recurrence prediction (15). In this study, we used 517 endometrial cancer patients who visited Nanjing Drum Tower Hospital from 2008 to 2018 to establish a nomogram tumor recurrence prediction model for EC.

Materials and methods

Study population

The only participants in this retrospective cohort research were endometrial cancer patients who attended Nanjing Drum Tower Hospital between 2008 and 2018. The following are the requirements for inclusion: (1) patients diagnosed with endometrial cancer based on clinical manifestations, auxiliary examinations, and postoperative pathology; and (2) case records including age, menopausal status, clinical stage, histological tumor grade, radiotherapy history, chemotherapy history, preoperative CA125, preoperative ultrasound results, postoperative CA125, reproductive history, histological type, cervical infiltration, vascular infiltration, metastasis, and surgical approach. The exclusion criteria included patients who were not regularly followed up.

Cohort partitioning and variable filtering

All patient data were used for the training group, and 60% and 70% were randomly selected as validation group 1 and validation group 2. For the first cohort, it intended to filter the factors for model production. Meanwhile, validation sets verify the former group's outcomes. The collected data included 15 variables: age, menopausal status, clinical stage, histological tumor grade, radiotherapy history, chemotherapy history, preoperative CA125, preoperative ultrasound results, postoperative CA125, reproductive history, histological type, cervical invasion, vascular invasion, metastasis, and surgical approach. Among them, the diagnostic criteria of CA125 in Nanjing Drum Tower hospital was the normal range of 0-30.2U/ml, when the serum CA125 level >30.2U/ml, it was defined as an elevated CA125 level. Surgical approaches were divided into non-surgical treatments based on radiotherapy and chemotherapy, minimally invasive surgery (laparoscopic/Da Vinci robotic), or open surgery. Univariate logistic regression was performed on all 15 variables; those part of stepwise regression and multivariate logistic regression analyses were those having $p < 0.1$. In latter analysis, those having $p < 0.05$ were deemed independent risk factors, and regression analysis selected variables for the nomogram based on the Akaike information criterion (AIC). Finally, a total of eight variables were screened out.

Statistical analysis

The percentage of missing data was 4.20%, and the missing ratio of each item was less than 20%. Missing data was performed with multiple imputations using IBM SPSS 26 with complete conditional specification (MCMC). The model type for scale variables was predicted mean matching (PMM), and all variables were used as predictors. To evaluate the recurrence probability of EC, factors were included in the nomogram using univariate logistic regression, multivariate logistic regression, and stepwise regression based on the minimal value of the AIC. The capacity to recognize was examined using the consistency index (C-index)/receiver operating characteristic curve (ROC), as well as related ability to calibrate was evaluated with the calibration chart. Related values have a 0.5-1.0 range denoting random to perfect probability. In general, those values > 0.7 imply acceptable estimations. For assessing the nomogram's effectiveness with respect to the ESMO-ESGO-ESTRO pattern, integrated discrimination improvement (IDI), net reclassification index (NRI), and decision curve analysis (DCA) were utilized. NRI and IDI examine prediction advancements involving risks as well as novel models' utility (20, 21); the other assesses predictive models' viability (22, 23) through calculating the net benefit at various threshold likelihoods on the nomogram.

10.3389/fendo.2023.1156169

We evaluated age distributions across the training and validation groups using one-way ANOVA, and other clinicopathological parameters, such as clinical stage, were compared using cross-tab chi-square tests. Two-sided P values were considered, with values < 0.05 deemed to have significance statistically. For all statistical studies, R v.4.0.2 or SPSS 26 were used. The main endpoint of the trial was relapse, as measured by the period between diagnosis and all-cause recurrence or the date of the final follow-up in 2018. The nomogram risk was divided into low, medium, media-high, and high risk using the 0.25, 0.5, and 0.75 cutoffs, using the model predicted value. Risk stratification based on ESMO-ESGO-ESTRO was utilized for categorizing patients as low-risk, intermediate-risk, intermediate-high-risk, or high-risk. The Kaplan-Meier curve of recurrence-free survival (RFS) was developed for assessing accuracy in prediction on each approach.

Results

Patients' characteristics

Between 2008 and 2018, a total of 671 patients were diagnosed with endometrial cancer at Nanjing Drum Tower Hospital, of whom 517 were qualified for the research. Patients lost to follow-up were excluded. All 517 patients were applied to the training cohort to construct a predictive mode. Of these patients, 70% (364) and 60% (312) were randomly selected for the validation cohort. Comparable clinical features existed across the training and validation groups (P>0.05) according to Table 1, which summarizes the clinical features of these EC patients.

In the training cohort, validation cohort 1 and validation cohort 2, the median EC patient age in years was 58. Relapsed patients accounted

TABLE 1 Clinical demographics of EC patients.

Characteristic	Whole population [cases (%)]	Validation cohort1 [cases (%)]	Validation cohort2 [cases (%)]	P value
Total	517	364	312	
Age				
Median	58	58	58	0.943
Mean	57.29	57.46	57.56	
Clinical stage				
I	398 (77.0)	286 (78.6)	244 (78.2)	0.998
II	41 (7.9)	27 (7.4)	22 (7.1)	
III	55 (10.6)	36 (9.9)	32 (10.3)	
IV	23 (4.4)	15 (4.1)	14 (4.5)	
Menopause status				
Pre	91 (17.6)	63 (17.3)	58 (18.6)	0.979
Peri	87 (16.8)	63 (17.3)	49 (15.7)	
Post	339 (65.6)	238 (65.4)	205 (65.7)	
Histologic grade				
Low grade	175 (33.8)	121 (33.2)	102 (32.7)	0.699
Media grade	246 (47.6)	171 (47.0)	139 (44.6)	
High grade	96 (18.6)	72 (19.8)	71 (22.8)	
Radiation therapy				
No	273 (52.8)	195 (53.6)	170 (54.5)	0.894
Yes	244 (47.2)	169 (46.4)	142 (45.5)	
Chemotherapy				
No	292 (56.5)	204 (56.0)	174 (55.8)	0.979
Yes	225 (43.5)	160 (44.0)	138 (44.2)	
Preoperative CA125				
Negative	384 (74.3)	275 (75.5)	224 (71.8)	0.532

(Continued)

10.3389/fendo.2023.1156169

TABLE 1 Continued

Characteristic	Whole population [cases (%)]	Validation cohort1 [cases (%)]	Validation cohort2 [cases (%)]	P value
Positive	133 (25.7)	89 (24.5)	88 (28.2)	
Positive ultrasound				
Negative	40 (7.7)	29 (8.0)	26 (8.3)	0.954
Positive	477 (92.3)	335 (92.0)	286(91.7)	
Postoperative CA125				
Negative	482 (93.2)	338 (92.9)	295 (94.6)	0.648
Positive	35 (6.8)	26 (7.1)	17 (5.4)	
Reproductive history				
Yes	469 (90.7)	332 (91.2)	285 (91.3)	0.944
No	48 (9.3)	32 (8.8)	27 (8.7)	
Histological type				
Endometrioid adenocarcinoma	470 (90.9)	333 (91.5)	281 (90.1)	0.815
Others	47 (9.1)	31 (8.5)	31 (9.9)	
Cervical invasion				
Negative	498 (96.3)	351 (96.4)	298 (95.5)	0.794
Positive	19 (3.7)	13 (3.6)	14 (4.5)	
Vascular invasion				
Negative	430 (83.2)	295 (81.0)	249 (79.8)	0.450
Positive	87 (16.8)	69 (19.0)	63 (20.2)	
Metastasis				
Negative	475 (91.9)	335 (92.0)	287 (92.0)	0.996
Positive	42 (8.1)	29 (8.0)	25 (8.0)	
Surgical approach				
No surgery	21 (4.1)	12 (3.3)	11 (3.5)	0.967
Minimally invasive	340 (65.8)	239 (65.7)	202 (64.7)	
Open	156 (30.2)	113 (31.0)	99 (31.7)	
Recurrence				
No	455 (88.0)	321 (88.2)	275 (88.1)	0.996
Yes	62 (12.0)	43 (11.8)	37 (11.9)	

Pre (<6 months since last menstrual period (LMP) AND no prior bilateral ovariectomy AND not on estrogen replacement),Peri (6-12 months since LMP),Post (>12 months since LMP or prior bilateral ovariectomy).

for 12.0%; 8.1% of patients had metastases upon diagnosis; 4.1% of patients received radiotherapy or chemotherapy without surgery; 30.2% received laparotomy; 65.8% of patients underwent minimally invasive laparoscopic or da Vinci robotic surgery.

Nomogram variable screening

In multivariate logistic regression analysis, only those having P < 0.1 in univariate regression analysis had been included, including age, clinical stage, histological grade, radiotherapy history, chemotherapy history, preoperative CA125, preoperative ultrasound results, postoperative CA125, pathological type, cervical invasion, vascular invasion, and metastasis. In multivariate logistic regression analysis, age, clinical stage, CA125 levels after surgery and surgical technique were identified as independent predictive variables for EC. The findings of stepwise regression showed that, within the training cohort, the model, including age, chemotherapy history, preoperative ultrasound results, postoperative CA125, cervical invasion, vascular invasion,

10.3389/fendo.2023.1156169

and surgical approach, had the smallest AIC value, with an AIC value of 303.03. Therefore, we included eight factors, including age, clinical stage, history of chemotherapy, positive ultrasound, postoperative CA125, cervical invasion, vascular invasion, and surgical approach, in constructing the nomogram prediction model for EC recurrence (Table 2).

TABLE 2 Univariate and multivariate logistic analyses on variables for the prediction of recurrence of EC patients.

	Univariate logistic analysis			Multivariate logistic analysis		
Variable	HR	95% CI	P value	HR	95% CI	P value
Age	1.05	1.03-1.08	**<0.001**	1.06	1.03-1.09	**<0.001**
Clinical stage						
I	1.00			1.00		
II	2.62	1.00-6.14	**0.035**	2	0.67-5.38	0.2
III	5.22	2.57-10.38	**<0.001**	2.11	0.66-6.56	0.188
IV	9.79	3.88-24.25	**<0.001**	4.09	1.19-13.81	**0.023**
Menopause_status						
Pre	1.00					
Peri	0.8	0.27-2.24	0.668			
Post	1.43	0.70-3.23	0.353			
Histologic grade						
Low grade	1.00			1.00		
Media grade	1.15	0.59-2.31	0.680	1.09	0.48-2.53	0.839
High grade	3.36	1.67-6.94	**0.001**	1.83	0.76-4.47	0.179
Radiation therapy						
No	1.00			1.00		
Yes	0.35	0.19-0.62	**0.001**	0.71	0.31-1.62	0.422
Chemotherapy						
No	1.00			1.00		
Yes	2.88	1.66-5.11	**<0.001**	1.54	0.68-3.57	0.306
Preoperative CA125						
Negative	1.00			1.00		
Positive	2.35	1.35-4.05	**0.002**	1.14	0.56-2.25	0.706
Positive ultrasound						
Negative	1.00			1.00		
Positive	0.17	0.01-0.83	**0.088**	0.3	0.02-1.63	0.258
Postoperative CA125						
Negative	1.00			1.00		
Positive	6.94	3.29-14.43	**<0.001**	5.82	2.27-14.95	**<0.001**
Reproductive history						
Yes	1.00					
No	0.78	0.35-1.97	0.563			

(Continued)

10.3389/fendo.2023.1156169

TABLE 2 Continued

	Univariate logistic analysis			Multivariate logistic analysis		
Variable	HR	95% CI	P value	HR	95% CI	P value
Histological type						
Endometrioid adenocarcinoma	1.00			1.00		
Others	0.35	0.17-0.74	**0.004**	1.06	0.43-2.81	0.897
Cervical invasion						
Negative	1.00			1.00		
Positive	5.98	2.23-15.43	**<0.001**	2.3	0.59-9.08	0.228
Vascular invasion						
Negative	1.00			1.00		
Positive	3.3	1.82-5.87	**<0.001**	1.86	0.87-3.92	0.105
Metastasis						
Negative	1.00			1.00		
Positive	2.54	1.13-5.31	**0.017**	0.49	0.15-1.51	0.221
Surgical approach						
No surgery	1.00			1.00		
Minimally invasive	0.16	0.06-0.50	**0.001**	0.11	0.03-0.44	**0.001**
Open	0.72	0.27-2.15	0.533	0.42	0.12-1.54	0.174

P values that have statistical significance (less than 0.05) were in bold. Confidence interval is denoted by CI, while hazard ratio is represented by HR.

Prediction of recurrence in women with endometrial cancer

We estimated the probability of EC recurrence by constructing two nomograms for EC recurrence based on total variables and eight screened covariates (Figure 1) that were independently associated with EC recurrence. In the nomogram model, Figure 1 demonstrates how a nomogram may be used to forecast a patient's chance of recurrence. Individual scores derived from a nomogram are used to compute total scores. The C-index value for the training cohort's nomogram was 0.851 (95% confidence interval = 0.803-0.899) (Figure 2A), while the Hosmer-Lemeshow test indicated the existence of statistical significance on the two models' difference (p = 0.850).

Nomogram validation

The C-index value for the training cohort was 0.851 (95% confidence interval = 0.803-0.899); for validation cohort 1, it was 0.847 (95% confidence interval = 0.790-0.903); for validation cohort 2, it was 0.848 (95% confidence interval = 0.786-0.911) (Figure 2B, C). In all cohorts, the curves indicating nomogram calibration demonstrated significant correlation for anticipated and experimental recurrence probabilities (Figures 2D-F). In addition, the DCA curves indicated that the nomogram predicted recurrence in EC patients with a good level of discriminating (Figures 2G-I). Taken together, our constructed nomogram for predicting EC recurrence has considerable discriminative and calibration power.

Nomogram clinical value against the ESMO-ESGO-ESTRO pattern

E. Vizza (24) et al. judged the risk of recurrence according to ESMO-ESGO-ESTRO risk classes (low, intermediate, intermediate-high, and high-risk). We compare the accuracy of the nomograph and ESMO-ESGO-ESTRO risk models using the C-index, NRI, and IDI. The ESMO-ESGO-ESTRO risk class predicted recurrence with a C-index of 0.756 (95% CI = 0.701-0.812). Therefore, our newly constructed model was more accurate in predicting recurrence than the ESMO-ESGO-ESTRO risk class (P = 0.002). It was 0.406 (95% CI = 0.058-0.576) for NRI, while it was 0.11 (95%CI = 0.058-0.162, P < 0.05) for IDI. With validation from the validation cohort (Table 3), there was greater accuracy in the nomogram than ESMO-ESGO-ESTRO-based recurrence assessment of risk for predicting EC recurrence. The DCA curves indicated that the nomogram improved recurrence prediction in both the training and validation groups of EC patients, adding more net benefit than risk classes based on ESMO-ESGO-ESTRO (Figures 3A-D). Hence, the nomogram has a good discriminative potential for predicting the recurrence of EC in patients.

Finally, risk stratification was performed according to the recurrence probability calculated from the nomogram. The LG-ESS patients were

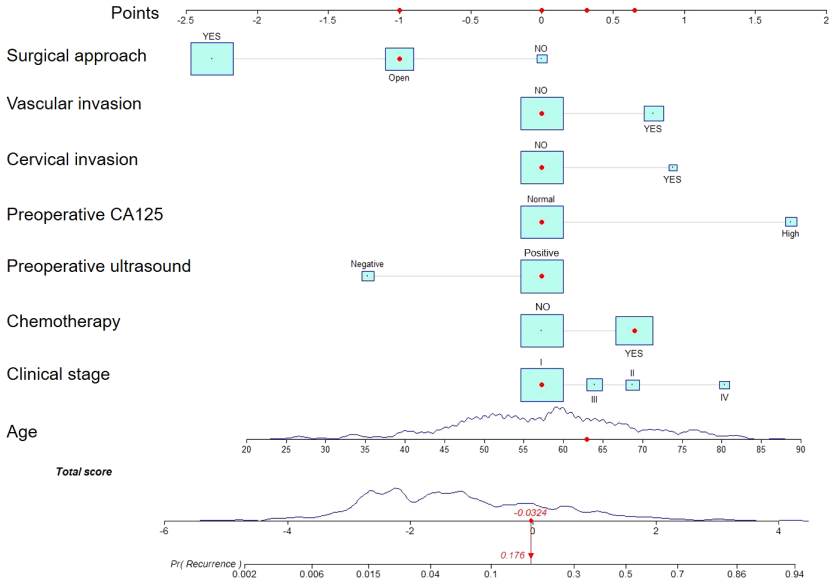


FIGURE 1
A nomogram for predicting endometrial cancer recurrence in patients. Estimating risk requires drawing a line from the patient's variable value to the "Points" axis and counting the number of points for each variable. To establish the recurrence likelihood for this patient, the total score was computed by summing the points of all factors, and a straight line was formed between the total score axis and the recurrence prediction axis.

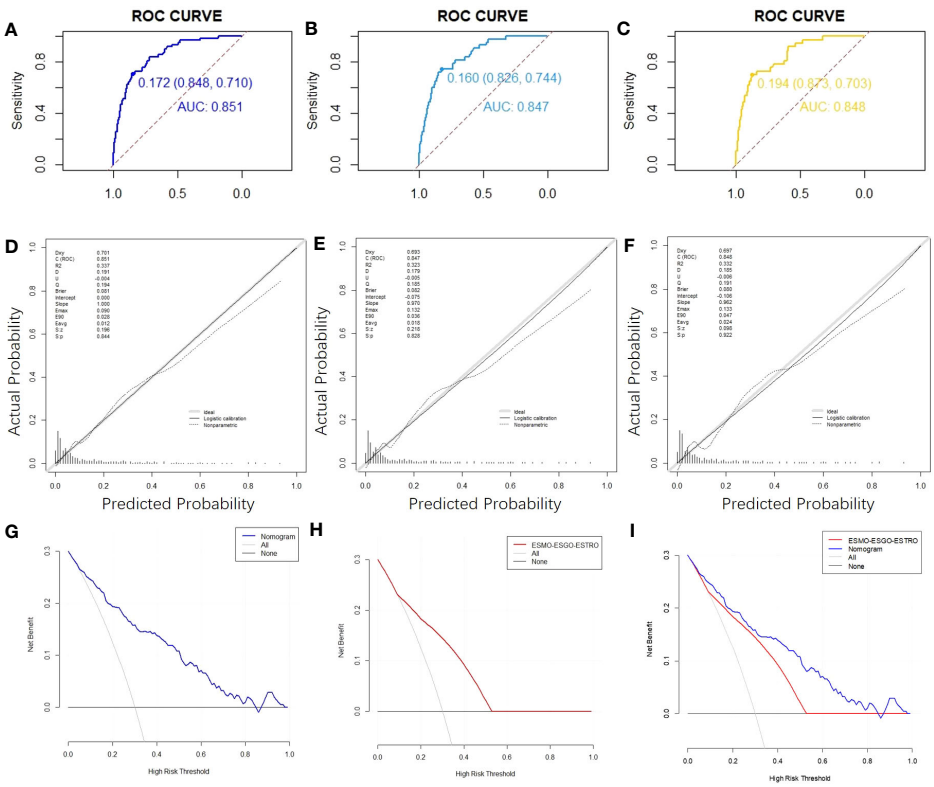


FIGURE 2
ROC curves,calibration charts and decision curve analysis of the recurrence prediction of patients with EC. **(A)** The ROC curve of the nomogram in the training cohort. **(B)** The nomogram's ROC curves in validation cohort 1. **(C)** Nomogram ROC curves in validation cohort 2. **(D)** Calibration chart of the nomogram for the training cohort's recurrence prediction of EC. **(E)** Nomogram calibration on EC recurrence prediction in validation cohort 1. **(F)** Calibration chart on EC recurrence prediction in validation cohort 2. **(G)** The nomogram's DCA curve on training cohort's EC recurrence prediction. **(H)** DCA curve of the ESMO-ESGO-ESTRO pattern for the recurrence prediction of EC in a training cohort. **(I)** Nomogram and ESMO-ESGO-ESTRO pattern comparison. DCA: Decision Curve Analysis.

10.3389/fendo.2023.1156169

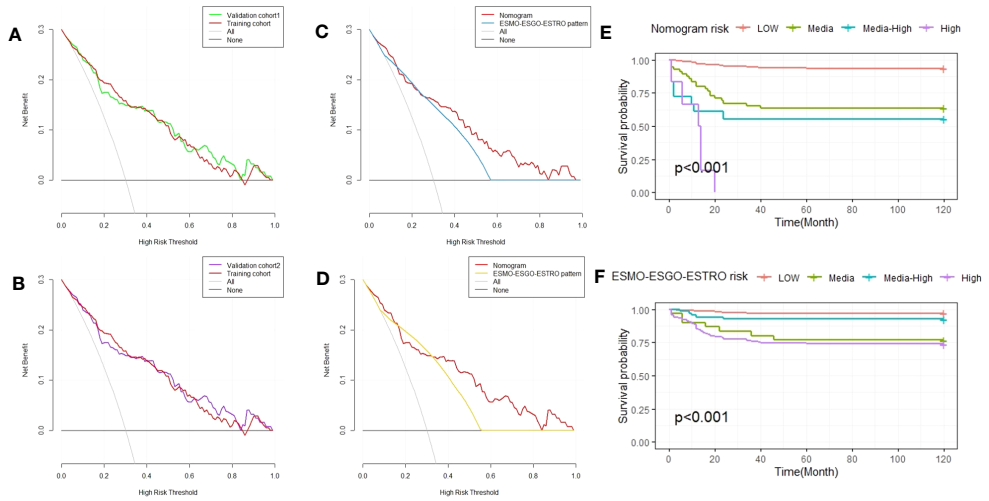


FIGURE 3
Decision curve analysis of the nomogram and ESMO-ESGO-ESTRO pattern for predicting EC recurrence, as well as Kaplan-Meier RFS curves for EC patients with varied risks. **(A)** A comparison of the validation 1 and training cohorts. **(B)** A comparison of the validation 2 and training cohorts. **(C)** Comparison of the nomogram and ESMO-ESGO-ESTRO pattern in validation cohort 1. **(D)** Comparison of the nomogram and the ESMO-ESGO-ESTRO pattern in validation cohort 2. **(E)** Nomogram-stratified Kaplan-Meier RFS curves for EC patients with varying risk levels in the training cohort. **(F)** Kaplan-Meier RFS curves of EC patients in the training group with differing risks according to classification using the ESMO-ESGO-ESTRO pattern. ESMO-ESGO-ESTRO refers to Medical Oncology, European Society of Gynaecological Oncology and European SocieTy for Radiotherapy & Oncology, while RFS denotes recurrence free survival.

separated into three groups: those at low risk (probability<0.25), those at intermediate-risk (0.25≤probability<0.5), those at intermediate-high risk (0.5≤probability<0.75), and those at high-risk (probability≥0.75). The Kaplan-Meier RFS curve demonstrated substantial difference among the four risk categories (P < 0.001). The nomogram outperforms the ESMO-ESGO-ESTRO pattern in identifying groups at high risk (Figures 3E, F).

Discussion

This research aims to develop a nomogram prediction model for endometrial cancer recurrence by gathering patient data. Using multivariate logistic regression and stepwise regression, eight variables were selected based on AIC minima and integrated into the nomogram design. In this study, all cases were used to build the endometrial cancer recurrence model, and the validation cohort was constructed by randomly selecting 70% and 60% of the patients. Previous studies have shown that age, histopathological type, myometrial invasion, FIGO stage, lymph node metastasis, lymphovascular invasion, and tumor grade are endometrial cancer recurrence risk factors (12, 25, 26). This study considers these influencing factors consistent with our model's prediction results.

However, there is still some disagreement about the impact of surgical methods. For example, previous studies have found no statistically significant difference in the recurrence risk of early-stage endometrial cancer between laparoscopic and open surgery (27), while it is surgery in our nomogram model. Modality is deemed a risk factor for recurrence of endometrial cancer. This might be since our model now includes patients with all stages of endometrial cancer, not only the early stage.

Additionally, adjuvant treatment is a disputed subject. Patients with a high risk of recurrence following surgical resection and staging of endometrial cancer may be given adjuvant chemotherapy with carboplatin and paclitaxel (28). According to Nick Johnson et al. (29), adjuvant chemotherapy alone may greatly lower the chance of endometrial cancer recurrence, especially the probability of a first recurrence beyond the pelvis, which is consistent with the findings of our predictive model. Chemotherapy considerably reduced the nomogram's AIC on our predictive model, indicating its value in predicting RFS in endometrial cancer. In contrast, radiotherapy had no significant effect on predicting the recurrence of endometrial cancer. It may be because radiotherapy patients are more advanced or have higher risk factors for recurrence. In addition, some patients received both radiotherapy and chemotherapy, which shows that excessive adjuvant therapy in endometrial cancer patients may not be beneficial.

Traditionally, the initial choice for predicting recurrence in patients with endometrial cancer has been to stratify the risk of recurrence of endometrial cancer based on the ESMO-ESGO-ESTRO consensus. Usually, this stratified model cannot accurately predict the recurrence of endometrial cancer. Such phenomena may be attributable to age, adjuvant treatment, and other characteristics not included in the ESMO-ESGO-ESTRO consensus recurrence risk categorization. Thus, we compared the variable-richer nomogram to the conventional ESMO-ESGO-ESTRO consensus conference-based recurrence risk categorization. Through C-index, NRI, IDI, and DCA curves, our nomogram predicts recurrence probability alongside a much greater clinical advantage and utilization compared to the ESMO-ESGO-ESTRO risk stratification system and can better identify high-risk groups.

TABLE 3 NRI, IDI, and C-index of the nomogram and the ESMO-ESGO-ESTRO risk classes alone in recurrence prediction for EC patients.

	Training cohort			Validation cohort1			Validation cohort2		
Index	Estimate	95% CI	P value	Estimate	95% CI	P value	Estimate	95% CI	P value
NRI	0.406	0.058-0.576		0.347	0.008-0.590		0.340	0.047-0.643	
IDI	0.110	0.058-0.162	**<0.001**	0.070	0.007-0.132	**0.028**	0.103	0.030-0.175	**0.006**
C-index(ROC)									
The nomogram	0.851	0.803-0.899	**0.002**	0.847	0.790-0.903	0.088	0.848	0.786-0.911	**0.035**
The ESMO-ESGO-ESTRO risk classes	0.756	0.701-0.812		0.787	0.725-0.849		0.778	0.710-0.846	

NRI, net reclassification index; IDI, integrated discrimination improvement, C-index, concordance index.
P values that have statistical significance (less than 0.05) were in bold.

The nomogram has shown possible clinical capacity. Data from all patients diagnosed with endometrial cancer in Nanjing Drum Tower Hospital between 2008 and 2018 were used, which represents different types of populations. We calculated the C-index or AUC, calibration curve, DCA curve, and others to evaluate the model, validating these results with a validation cohort. Taken together, our nomogram is a viable tool for determining the recurrence likelihood of endometrial cancer patients, and it may have therapeutic relevance for the postoperative monitoring and early diagnosis of disease recurrence in endometrial cancer patients. Despite the excellent performance of the nomogram, there are limits to this research. For example, some of the collected information is missing. In addition, clinical validation across multiple centers may be necessary for assessing nomograms' external usefulness.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The data set is from a single medical center and has limitations. Requests to access these datasets should be directed to Mengdan Miao, mengdan_miao@njmu.edu.cn.

Ethics statement

The studies involving humans were approved by The Medical Ethics Committee of Affiliated Drum Tower Hospital, Medical School, Nanjing University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

MM prepared the manuscript. All authors analyzed the data, read, and approved the final manuscript. HZ conceived and supervised the project. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (NSFC 81972434,NSFC 82172819) to HZ and Jiangsu Province Association of Maternal and Child Health (FYX202030) to RL.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Crosbie EJ, Kitson SJ, McAlpine JN, Mukhopadhyay A, Powell ME, Singh N. Endometrial cancer. *Lancet* (2022) 399(10333):1412–28. doi: 10.1016/S0140-6736(22)00323-3

2. Brooks RA, Fleming GF, Lastra RR, Lee NK, Moroney JW, Son CH, et al. Current recommendations and recent progress in endometrial cancer. *CA Cancer J Clin* (2019) 69(4):258–79. doi: 10.3322/caac.21561

3. Lortet-Tieulent J, Ferlay J, Bray F, Jemal A. International patterns and trends in endometrial cancer incidence, 1978-2013. *J Natl Cancer Inst* (2018) 110(4):354–61. doi: 10.1093/jnci/djx214

4. Islami F, Ward EM, Sung H, Cronin KA, Tangka FKL, Sherman RL, et al. Annual report to the nation on the status of cancer, part 1: national cancer statistics. *J Natl Cancer Inst* (2021) 113(12):1648–69. doi: 10.1093/jnci/djab131

5. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2021. *CA Cancer J Clin* (2021) 71(1):7–33. doi: 10.3322/caac.21654

6. World Health Organization (WHO). *World cancer report 2020* (2020). Available at: https://www.iarc.fr/cards_page/world-cancer-report/.

7. Hopkins BD, Goncalves MD, Cantley LC. Obesity and cancer mechanisms: cancer metabolism. *J Clin Oncol* (2016) 34(35):4277–83. doi: 10.1200/JCO.2016.67.9712

8. Colombo N, Creutzberg C, Amant F, Bosse T, González-Martín A, Ledermann J, et al. ESMO-ESGO-ESTRO Consensus Conference on Endometrial Cancer: diagnosis, treatment and follow-up. *Ann Oncol* (2016) 27(1):16–41. doi: 10.1093/annonc/mdv484

9. Siegenthaler F, Lindemann K, Epstein E, Rau TT, Nastic D, Ghaderi M, et al. Time to first recurrence, pattern of recurrence, and survival after recurrence in endometrial cancer according to the molecular classification. *Gynecol Oncol* (2022) 165(2):230–8. doi: 10.1016/j.ygyno.2022.02.024

10. Beavis AL, Yen TT, Stone RL, Wethington SL, Carr C, Son J, et al. Adjuvant therapy for early stage, endometrial cancer with lymphovascular space invasion: Is there a role for chemotherapy? *Gynecol Oncol* (2020) 156(3):568–74. doi: 10.1016/j.ygyno.2019.12.028

11. Ouldamer L, Bendifallah S, Body G, Touboul C, Graesslin O, Raimond E, et al. Predicting poor prognosis recurrence in women with endometrial cancer: a nomogram developed by the FRANCOGYN study group. *Br J Cancer* (2016) 115(11):1296–303. doi: 10.1038/bjc.2016.337

12. Oaknin A, Bosse TJ, Creutzberg CL, Giornelli G, Harter P, Joly F, et al. Endometrial cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol* (2022) 33(9):860–77. doi: 10.1016/j.annonc.2022.05.009

13. Bendifallah S, Ouldamer L, Lavoue V, Canlorbe G, Raimond E, Coutant C, et al. Patterns of recurrence and outcomes in surgically treated women with endometrial cancer according to ESMO-ESGO-ESTRO Consensus Conference risk groups: Results from the FRANCOGYN study Group. *Gynecol Oncol* (2017) 144(1):107–12. doi: 10.1016/j.ygyno.2016.10.025

14. Colombo N, Creutzberg C, Amant F, Bosse T, González-Martín A, Ledermann J, et al. ESMO-ESGO-ESTRO consensus conference on endometrial cancer: Diagnosis, treatment and follow-up. *Radiother Oncol* (2015) 117(3):559–81. doi: 10.1016/j.radonc.2015.11.013

15. Balachandran VP, Gonen M, Smith JJ, DeMatteo RP. Nomograms in oncology: more than meets the eye. *Lancet Oncol* (2015) 16(4):e173–80. doi: 10.1016/S1470-2045(14)71116-7

16. Gafita A, Calais J, Grogan TR, Hadaschik B, Wang H, Weber M, et al. Nomograms to predict outcomes after (177)Lu-PSMA therapy in men with metastatic castration-resistant prostate cancer: an international, multicentre, retrospective study. *Lancet Oncol* (2021) 22(8):1115–25. doi: 10.1016/S1470-2045(21)00274-6

17. Wang R, Dai W, Gong J, Huang M, Hu T, Li H, et al. Development of a novel combined nomogram model integrating deep learning-pathomics, radiomics and immunoscore to predict postoperative outcome of colorectal cancer lung metastasis patients. *J Hematol Oncol* (2022) 15(1):11. doi: 10.1186/s13045-022-01225-3

18. Khosrotehrani K, Ploeg der van AP, Siskind V, Hughes MC, Wright A, Thomas J, et al. Nomograms to predict recurrence and survival in stage IIIB and IIIC melanoma after therapeutic lymphadenectomy. *Eur J Cancer* (2014) 50(7):1301–9. doi: 10.1016/j.ejca.2014.02.010

19. Pan YX, Chen JC, Fang AP, Wang XH, Chen JB, Wang JC, et al. A nomogram predicting the recurrence of hepatocellular carcinoma in patients after laparoscopic hepatectomy. *Cancer Commun (Lond)* (2019) 39(1):55. doi: 10.1186/s40880-019-0404-6

20. Uno H, Tian L, Cai T, Kohane IS, Wei LJ, et al. A unified inference procedure for a class of measures to assess improvement in risk prediction systems with survival data. *Stat Med* (2013) 32(14):2430–42. doi: 10.1002/sim.5647

21. Pencina MJ, Agostino RBD', Steyerberg EW. Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. *Stat Med* (2011) 30(1):11–21. doi: 10.1002/sim.4085

22. Wu J, Zhang H, Li L, Hu M, Chen L, Xu B, et al. A nomogram for predicting overall survival in patients with low-grade endometrial stromal sarcoma: A population-based analysis. *Cancer Commun (Lond)* (2020) 40(7):301–12. doi: 10.1002/cac2.12067

23. Van Calster B, Wynants L, Verbeek JFM, Verbakel JY, Christodoulou E, Vickers AJ, et al. Reporting and interpreting decision curve analysis: A guide for investigators. *Eur Urol* (2018) 74(6):796–804. doi: 10.1016/j.eururo.2018.08.038

24. Vizza E, Cutillo G, Bruno V, Sperduti I, Mancini E, Baiocco E, et al. Pattern of recurrence in patients with endometrial cancer: A retrospective study. *Eur J Surg Oncol* (2020) 46(9):1697–702. doi: 10.1016/j.ejso.2020.03.203

25. Restaino S, Tortorella L, Dinoi G, Zannoni GF, Baroni A, Capasso I, et al. Semiquantitative evaluation of lymph-vascular space invasion in patients affected by endometrial cancer: Prognostic and clinical implications. *Eur J Cancer* (2021) 142:29–37. doi: 10.1016/j.ejca.2020.10.011

26. Viola L, Londero AP, Bertozzi S, Orsaria M, Marzinotto S, Antoniazzi F, et al. Prognostic role of krüppel-like factors 5, 9, and 11 in endometrial endometrioid cancer. *Pathol Oncol Res* (2020) 26(4):2265–72. doi: 10.1007/s12253-020-00817-z

27. Galaal K, Donkers H, Bryant A, Lopes AD. Laparoscopy versus laparotomy for the management of early stage endometrial cancer. *Cochrane Database Syst Rev* (2018) 10(10):Cd006655. doi: 10.1002/14651858.CD006655.pub3

28. Bestvina CM, Fleming GF. Chemotherapy for endometrial cancer in adjuvant and advanced disease settings. *Oncologist* (2016) 21(10):1250–9. doi: 10.1634/theoncologist.2016-0062

29. Johnson N, Bryant A, Miles T, Hogberg T, Cornes P. Adjuvant chemotherapy for endometrial cancer after hysterectomy. *Cochrane Database Syst Rev* (2011) 2011(10):Cd003175. doi: 10.1002/14651858.CD003175.pub2

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 16 November 2023
DOI 10.3389/fendo.2023.1272200

OPEN ACCESS

EDITED BY
Iveta Yotova,
Medical University of Vienna, Austria

REVIEWED BY
Abdulsamed Kükürt,
Kafkas University, Türkiye
Carla Azevedo Piccinato,
University of Sao Paulo, Brazil

*CORRESPONDENCE
Songling Zhang
slzhang@jlu.edu.cn

†These authors have contributed equally to this work and share first authorship

RECEIVED 03 August 2023
ACCEPTED 30 October 2023
PUBLISHED 16 November 2023

CITATION
Bai C, Shen Z, Qiu B and Zhang S (2023) Leukocyte telomere length is associated with increased risk of endometriosis: a bidirectional two-sample Mendelian randomization study.
Front. Endocrinol. 14:1272200.
doi: 10.3389/fendo.2023.1272200

Leukocyte telomere length is associated with increased risk of endometriosis: a bidirectional two-sample Mendelian randomization study

Chenxue Bai[1†], Zixiong Shen[2†], Binxu Qiu[3] and Songling Zhang[1*]

[1]Department of Obstetrics and Gynecology, The First Hospital of Jilin University, Changchun, China,
[2]Department of Thoracic Surgery, The First Hospital of Jilin University, Changchun, China,
[3]Department of Gastrointestinal Surgery, The First Hospital of Jilin University, Changchun, China

Background: Endometriosis (EMs) is a common gynecological disorder. Observational studies on the relationship between leukocyte telomere length (LTL) and EMs have shown conflicting results. The purpose of this study was to evaluate the precise causal relationship between LTL and EMs using Mendelian randomization (MR) methodology.

Methods: We employed MR to assess the causal relationship between LTL and EMs. Summary data from several large-scale genome-wide association studies (GWAS) were used for bidirectional two-sample MR analysis. Sensitivity analyses were conducted to ensure the robustness of our results. All analyses were also replicated in another completely independent EMs dataset.

Results: Our MR analysis indicated that genetically predicted longer LTL increased the risk of EMs (IVW: discovery, OR=1.169, 95%CI: 1.059-1.290, p=0.002; validation, OR=1.302, 95%CI: 1.140-1.487, p=0.000), while EMs had no causal impact on LTL (IVW: discovery, OR=1.013, 95%CI: 1.000-1.027, p=0.056; IVW: validation, OR=1.005, 95%CI: 0.995-1.015, p=0.363). Causal estimates were supported by various calculation models (including MR-Egger, Weighted median, MR-PRESSO, and MR-RAPS). Heterogeneity and pleiotropy analyses also indicated robustness of the results.

Conclusion: Our findings substantiate the idea that a genetically predicted longer LTL elevates the risk of EMs, with no influence of EMs on LTL risk. This research bolsters the causal link between LTL and EMs, overcoming the constraints of earlier observational studies. It implies that LTL may potentially function as a biomarker for EMs, opening up novel possibilities for EMs prevention and treatment.

KEYWORDS

endometriosis, telomere length, leukocyte telomere length, Mendelian randomization, endometrium

Introduction

Endometriosis (EMs) is a prevalent and intricate gynecological ailment characterized by the growth of endometrial-like tissue outside the uterine cavity, such as in the pelvic area, ovaries, and fallopian tubes (1). EMs poses a challenge for 5-10% of reproductive-aged women, often manifesting as pain, dysfertility, and discomfort during intercourse (2). The exact etiology of EMs remains unclear, with theories encompassing retrograde endometrial transplantation, embryonic developmental abnormalities, immune system aberrations, and genetic factors (3).

Telomeres are repetitive DNA sequences and associated proteins located at the ends of chromosomes (4). During cell division, a small portion of DNA is lost from the ends of chromosomes (4). Telomeres play a vital role in preserving the stability of crucial genes on chromosomes, and their length shortens with an increasing number of cell divisions (5). Critically short telomeres may lead cells to enter a state of aging or cease division, believed to be closely related to organismal aging (6). Conversely, longer telomeres play a significant role in maintaining cellular stability, delaying cell aging, sustaining stem cell function, and preventing cell apoptosis (6).

However, the relationship between TL and EMs has been a topic of debate. Studies suggest that TL may be associated with various gynecological diseases, including EMs (7). Additionally, the chronic inflammation associated with EMs may have an adverse effect on TL (8). One study (9) collected data from 877 women in New England (53 cases and 824 controls), revealing an association between shorter LTL and EMs (OR=2.56, 95%CI: 1.16-5.63; p=0.02). Conversely, another study (86 cases and 21 controls) found that EMs patients had higher peripheral blood LTL compared to the control group (OR=8.1, 95%CI: 1.28-51.57; p=0.0264) (10). A recent machine learning study (11) also identified telomere-related genes associated with EMs development, although their EMs sample was limited to 28 cases. These observational studies provide clinical evidence for the correlation between LTL and EMs, but unfortunately, their conclusions are not consistent. Moreover, their sample sizes are generally small, posing a risk of low statistical power. Therefore, conducting a large-scale study to explore the correlation between TL and EMs is necessary. Rahmioglu et al. (12) conducted a genome-wide association study (GWAS) meta-analysis of EMs, identifying 42 single nucleotide polymorphisms (SNPs) significantly associated with EMs. They comprehensively analyzed the genetic correlations between EMs and various pain and inflammatory diseases. However, they did not analyze the correlation between LTL and EMs. Nevertheless, their study provides data support for our research.

Mendelian randomization (MR) can be considered a natural randomized controlled trial (RCT) using genetic variations (typically SNPs) as instrumental variables (IV) for causal inference (13). MR is less susceptible to environmental influences because genetic variations are randomly allocated during meiosis and persist throughout a person's lifetime (14). MR effectively circumvents confounding and reverse causation in observational studies and addresses the challenges of implementing RCTs (13, 14). By leveraging genetic information and large-scale GWAS, MR allows us to explore whether genetically predicted LTL contributes to the development of EMs and whether EMs, in turn, causally affect LTL.

To overcome the limitations of existing observational studies, we conducted a bidirectional two-sample MR study using large-scale GWAS data to reveal the causal relationship between LTL and EMs. Our study results, based on robust statistical methods and replication in an independent EMs dataset, provide compelling evidence. Our findings suggest that genetically predicted longer LTL increases the risk of EMs, while EMs do not causally impact LTL. These results not only enhance our understanding of the interplay between LTL and EMs but also emphasize the potential of LTL as a valuable biomarker for EMs. These insights could potentially alter our approaches to preventing and treating EMs, providing new pathways for therapeutic interventions and personalized care strategies.

Method

Study design

Figure 1 is a brief description of this study. This study is based on three basic assumptions of MR (15): I) The IV is associated with the exposure; II) The instrumental variables are independent of any known or unknown confounders that mediate the exposure to the outcome; III) The outcome is associated with the genetic instrument only through the effect of the exposure.

Data Source

We utilized summary data from several large-scale GWAS studies in this research. Summary data for LTL were obtained from the UK Biobank (16), which comprised 472,174 individuals of European ancestry. LTL was defined as the average leukocyte telomere length measured using a multiplex quantitative polymerase chain reaction assay in a mixed leukocyte population, and then log-transformed to approximate a normal distribution (16). The GWAS data for EMs (discovery) was sourced from the meta-analysis conducted by Rahmioglu et al. (12) and included 21,779 European ancestry EMs cases and 449,087 European ancestry controls. The definition of EMs (discovery) encompassed a mix of surgically confirmed cases, medical records, and self-reported cases. For EMs (validation), the data were obtained from

Abbreviations: EMs, endometriosis; TL, telomere length; LTL, leukocyte telomere length; GWAS, genome-wide association studies; MR, Mendelian randomization; RCT, randomized controlled trial; IV, instrumental variable; SNP, single nucleotide polymorphism; OR, odds ratios; IVW, inverse variance weighting; MR-Egger, MR-Egger regression; MR-PRESSO, Mendelian Randomization Pleiotropy RESidual Sum and Outlier test; MR-RAPS, Mendelian Randomization Robust Adjusted Profile Score.

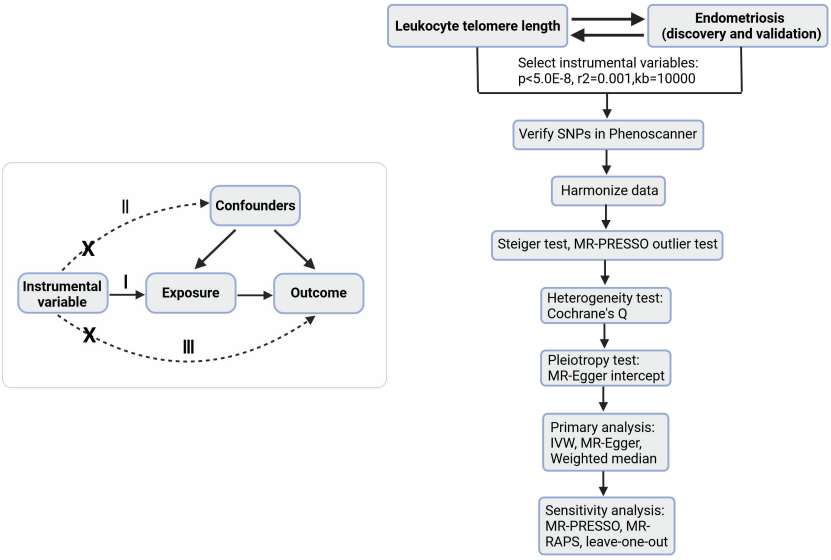


FIGURE 1
A brief description of the study. On the left is a bidirectional acyclic graph, on the right is the analysis flow of this study. I, assumption I; II, assumption II; III, assumption III; MR-Egger, MR-Egger regression; MR-PRESSO, Mendelian Randomization Pleiotropy RESidual Sum and Outlier test; MR-RAPS, Mendelian Randomization Robust Adjusted Profile Score.

FinnGen (17) and comprised 15,088 cases of European ancestry and 107,564 controls of European ancestry. The definition of EMs (validation) was based on a mix of International Classification of Diseases 10 (ICD-10), ICD-9, and ICD-8 codes. Ethical approvals had been obtained for each GWAS dataset in their original studies, and our study solely utilized summary data, obviating the need for additional ethical approval. Table 1 provides a brief overview of the GWAS data utilized in this study and how it was acquired.

Instrumental variable

We employed a significance threshold of p<5.0E-08 to identify SNPs significantly associated with both LTL and EMs. Stringent criteria were applied to remove linkage disequilibrium, with an aggregation window set at 10,000 kb and an r2 threshold set at 0.001. We calculated the F-statistic for each SNP and the overall F-statistic for the set of SNPs. The F-statistic for an individual SNP was determined using the following formula (18): $F = \frac{beta^2}{se^2}$, where "beta" is the effect of the instrumental variable (IV) on the exposure, and "se" is the standard error of "beta." The overall F-statistic was calculated using the following formula (18): $F = \frac{N-K-1}{K} \times \frac{R^2}{1-R^2}$ $R^2 = 2 \times eaf \times (1 - eaf) \times beta^2$, where "N" is the sample size for the exposure, "K" is the number of SNPs, "R^2" is the proportion of exposure variance explained by SNPs, "eaf" is the effect allele frequency of the SNP, and "beta" is the effect of the SNP on the exposure. An F-statistic greater than 10 indicates a robust association between the SNP and the phenotype (19). We searched all SNPs in PhenoScanner to identify any SNPs related to potential confounders or outcomes. We then harmonized exposure and outcome data and excluded palindromic SNPs with moderate allele frequencies. Finally, we conducted an MR Steiger test to ensure the correct direction of causality (20) and removed SNPs that had a greater impact on the outcome than the exposure.

Statistical analysis

We conducted a bidirectional two-sample MR analysis using LTL and Ems (discovery and validation). The primary analysis utilized the Inverse Variance Weighting (IVW) random-effects model, and we used MR-Egger regression and Weighted Median as validation methods. Heterogeneity was assessed using I^2 and Cochran's Q-value (21, 22), with I^2>90% indicating reliable results (21). Assessment of the magnitude of pleiotropy was done by examining funnel plot symmetry and the difference in the intercept of MR-Egger regression from zero (23). Further sensitivity analysis was performed using Mendelian Randomization Pleiotropy RESidual Sum and Outlier test (MR-PRESSO) (24) and Mendelian Randomization Robust Adjusted Profile Score (MR-RAPS) (25) to address potential pleiotropy and weak instrument bias. MR-PRESSO was used to detect and correct for horizontal pleiotropy, with a distribution of MR-PRESSO set to 5000, and signifthreshold set to 0.05 in this study (24). MR-RAPS allowed for causal reevaluation after accounting for residual variance, effectively addressing horizontal pleiotropy and weak instrument bias (25). Finally, we conducted a leave-one-out analysis to identify individual SNPs that significantly affected the causal estimates.

MR results were presented in the form of odds ratios (OR) to establish the direction of causality (26). All analyses were performed using R software version 4.2.3 (https://www.r-project.org/). We used R packages such as "TwoSampleMR," "MR-PRESSO," and "mr.raps" for MR analysis, and data visualization was carried out using "TwoSampleMR" and "forestploter." Instructions for using these packages can be found on GitHub (https://github.com).

TABLE 1 A brief description of each GWAS summary statistics.

Trait	Study/Consortium	Ancestry	Sample size	Cases definition	Data available
LTL	UK Biobank	European	472,174	The average leukocyte telomere length in a mixed white blood cell population measured using multiplex quantitative polymerase chain reaction technology.	"https://gwas.mrcieu.ac.uk/"; ID: "ieu-b-4879"
EMs (discovery)	The Women's Health Study: From Adolescence to Adulthood; Crete dataset; DeCODE Genetics; The ENDOX study and Liverpool datasets; The ENDOX study part 2 and Liverpool and Edinburgh datasets; Leuven dataset; Lodz dataset; Melbourne dataset; Oxford Endometriosis Gene Study; Queensland Institute of Medical Research and Hunter; Community Study; University of California, San Francisco; Vanderbilt Biorepository; Danish Blood Donor Study; Generation Scotland: Scottish Family Health Study; The Estonian Biobank Cohort; Northern Finland Birth Cohort; Nurses' Health Study II; UK Biobank dataset; QSkin Sun and Health Study; Twins UK; The Women's Genome Health Study.	European	21,779 cases and 449,087 controls	Surgically confirmed (7593 cases); Medical records (797 cases); Self-reported (2791 cases); Mixed: Surgically confirmed + medical records (1716 cases); Mixed: Surgically confirmed + self-reported (2104 cases); Mixed: Medical records + self-reported (6778 cases).	"https://www.ebi.ac.uk/gwas"; ID: "GCST90269970"
EMs (validation)	FinnGen	European	15,088 cases and 107,564 controls	ICD-10-N80, ICD-9-617, ICD-8-6253	"https://www.finngen.fi/en"; ID: "N14_ENDOMETRIOSIS"

LTL, leukocyte telomere length; EMs, Endometriosis; ICD, International Classification of Diseases.

Lastly, we used the mRnd tool (27) to calculate the statistical power of the MR analysis (https://shiny.cnsgenomics.com/mRnd/).

Results

Instrumental variable

In the MR analysis with LTL as the exposure, initially, 154 SNPs were selected as Ivs for LTL. In PhenoScanner, these SNPs were not found to be associated with any potential confounders or outcomes. After removing palindromic SNPs and those missing in the outcome, 125 and 117 SNPs remained for the discovery and validation analyses, respectively. The Steiger test and MR-PRESSO outlier test did not identify any anomalous SNPs. Each SNP in this subset had an F-statistic greater than 10. The R^2 for the 125 SNPs was 3.16%, with a total F-statistic of 123.33. For the 117 SNPs, the R^2 was 2.93%, with a total F-statistic of 121.88.

In the MR analysis with Ems as the exposure, initially, 22 and 27 SNPs were selected as Ivs for Ems (discovery and validation, respectively). In PhenoScanner, these SNPs were not found to be associated with any potential confounders or outcomes. After removing palindromic SNPs and those missing in the outcome, 19 and 23 SNPs remained for the discovery and validation analyses. The Steiger test did not identify any anomalous SNPs. The MR-PRESSO outlier test detected 1 anomalous SNP in the Ems(validation) analysis, which was excluded from subsequent analysis. In the end, 19 and 22 SNPs were used for the discovery and validation analyses of Ems as the exposure. Each SNP in this subset had an F-statistic greater than 10. The R2 for the 19 SNPs was 6.41%, with a total F-statistic of 1696.01, and for the 22 SNPs, the R2 was 9.19%, with a total F-statistic of 564.23.

Table S1, Table S2 and Table S3 of supplementary tables provide the details of the initial selection of all SNPs in PhenoScanner in this study. Table S4 contains information on SNPs that were not used in the final analysis. Table S5 presents details on the SNPs used in the final analysis of this study.

MR results and sensitivity analysis

Causal estimates

In summary, as depicted in Figures 2, 3, our results suggest that genetically predicted longer LTL increases the risk of Ems, while Ems does not have a causal impact on LTL. The three primary methods, IVW, MR-Egger, and Weighted Median, consistently support the direction of causality (Figure 3). Our causal estimates were further validated in another entirely independent dataset of Ems. A series of sensitivity analyses further underline the robustness of our findings.

When LTL was considered as the exposure, the IVW analysis revealed a significant positive causal relationship between genetically predicted LTL and Ems (discovery, OR=1.169, 95%CI: 1.059-1.290, p=0.002; validation, OR=1.302, 95%CI: 1.140-1.487, p=1.02E-04). The IVW results in the discovery were supported by MR-Egger (OR=1.279, 95%CI: 1.075-1.521, p=0.006), and the

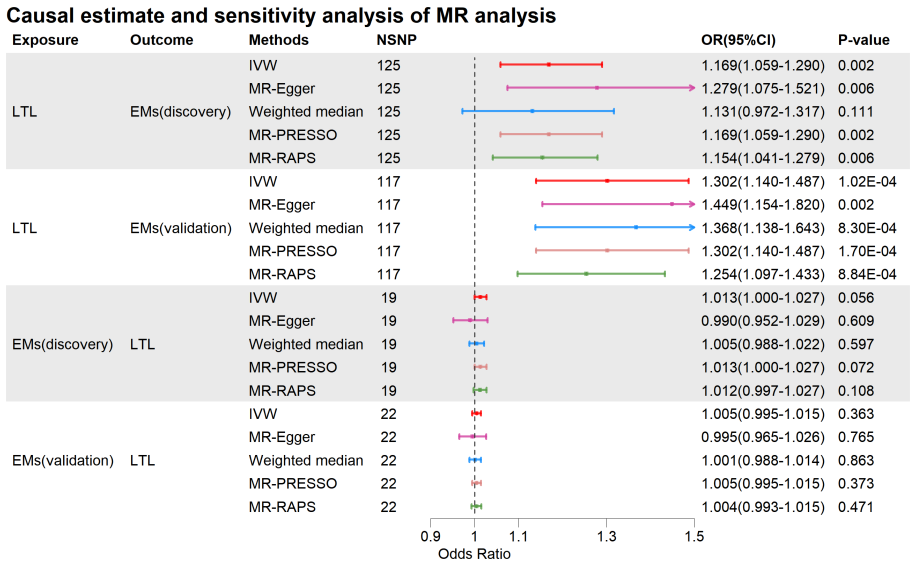


FIGURE 2
Causal estimate and sensitivity analysis of MR analysis. MR, Mendelian Randomization; NSNP, Number of SNPs; OR, odds ratio; LTL, leukocyte telomere length; Ems, Endometriosis; IVW, inverse variance weighting; MR-Egger, MR-Egger regression; MR-PRESSO, Mendelian Randomization Pleiotropy RESidual Sum and Outlier test; MR-RAPS, Mendelian Randomization Robust Adjusted Profile Score.

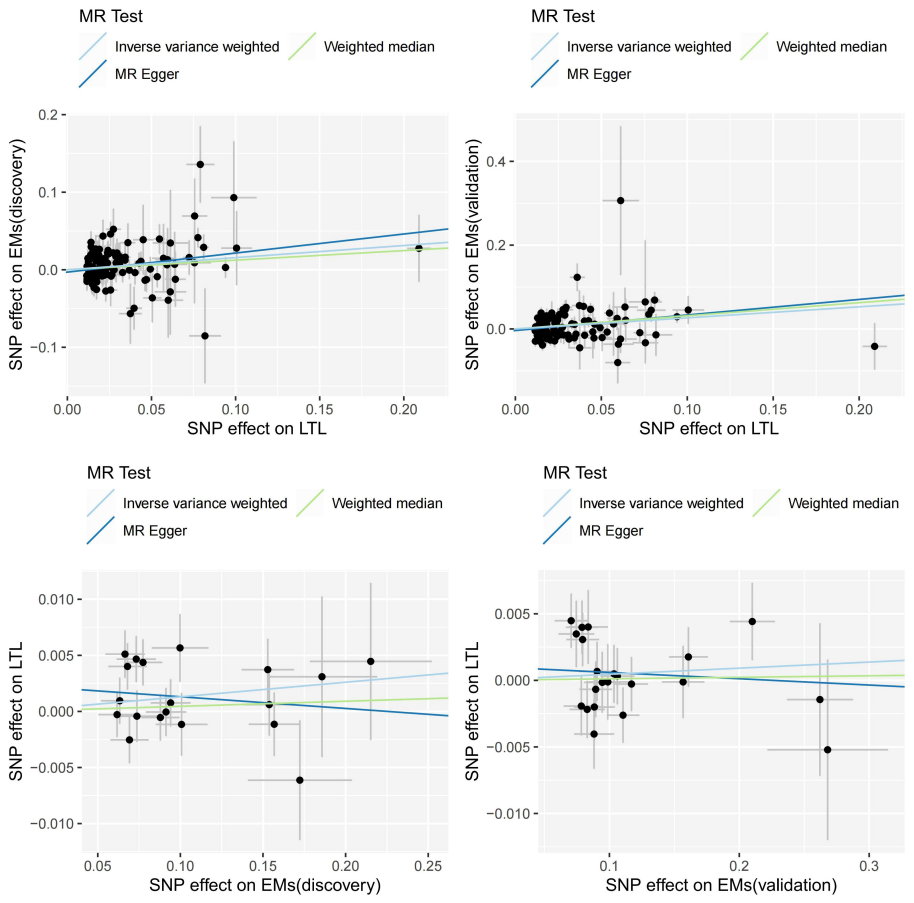


FIGURE 3
Scatter plots of MR analysis. MR, Mendelian Randomization; LTL, leukocyte telomere length; Ems, Endometriosis; SNP, single nucleotide polymorphism.

Weighted Median maintained consistency with IVW in the direction of causal estimation (OR=1.131, 95%CI: 0.972-1.317, p=0.111). The IVW results in validation were supported by MR-Egger (OR=1.449, 95%CI: 1.154-1.820, p=0.002) and Weighted Median (OR=1.368, 95%CI: 1.138-1.643, p=8.30E-04). Furthermore, in both the discovery and validation, MR-PRESSO and MR-RAPS also supported the positive causal relationship between genetically predicted LTL and Ems. The mRnd tool calculated a statistical power of 100% for the MR analysis of LTL to Ems (discovery) and Ems (validation).

When Ems was used as the exposure, there was no evidence of a causal impact of Ems on LTL (IVW: discovery, OR=1.013, 95%CI: 1.000-1.027, p=0.056; validation, OR=1.005, 95%CI: 0.995-1.015, p=0.363). MR-Egger, Weighted Median, MR-PRESSO, and MR-RAPS also indicated that Ems did not causally affect LTL. More detailed MR causal estimation results are provided in Supplementary Table S6.

Sensitivity Analysis

We assessed the heterogeneity and horizontal pleiotropy in our MR analysis. The results of heterogeneity and pleiotropy analyses in this study are presented in Table 2. Figures S1 and S2 depict funnel plots and leave-one-out analyses for Ems as both the outcome and exposure.

In MR analyses with LTL as exposure or outcome, some degree of heterogeneity was observed only in the MR analysis of LTL to Ems (validation). However, the MR-Egger intercept did not significantly differ from zero, and the leave-one-out analysis did not identify any single SNP with a significant impact on causal estimation results. This suggests that the presence of heterogeneity does not significantly affect the causal estimation results, and our results remain reliable. Additionally, in all other MR analyses, significant heterogeneity and horizontal pleiotropy were not observed, indicating high reliability and reproducibility of the results.

Discussion

The exact cause of Ems remains uncertain. Current perspectives suggest it may be associated with embryonic development, degenerative physiological changes, immune factors, and genetic factors (3). Ems poses significant psychological and physiological burdens on women worldwide and their families. Over the past decade, numerous treatment approaches for Ems have emerged. Hormone therapy has shown promise (28), but it can lead to menstrual cycle changes, breast tenderness, mood swings, and headaches, and relapse is common upon discontinuation. Surgical intervention combined with medication is considered the gold standard for Ems treatment (29). However, surgery doesn't address the root cause of Ems and can bring about tissue damage and a substantial economic burden that many patients find challenging to bear (29). Therefore, further research into the etiology of Ems can aid in disease prevention and early intervention, helping identify high-risk individuals. This is also of significant importance for drug development and adjusting treatment strategies to provide better medical care and psychological support for patients.

Rahmioglu et al. (12) conducted a comprehensive GWAS meta-analysis of Ems, identifying 42 significantly associated SNPs. They also analyzed the genetic correlations between Ems and various pain and inflammatory disorders, providing comprehensive insights into the associations between Ems and many diseases. However, there is currently no large-sample study on the correlation between LTL and Ems. Some previous observational studies suggested a link between LTL and Ems, but their conclusions were inconsistent. A case-control study included two large population studies to investigate the association between LTL and Ems (9). One group from New England, comprising 877 women (53 cases and 824 controls), showed a significant association with shorter LTL (OR=2.56, 95% CI: 1.16-5.63; p=0.02). The other group from the National Health and Nutrition Examination Survey, including 2268 women (151 cases and 2117 controls), indicated a similar but weaker association (OR=1.29, 95% CI: 0.85-1.96, p=0.22). Gleason et al. (30) reviewed data from the 1999-2002 National Health and Nutrition Examination Survey in the United States, finding that Ems patients had a shorter average LTL (-3.4, 95%CI: -7.3 to -0.3, p<0.05), and the LTL of Ems patients shortened by 1% per year. However, another observational study (86 cases and 21 controls) found that peripheral blood LTL in Ems patients was higher than in the control group (OR=8.1, 95%CI: 1.28-51.57; p=0.0264) (10). Some studies also investigated the association between TL in endometrial cells themselves and Ems. One study involving 29 cases and 27 controls measured the average TL of endometrial cells (31), and the results showed significantly longer TL in the Ems group (p = 0.005). Another study (32) measured replication characteristics and telomere length in endometrial cells of 38 Ems patients, indicating stronger replication status and longer average TL (p<0.05). These observational studies suggest that the causal direction between LTL and Ems remains unclear.

We addressed some limitations of observational studies in this research, providing new evidence to clarify the causal relationship

TABLE 2 Heterogeneity and pleiotropy of MR analysis.

Exposure	Outcome	Q	P-value	I^2(%)	PRESSO-RSSobs	P-PRESSO	Egger-intercept	P-Egger
LTL	EMs(discovery)	148.22	0.068	99.20	151.09	0.062	-0.0031	0.221
LTL	EMs(validation)	202.03	0.000	99.18	205.70	0.000	-0.0038	0.259
EMs(discovery)	LTL	24.10	0.152	98.05	26.53	0.175	0.0024	0.231
EMs(validation)	LTL	24.54	0.268	98.35	26.54	0.288	0.0011	0.535

Q, Cochrane's Q; I^2, I squared; P-value, p-value of Q; PRESSO-RSSobs, RSSobs of Global Test in MR-PRESSO; P-PRESSO, p-value of PRESSO-RSSobs; Egger-intercept, intercept of MR-Egger; P-Egger, p-value of Egger-intercept; LTL, leukocyte telomere length; EMs, Endometriosis.

between LTL and Ems. Our findings support a causal impact of LTL on Ems, rather than the other way around. Compared to previous observational studies, this research boasts several unique advantages. MR analysis is an effective epidemiological method that can overcome issues like confounding bias and reverse causality, which are challenging to resolve in some observational studies. This study also mitigated the limitations of smaller sample sizes in previous observational studies, offering more reliable causal inferences. Our study had a sufficiently large sample size, and we utilized a validation cohort, enhancing our statistical power.

Explaining how an increase in LTL raises the risk of Ems is indeed a challenge, and several potential mechanisms can elucidate this association. Firstly, previous study (33) has indicated a positive correlation between longer telomere length and enhanced cell proliferation and repair capabilities, along with the inhibition of apoptosis. Telomere length is also particularly closely associated with the division, growth, and maintenance of stem cells (34). Ems is believed to be linked to an excessive response of cyclic epithelial progenitor cells or stem cells related to endometrial regeneration after menstruation (35, 36). Therefore, we speculate that when endometrial tissue, carrying peripheral blood leukocytes, reach locations outside the uterine body, longer LTL may inhibit the apoptosis of ectopic endometrial cells and promote the cloning and differentiation of progenitor or stem cells. This could potentially facilitate the infiltration, survival, and unrestricted growth of endometrial cells in ectopic sites. Secondly, studies have shown a positive correlation between longer telomere length and estrogen levels (37), and estrogen plays a significant role in the pathological process of Ems (3). Excessive estrogen stimulation may lead to the growth and proliferation of ectopic endometrial tissues, thereby increasing the risk of Ems. Finally, LTL is influenced by genetic factors, resulting in variations in LTL between different individuals (38). Ems also exhibits certain features influenced by genetic factors (3), and some genetic factors may simultaneously affect both LTL and the development of Ems.

Research indicates that long-term chronic inflammation can lead to telomere shortening (39, 40), and Ems is a chronic inflammatory disease (3), providing a theoretical basis for how Ems may impact LTL. Previous observational studies have also observed telomere shortening in Ems patients (9, 30). However, our study did not find evidence of Ems causally affecting LTL. Nevertheless, negative results in MR studies cannot entirely exclude a causal relationship because genetic determinants of exposure may not represent the true exposure.

In summary, our study provides strong evidence regarding the association of increased Ems risk with longer LTL. These research findings may hold crucial clinical significance, particularly in the context of women's health and patient care. They also provide valuable directions for future research. First, if LTL becomes an effective biomarker for Ems, it can aid in early diagnosis and intervention, ultimately reducing the severity of the disease and the suffering of patients. Second, for those already diagnosed with Ems, monitoring their peripheral blood LTL could assist in better disease management. Additionally, future research can explore how peripheral blood LTL influences the mechanisms behind Ems development. Finally, future studies can investigate whether adjusting LTL can reduce the risk of Ems or improve treatment outcomes. This could encompass interventions such as nutritional changes, lifestyle modifications, or drug therapies.

However, this study has limitations. Firstly, despite our efforts to mitigate pleiotropic bias using various methods, there is still a risk of potential pleiotropic bias, inherent to the limitations of the MR method itself (13). Secondly, all the summary data we used are from European populations, which limits the generalizability of the causal relationship to different ethnicities. Furthermore, our research focused on peripheral blood LTL and its relation to Ems, and the results may not represent the causal association of TL, particularly in endometrial cells themselves, with Ems. Lastly, we were unable to perform gender-stratified analyses due to the lack of appropriate data.

Conclusion

In conclusion, our study strengthens the causal inference between LTL and Ems, supporting a positive causal impact of LTL on Ems, rather than Ems affecting LTL causally. This holds vital importance for deepening our understanding of the disease's pathogenesis, offering potential avenues for Ems prevention and treatment. LTL may emerge as a potential biomarker for the disease, and future research can delve further into the exact role and impact mechanisms of LTL in Ems occurrence, including investigating interventions targeting LTL and related treatment methods.

Data availability statement

Publicly available datasets were analyzed in this study. Leukocyte telomere length summary data can be obtained at "https://gwas.mrcieu.ac.uk/", ID is "ieu-b-4879". Endometriosis (discovery) summary data is available at "https://www.ebi.ac.uk/gwas" with ID "GCST90269970". Endometriosis (validation) summary data are available at "https://www.finngen.fi/en /access_results".

Ethics statement

This study used summary data from genome-wide association studies and did not involve data from any individual individual. All data had received appropriate ethical approval in their original studies. No additional ethical approval was required to conduct this study.

Author contributions

CB: Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. ZS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Software, Validation, Writing – original draft, Writing – review & editing. BQ: Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. SZ: Data curation,

Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1272200/full#supplementary-material

References

1. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *N Engl J Med* (2020) 382 (13):1244–56. doi: 10.1056/NEJMra1810764

2. Horne AW, Saunders PTK. SnapShot: endometriosis. *Cell* (2019) 179(7):1677–1677 e1. doi: 10.1016/j.cell.2019.11.033

3. Saunders PTK, Horne AW. Endometriosis: Etiology, pathobiology, and therapeutic prospects. *Cell* (2021) 184(11):2807–24. doi: 10.1016/j.cell.2021.04.041

4. Sfeir A, de Lange T. Removal of shelterin reveals the telomere end-protection problem. *Science* (2012) 336(6081):593–7. doi: 10.1126/science.1218498

5. Chakravarti D, LaBella KA, DePinho RA. Telomeres: history, health, and hallmarks of aging. *Cell* (2021) 184(2):306–22. doi: 10.1016/j.cell.2020.12.028

6. Shay JW, Wright WE. Telomeres and telomerase: three decades of progress. *Nat Rev Genet* (2019) 20(5):299–309. doi: 10.1038/s41576-019-0099-1

7. Giri N, Alter BP, Savage SA, Stratton P. Gynaecological and reproductive health of women with telomere biology disorders. *Br J Haematol* (2021) 193(6):1238–46. doi: 10.1111/bjh.17545

8. Zhang J, Rane G, Dai X, Shanmugam MK, Arfuso F, Samy RP, et al. Ageing and the telomere connection: An intimate relationship with inflammation. *Ageing Res Rev* (2016) 25:55–69. doi: 10.1016/j.arr.2015.11.006

9. Sasamoto N, Yland J, Vitonis AF, Cramer DW, Titus LJ, De Vivo I, et al. Peripheral blood leukocyte telomere length and endometriosis. *Reprod Sci* (2020) 27 (10):1951–9. doi: 10.1007/s43032-020-00214-6

10. Dracxler RC, Oh C, Kalmbach K, Wang F, Liu L, Kallas EG, et al. Peripheral blood telomere content is greater in patients with endometriosis than in controls. *Reprod Sci* (2014) 21(12):1465–71. doi: 10.1177/1933719114527353

11. Zhang H, Kong W, Xie Y, Zhao X, Luo D, Chen S, et al. Telomere-related genes as potential biomarkers to predict endometriosis and immune response: Development of a machine learning-based risk model. *Front Med (Lausanne)* (2023) 10:1132676. doi: 10.3389/fmed.2023.1132676

12. Rahmioglu N, Mortlock S, Ghiasi M, Moller PL, Stefansdottir L, Galarneau G, et al. The genetic basis of endometriosis and comorbidity with other pain and inflammatory conditions. *Nat Genet* (2023) 55(3):423–36. doi: 10.1038/s41588-023-01323-z

13. Skrivankova VW, Richmond RC, Woolf BAR, Davies NM, Swanson SA, VanderWeele TJ, et al. Strengthening the reporting of observational studies in epidemiology using mendelian randomisation (STROBE-MR): explanation and elaboration. *BMJ* (2021) 375:n2233. doi: 10.1136/bmj.n2233

14. Sleiman PM, Grant SF. Mendelian randomization in the era of genomewide association studies. *Clin Chem* (2010) 56(5):723–8. doi: 10.1373/clinchem.2009.141564

15. Haycock PC, Burgess S, Wade KH, Bowden J, Relton C, Davey Smith G. Best (but oft-forgotten) practices: the design, analysis, and interpretation of Mendelian randomization studies. *Am J Clin Nutr* (2016) 103(4):965–78. doi: 10.3945/ajcn.115.118216

16. Codd V, Denniff M, Swinfield C, Warner SC, Papakonstantinou M, Sheth S, et al. Measurement and initial characterization of leukocyte telomere length in 474,074 participants in UK Biobank. *Nat Aging* (2022) 2(2):170–9. doi: 10.1038/s43587-021-00166-9

17. Kurki MI, Karjalainen J, Palta P, Sipila TP, Kristiansson K, Donner KM, et al. FinnGen provides genetic insights from a well-phenotyped isolated population. *Nature* (2023) 613(7944):508–18. doi: 10.1038/s41586-022-05473-8

18. Heid IM, Jackson AU, Randall JC, Winkler TW, Qi L, Steinthorsdottir V, et al. Meta-analysis identifies 13 new loci associated with waist-hip ratio and reveals sexual dimorphism in the genetic basis of fat distribution. *Nat Genet* (2010) 42(11):949–60. doi: 10.1038/ng.685

19. Lawlor DA, Harbord RM, Sterne JA, Timpson N, Davey Smith G. Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. *Stat Med* (2008) 27(8):1133–63. doi: 10.1002/sim.3034

20. Hemani G, Tilling K, Davey Smith G. Orienting the causal relationship between imprecisely measured traits using GWAS summary data. *PloS Genet* (2017) 13(11): e1007081. doi: 10.1371/journal.pgen.1007081

21. Bowden J, Del Greco MF, Minelli C, Davey Smith G, Sheehan NA, Thompson JR. Assessing the suitability of summary data for two-sample Mendelian randomization analyses using MR-Egger regression: the role of the I2 statistic. *Int J Epidemiol* (2016) 45(6):1961–74. doi: 10.1093/ije/dyw220

22. Bowden J, Del Greco MF, Minelli C, Zhao Q, Lawlor DA, Sheehan NA, et al. Improving the accuracy of two-sample summary-data Mendelian randomization: moving beyond the NOME assumption. *Int J Epidemiol* (2019) 48(3):728–42. doi: 10.1093/ije/dyy258

23. Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. *Int J Epidemiol* (2015) 44(2):512–25. doi: 10.1093/ije/dyv080

24. Verbanck M, Chen CY, Neale B, Do R. Detection of widespread horizontal pleiotropy in causal relationships inferred from Mendelian randomization between complex traits and diseases. *Nat Genet* (2018) 50(5):693–+. doi: 10.1038/s41588-018-0099-7

25. Zhao QY, Wang JS, Hemani G, Bowden J, Small DS. Statistical inference in two-sample summary-data mendelian randomization using robust adjusted profile score. *Ann Stat* (2020) 48(3):1742–69. doi: 10.1214/19-Aos1866

26. Burgess S, Labrecque JA. Mendelian randomization with a binary exposure variable: interpretation and presentation of causal estimates. *Eur J Epidemiol* (2018) 33 (10):947–52. doi: 10.1007/s10654-018-0424-6

27. Brion MJ, Shakhbazov K, Visscher PM. Calculating statistical power in Mendelian randomization studies. *Int J Epidemiol* (2013) 42(5):1497–501. doi: 10.1093/ije/dyt179

28. Horne AW, Missmer SA. Pathophysiology, diagnosis, and management of endometriosis. *BMJ* (2022) 379:e070750. doi: 10.1136/bmj-2022-070750

29. Kalaitzopoulos DR, Samartzis N, Kolovos GN, Mareti E, Samartzis EP, Eberhard M, et al. Treatment of endometriosis: a review with comparison of 8 guidelines. *BMC Womens Health* (2021) 21(1):397. doi: 10.1186/s12905-021-01545-5

30. Gleason JL, Thoma ME, Zukerman Willinger N, Shenassa ED. Endometriosis and uterine fibroids and their associations with elevated C-reactive protein and leukocyte telomere length among a representative sample of U.S. Women: data from the national health and nutrition examination survey, 1999-2002. *J Womens Health (Larchmt)* (2022) 31(7):1020–8. doi: 10.1089/jwh.2021.0044

31. Hapangama DK, Turner MA, Drury JA, Quenby S, Saretzki G, Martin-Ruiz C, et al. Endometriosis is associated with aberrant endometrial expression of telomerase and increased telomere length. *Hum Reprod* (2008) 23(7):1511–9. doi: 10.1093/humrep/den172

32. Hapangama DK, Turner MA, Drury JA, Quenby S, Hart A, Maddick M, et al. Sustained replication in endometrium of women with endometriosis occurs without evoking a DNA damage response. *Hum Reprod* (2009) 24(3):687–96. doi: 10.1093/humrep/den416

33. Codd V, Wang Q, Allara E, Musicha C, Kaptoge S, Stoma S, et al. Polygenic basis and biomedical consequences of telomere length variation. *Nat Genet* (2021) 53 (10):1425–33. doi: 10.1038/s41588-021-00944-6

34. Lee L, Perez Oliva AB, Martinez-Balsalobre E, Churikov D, Peter J, Rahmouni D, et al. UFMylation of MRE11 is essential for telomere length maintenance and hematopoietic stem cell survival. *Sci Adv* (2021) 7(39):eabc7371. doi: 10.1126/sciadv.abc7371

35. Wang Y, Nicholes K, Shih IM. The origin and pathogenesis of endometriosis. *Annu Rev Pathol* (2020) 15:71–95. doi: 10.1146/annurev-pathmechdis-012419-032654

36. Figueira PG, Abrao MS, Krikun G, Taylor HS. Stem cells in endometrium and their role in the pathogenesis of endometriosis. *Ann N Y Acad Sci* (2011) 1221(1):10–7. doi: 10.1111/j.1749-6632.2011.05969.x

37. Song Y, Cho M, Brennan KM, Chen BH, Song Y, Manson JE, et al. Relationships of sex hormone levels with leukocyte telomere length in Black, Hispanic, and Asian/Pacific Islander postmenopausal women. *J Diabetes* (2018) 10(6):502–11. doi: 10.1111/1753-0407.12577

38. Li C, Stoma S, Lotta LA, Warner S, Albrecht E, Allione A, et al. Genome-wide association analysis in humans links nucleotide metabolism to leukocyte telomere length. *Am J Hum Genet* (2020) 106(3):389–404. doi: 10.1016/j.ajhg.2020.02.006

39. Wu L, Fidan K, Um JY, Ahn KS. Telomerase: Key regulator of inflammation and cancer. *Pharmacol Res* (2020) 155:104726. doi: 10.1016/j.phrs.2020.104726

40. Lv Z, Cui J, Zhang J. Associations between serum urate and telomere length and inflammation markers: Evidence from UK Biobank cohort. *Front Immunol* (2022) 13:1065739. doi: 10.3389/fimmu.2022.1065739

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 21 November 2023
DOI 10.3389/fendo.2023.1218980

OPEN ACCESS

EDITED BY
Iveta Yotova,
Medical University of Vienna, Austria

REVIEWED BY
Mohd Faizal Ahmad,
National University of Malaysia, Malaysia
Andrei Tica,
University of Craiova, Romania

*CORRESPONDENCE
Magdalena Olszanecka-Glinianowicz
molszanecka@sum.edu.pl

RECEIVED 08 May 2023
ACCEPTED 11 September 2023
PUBLISHED 21 November 2023

CITATION
Wójtowicz M, Zdun D, Owczarek AJ, Skrzypulec-Plinta V and Olszanecka-Glinianowicz M (2023) Evaluation of adipokines concentrations in plasma, peritoneal, and endometrioma fluids in women operated on for ovarian endometriosis.
Front. Endocrinol. 14:1218980.
doi: 10.3389/fendo.2023.1218980

Evaluation of adipokines concentrations in plasma, peritoneal, and endometrioma fluids in women operated on for ovarian endometriosis

Mariusz Wójtowicz [1], Dariusz Zdun [1], Aleksander Jerzy Owczarek [2], Violetta Skrzypulec-Plinta [3] and Magdalena Olszanecka-Glinianowicz [2*]

[1]Clinical Department of Gynecology and Obstetrics, Faculty of Medical Sciences in Zabrze, Medical University of Silesia in Katowice, Katowice, Poland, [2]Health Promotion and Obesity Management Unit, Department of Pathophysiology, Faculty of Medical Sciences in Katowice, Medical University of Silesia in Katowice, Katowice, Poland, [3]Reproductive Health and Sexology Unit, Department of Women's Health School of Health Sciences in Katowice, Medical University of Silesia in Katowice, Katowice, Poland

Introduction: Some studies indicate the role of selected adipokines in the development of endometriosis. However, a comprehensive assessment of plasma, peritoneal, and endometrioma fluids adipokines concentrations in women with ovarian endometriosis has not yet been performed. Therefore, this study aimed to analyze plasma, peritoneal, and endometrioma fluids selected adipokines concentrations in women operated on for ovarian endometriosis.

Materials and methods: A cross-sectional cohort study involved 56 women operated on for ovarian endometriosis. Body mass, height, and waist circumference were measured, and BMI was calculated. Plasma, peritoneal, and endometrioma fluids adiponectin, leptin, omentin resistin, RBP4, and visfatin/NAMPT were determined by ELISA.

Results: The highest plasma levels of adiponectin, leptin, omentin, and RBP4 than in the endometrioma and peritoneal fluids were found, while levels of resistin and visfatin/NAMPT were significantly higher in endometrioma fluid than in plasma and peritoneal fluid. In addition, levels of visfatin/NAMPT were significantly higher in peritoneal fluid than in plasma. There were also positive correlations between leptin, RBP4, and adiponectin levels in endometrioma and peritoneal fluids ($\rho = 0.28$; $p < 0.05$; $\rho = 0.31$; $p < 0.05$; $\rho = 0.32$; $p < 0.05$, respectively). There were no associations between adipokines levels in plasma, endometrioma, and peritoneal fluids and endometriosis stage.

10.3389/fendo.2023.1218980

Conclusion: Our results show that visfatin/NAMPT and resistin may be locally secreted in endometrioma related to inflammation regardless of the stage of endometriosis.

KEYWORDS

adipokines, nutritional status, endometriosis, inflammation, ovarian endometriosis

Introduction

Endometriosis occurs in 2%–10% of women of reproductive age (1). The pathogenesis of endometriosis development is still unclear. The mechanism of pathogenesis of endometriosis is thought to be uterine tissue damage or scarring, the uterine microenvironment, stem cells, remnant cells from menstrual blood, hormones, gene products regulating inflammation, apoptosis, invasion, angiogenesis, autophagy, and oxidative stress (2–4). Macrophages, natural killer cells, T cells, and dendritic cells regulated by cytokines, prostaglandins, and chemokines participate in the initiation and adhesion of endometriosis as well as infertility and pain related to endometriosis (2, 5, 6). Excessive estrogens production play a role in inflammation development (6, 7).

Interestingly, although both obesity and endometriosis are associated with inflammation, the prevalence of endometriosis is inversely related to BMI. However, it has also been shown that abdominal fat distribution is associated with the development of endometriosis (8–10). Some studies showed a role for adipokines in the pathogenesis of endometriosis (8, 11).

The meta-analysis of 25 studies including 2,645 women (1,362 with endometriosis and 1,283 without) showed higher serum leptin levels and leptin/BMI ratio in women with endometriosis. In addition, the leptin levels were lower in women with advanced-stage disease than in women with early endometriosis (12). However, the analysis of data from 29,611 women from the Nurses' Health Study did not show associations between leptin levels and the development of endometriosis (13). Also, two other meta-analyses did not find differences in plasma leptin levels between women with and without endometriosis, while leptin levels were significantly higher in the peritoneal fluid (14, 15). In addition, higher expression of levels of leptin and leptin-receptor protein was shown in endometrial tissues of women with endometriosis. However, the endometrial leptin mRNA expression was similar in women with and without endometriosis (16). Experimental studies found that leptin enhances the proliferation of both eutrophic and ectopic endometrial stromal cells in endometriosis (17), stimulates the migration and invasion of endometrial cells (18), and was essential for angiogenesis in a mouse model of endometriosis (19). Recombinant adiponectin in levels significantly lower than in serum was found to inhibit the proliferation of primary stromal cells in human endometriosis (20). Moreover, adiponectin reduces the viability of normal endometrial stromal cells (21). However, adiponectin and adiponectin-receptor protein levels in endometrial tissues of women with and without endometriosis were similar (22). Adiponectin levels in the peritoneal fluid were lower in women with than without endometriosis (23) and decreased with the intensity of endometriosis (24). Furthermore, serum adiponectin levels were lower in women with endometriosis and correlated with endometriosis stages (24). However, the meta-analysis of 25 studies mentioned above did not show an association between peritoneal and circulating adiponectin and disease stages but found lower adiponectin levels in women with endometriosis (12). Recently, apelin receptor APLNR was identified as one of three key genes in endometriosis (25).

As was described above, some studies indicated that selected adipokines play a role in the pathogenesis of endometriosis. However, a comprehensive assessment of plasma, peritoneal, and endometrioma fluids adipokines concentrations in women with ovarian endometriosis has not yet been performed. Understanding the relationship between the concentration of adipokines in plasma, peritoneal, and endometrioma fluids can provide data helpful in the diagnosis of ovarian endometriosis. Therefore, this study aimed to analyze plasma, peritoneal, and endometrioma fluids adipokines concentrations in women operated on for ovarian endometriosis.

Materials and methods

A cross-sectional cohort study involved 56 women operated on for ovarian endometriosis in the Clinical Department of Gynecology and Obstetrics Faculty of Medical Sciences in Zabrze between 2018 and 2022. Inclusion criteria were at least 2 years primary infertility, stage from II to IV ovarian endometriosis, and regular cycles. Endometriosis was diagnosed by laparoscopy and histologically confirmed and classified according to the American Society of Reproductive Medicine classification (26). The exclusion criteria included, other than ovarian localizations of endometriosis, additional extraovarian endometriosis, hormonal disturbances including thyroid dysfunction, Cushing's syndrome, type 1 and 2 diabetes, smoking and alcohol abuse, changes of body mass during the last 3-month period, and any pharmacological therapy. The study was conducted after obtaining informed consent of each participant, based on the study protocol, approved by the Bioethical Committee of the Medical University of Silesia.

Body mass, height, and waist circumference were measured, and body mass index (BMI) was calculated according to the standard

formula. During the morning between 6:00 and 7:00 a.m., after an overnight fast (14 h), 15 mL of venous blood samples were withdrawn. During laparoscopic operation, peritoneal fluid from Douglas' sinus and endometrioma fluid were collected according to recommendations of the kit manufacturers. Plasma and fluid aliquots were frozen and stored at −70°C.

Laboratory procedures

Blood morphology and serum C-reactive protein (CRP) levels were assessed. CRP concentrations were assessed by an automated system (Modular PPE, Roche Diagnostics GmbH, Mannheim, Germany). The inter-assay coefficient of variability was 5.7%.

The ELISA method was used for measurements of plasma and fluids leptin (TECOmedical AG Sissach, Switzerland), adiponectin (TECOmedical AG Sissach, Switzerland), omentin (DRG Instruments GmbH, Marburg, Germany), RBP4 levels (Phoenix Pharmaceuticals, Burlingame, USA), resistin (R&D, Minneapolis, MN, USA), and visfatin/NAMPT (BioVendor, Brno, The Czech Republic) with the LoQ of 0.08 ng/mL, 0.11 ng/mL, 0.2 ng/mL, 0.6 ng/mL and 0.5 ng/mL, 2.17 ng/mL, 0.05 ng/mL, and 30 pg/mL respectively; intra- and inter-assay coefficients of variations were 4.6% and 7% for leptin, 5% and 6% for adiponectin, 3.7% and 4.6% for omentin-1, 5.0% and <14.0% for RBP4, <5.5% and <9.2% for resistin, and 5.6% and 5.9% for visfatin/NAMPT.

Statistical analysis

Statistical analysis was performed using STATISTICA 13.0 PL (TIBCO Software Inc., Palo Alto, CA, U.S.) and StataSE 13.0 (StataCorp LP, TX, U.S.). Statistical significance was set at a $p < 0.05$. All tests were two-tailed. Nominal and ordinal data were expressed as percentages. Interval data were expressed as mean ± standard deviation (normal distribution) or median (lower–upper quartiles). The distribution of variables was evaluated by the *W* Shapiro-Wilk test and the quantile–quantile (Q–Q) plot. Rank ANOVA was used to compare adipokines levels between plasma and endometrial/peritoneal fluids with Tukey as a *post-hoc* test. The homogeneity of variance was assessed by the *F* Fisher–Snedecor test. Correlation between variables was assessed with the ρ Spearman rank correlation coefficient.

TABLE 1 Baseline characteristics of the study group.

N	56
Age [years]	33 ± 6
CRP [mg/L]	0.6 (0.6–1.8)
HGB [g/dL]	13.1 ± 1.1
WBC [tys/μL]	6.8 (5.6–8.1)
RBC [mln/μL]	4.4 ± 0.4
PLT [tys/μL]	258 ± 51
BMI [kg/m^2]	22.6 ± 4.3
Stages of endometriosis [*N*(%)]	
II	11 (26.4)
III	28 (52.8)
IV	14 (26.4)

Mean ± standard deviation or median (lower quartile–upper quartile).

Results

The baseline characteristics of the study group are presented in Table 1.

Statistically significant higher plasma levels of adiponectin, leptin, omentin, and RBP4 compared to endometrioma and peritoneal fluids were found. Levels of resistin and visfatin/NAMPT were significantly higher in endometrioma fluid than in plasma and peritoneal fluid. In addition, levels of visfatin/NAMPT were significantly higher in peritoneal fluid than in plasma (Table 2).

Spearman rank correlation analysis showed a positive correlation between plasma leptin levels and BMI (ρ = 0.63; $p < 0.00$) and negative correlations between plasma omentin and BMI (ρ = −0.25; $p = 0.07$). There were no correlations between plasma levels of other adipokines levels and BMI. In addition, adipokines levels in endometrioma and peritoneal fluids did not correlate with BMI.

The positive correlation between visfatin/NAMPT levels in peritoneal fluids and WBC numbers was found (ρ = 0.31; $p < 0.05$). There were also positive correlations between leptin levels in endometrioma and peritoneal fluids (ρ = 0.28; $p < 0.05$), between leptin levels in endometrioma fluids and WBC numbers (ρ = 0.32; $p < 0.05$), and between plasm leptin levels and serum CRP levels (ρ = 0.33; $p < 0.05$). We observed positive correlations between RBP4 levels in endometrioma and peritoneal fluids (ρ = 0.31; $p < 0.05$) and between RBP4 levels in plasma and endometrioma fluids (ρ = 0.44; $p < 0.001$), as well as RBP4 levels in endometrioma and peritoneal fluids and WBC numbers (ρ = 0.33; $p < 0.05$ and ρ = 0.33; $p < 0.05$, respectively). In addition, there was also positive correlation between plasma and endometrioma fluid RBP4 levels and serum CRP levels (ρ = 0.37; $p < 0.05$ and ρ = 0.29; $p < 0.05$, respectively). A positive correlation between adiponectin levels in endometrioma and peritoneal fluids (ρ = 0.32; $p < 0.05$) was found. Moreover, a negative correlation between plasma omentin levels and serum CRP levels (ρ = −0.40; $p < 0.01$) was observed.

There were no associations between adipokines levels in both plasma and endometrioma and peritoneal fluids and endometriosis stage.

Discussion

To the best of our knowledge, this is the first study that assessed the levels of adipokines in plasma, endometrioma, and peritoneal fluids of women operated on for ovarian endometriosis. Our study showed significant higher plasma levels of adiponectin, leptin, omentin, and RBP4 than in endometrioma and peritoneal fluids.

10.3389/fendo.2023.1218980

TABLE 2 Adipokines levels in plasma and endometrioma and peritoneal fluids.

	Plasma	Endometrioma fluid	Peritoneal fluid	*p*
Adiponectin [µg/mL]	9.8 (7.3–13.1)	1.2$^{\#}$ (0.1–2.4)	0.1$^{\#}$ (0.0–1.3)	<0.001
Leptin [ng/mL]	16.6 (10.1–29.5)	2.2$^{\#}$ (1.0–4.4)	1.6$^{\#}$ (0.4–4.1)	<0.001
Omentin [mg/mL]	498.6 (388.9–631.9)	228.0* (90.1–702.9)	230.9 (83.0–945.2)	<0.01
Resistin [ng/mL]	5.6 (4.6–6.8)	71.0$^{\#}$ (44.7–96.4)	3.9$^{\$}$ (1.0–42.6)	<0.001
RBP4 [µg/L]	29.4 (24.0–34.0)	5.5$^{\#}$ (2.9–8.7)	1.8$^{\#}$ (1.1–3.8)	<0.001
Visfatin/NAMPT [ng/mL]	1.2 (0.4–2.3)	12.0$^{\#}$ (8.1–13.4)	7.0$^{\#\$}$ (1.4–12.1)	<0.001

*$p < 0.05$, $^{\#}p < 0.001$ in comparison to plasma levels; $^{\$}p < 0.01$ in comparison to endometrial fluid; median (lower quartile–upper quartile).

In addition, there were no associations between adiponectin, leptin, omentin, and RBP4 levels in either plasma, endometrioma, or peritoneal fluids and endometriosis stage. Our results contradict previous studies that showed significantly higher leptin levels in peritoneal fluid than in plasma of women with endometriosis (14, 15) and higher levels of leptin in endometrial tissues (16). Moreover, contradictory to a study that showed a decrease of adiponectin levels in peritoneal fluid with the intensity of endometriosis (24), we did not observe any associations between adiponectin levels in either plasma, endometrioma, or peritoneal fluids and endometriosis stage. However, similar to a meta-analysis of 25 studies (12), we did not observe associations between plasma and peritoneal fluid adiponectin levels and disease stage. It should be noted that our study showed positive correlations between adiponectin, leptin, and RBP4 levels in endometrioma and peritoneal fluids. In addition, only plasma RBP4 levels correlated with RBP4 levels in endometrioma fluid. Moreover, the positive correlation between RBP4 levels in both endometrioma and peritoneal fluids and WBC numbers and between RBP4 levels in both plasma and endometrioma fluid and serum CRP levels was observed. So far, only one study showed higher RBP4 levels in peritoneal fluid in the women with than without endometriosis. Moreover, RBP4 immunoreactivity was significantly higher in ovarian endometriomas of women with advanced-stage endometriosis compared to women without endometriosis. Furthermore, *in vitro*, human recombinant-RBP4 increased the invasiveness of endometrial stromal cells. Transfection with RBP4 siRNA reduced the viability and invasiveness of endometrial stromal cells (27). Further studies are necessary to clarify the role of RBP4 in the pathogenesis of endometriosis. Although our study suggests that RBP4 levels in both endometrioma and peritoneal fluids reflect the severity of inflammation in endometriosis, it also seems that leptin levels in endometrioma fluid are an inflammation marker because our study has shown correlation between its levels in endometrioma fluid and WBC number.

Of interest, our study found significantly higher levels of resistin and visfatin/NAMPT in endometrioma fluid than in plasma and peritoneal fluid. Moreover, levels of visfatin/NAMPT were significantly higher in peritoneal fluid than in plasma. Furthermore, a positive correlation between visfatin/NAMPT levels in peritoneal fluids and WBC numbers was found. Visfatin is an adipokine mainly expressed and secreted by macrophages and adipocytes of visceral adipose tissue, and is also known as nicotinamide phosphoribosyltransferase (NAMPT) and pre-B-cell colony enhancing factor (PCEF) produced by lymphocytes (28–30). Thus, the higher visfatin/NAMPT levels in endometrioma and peritoneal fluids and their correlation with WBC numbers may indicate that it is an indicator of inflammation associated with endometriosis. Our results are contrary to a study that has shown lower visfatin levels in peritoneal fluid in women with than without endometriosis (31). However, the results of another study that found significantly lower visfatin gene expression in whole blood samples in women with than without endometriosis (32) seem to support our hypothesis that increased visfatin concentrations in endometrioma and peritoneal fluids are associated with its local production in endometrioma cells. Resistin is the adipokine produced by macrophages of visceral adipose tissue. However, the primary sources of circulating resistin in humans are peripheral blood mononuclear cells (PBMCs), macrophages, and bone marrow cells (33). Thus, we hypothesized that higher resistin levels in endometrioma fluid is similar to visfatin/NAMPT is an indicator of local inflammation in endometriosis. Our hypothesis is supported by studies that showed higher resistin levels in peritoneal fluid of women with than without endometriosis (34) and higher resistin mRNA and protein levels in ectopic endometrial tissue of patients with endometriosis compared to normal eutopic endometrial tissue (35).

The limitations of our study are the size of the study group and the lack of a control group without endometriosis. Furthermore, the distribution of body fat and its visceral deposits was not directly assessed. Moreover, in our study, only selected adipokines were analyzed.

The strengths of the study include the demonstration that the analysis of visfatin/NAMPT and resistin concentrations in the peritoneal fluid may be clinically useful in assessing the severity of inflammation in ovarian endometriosis. A pouch of Douglas fluid

collection is an easier procedure than an ovarian endometriosis fluid collection. Inflammation can translate into increased pain and fertility disturbances. However, it should be emphasized that our study opens a new direction of research on ovarian endometriosis, and at this stage, it cannot be used as guidelines for clinical management. More research is needed to translate the evidence into clinical utility.

Conclusions

Our results show that visfatin/NAMPT and resistin may be locally secreted in endometrioma related to inflammation regardless of the stage of endometriosis.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Medical University of Silesia Katowice Poland. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MW has initial idea designed the study protocol, performed an operation, supervised the blood and fluids collections, and wrote the manuscript; DZ performed an operation, collected the blood and fluids, collected informed consents from the participants, and search literature, AO performed the statistical analysis and prepared the tables and figures, VS-P has initial idea, received grant, and revised the manuscript; MO-G has an initial idea, supervised the study, performed the data analysis and revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

The study was funded by Medical University of Silesia grant no. PCN-1-210/N/9/Z.

Acknowledgments

The authors would like to thank native speaker Mike Smertka for the linguistic revision.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Sampson JA. Metastaticor embolic endometriosis, due to the menstrual dissemination of endometrial tissue in to the venous circulation. *Am J Pathol* (1927) 3:93–110.

2. Kapoor R, Stratopoulou CA, Dolmans M. Pathogenesis of endometriosis: new insights into prospective therapies. *Int J Mol Sci* (2021) 22:1700. doi: 10.3390/ijms222111700

3. Giudice LC, Kao LC. Endometriosis. *Lancet* (2004) 364:1789–99. doi: 10.1016/S0140-6736(04)17403-5

4. Taylor HS, Kotlyar AM, Flores VA. Endometriosis is a chronic systemic disease: Clinical challenges and novel innovations. *Lancet* (2021) 397:839–52. doi: 10.1016/S0140-6736(21)00389-5

5. Giacomini E, Minetto S, LiPiani L, Pagliardini L, Somigliana E, Viganó P. Genetics and inflammation in endometriosis: improving knowledge for development of new pharmacological strategies. *Int J Mol Sci* (2021) 22:9033. doi: 10.3390/ijms22169033

6. García-Gómez E, Vázquez-Martínez ER, Reyes-Mayoral C, Cruz-Orozco OP, Camacho-Arroyo I, Cerbón M. Regulation of inflammation pathways and inflammasome by sex steroid hormones in endometriosis. *Front Endocrinol* (2019) 10:935. doi: 10.3389/fendo.2019.00935

7. Yilmaz BD, Bulun SE. Endometriosis and nuclear receptors. *Hum Reprod Update* (2019) 25:473–85. doi: 10.1093/humupd/dmz005

8. Pantelis A, Machairiotis N, Lapatsanis DP. The formidable yet unresolved interplay between endometriosis and obesity. *Sci World J* (2021) 2021:6653677. doi: 10.1155/2021/6653677

9. Backonja U, BuckLouis GM, Lauver DR. Overall adiposity, adipose tissue distribution, and endometriosis: A systematic review. *Nurs Res* (2016) 65:151–66. doi: 10.1097/NNR.0000000000000146

10. Venkatesh SS, Ferreira T, Benonisdottir S, Rahmioglu N, Becker CM, Granne I, et al. Obesity and risk of female reproductive conditions: A Mendelian randomisation study. *PloS Med* (2022) 19:e1003679. doi: 10.1371/journal.pmed.1003679

11. Hong J, Yi KW. What is the link between endometriosis and adiposity? Obstet. *Gynecol Sci* (2022) 65:227–33. doi: 10.5468/ogs.21343

12. Zhao Z, Wu Y, Zhang H, Wang X, Tian X, Wang Y, et al. Association of leptin and adiponectin levels with endometriosis: A systematic review and meta-analysis. *Gynecol Endocrinol* (2021) 37:591–9. doi: 10.1080/09513590.2021.1878139

13. Shah DK, Correia KF, Harris HR, Missmer SA. Plasma adipokines and endometriosis risk: A prospective nested case-control investigation from the Nurses' Health Study II. *Hum Reprod* (2013) 28:315–21. doi: 10.1093/humrep/des411

14. Kalaitzopoulos DR, Lempesis IG, Samartzis N, Kolovos G, Dedes I, Daniilidis A, et al. Leptin concentrations in endometriosis: A systematic review and meta-analysis. *J Reprod Immunol* (2021) 146:103338. doi: 10.1016/j.jri.2021.103338

15. Tian Z, Wang Y, Zhao Y, Chang X-H, Zhu H-L. Serum and peritoneal fluid leptin levels in endometriosis: A systematic review and meta-analysis. *Gynecol Endocrinol* (2021) 37:689–93. doi: 10.1080/09513590.2020.1862789

16. Lima-Couy I, Cervero A, Bonilla-Musoles F, Pellicer A, SimoŐĀn C. Endometrial leptin and leptin receptor expression in women with severe/moderate endometriosis. *Mol Hum Reprod* (2004) 10:777–82. doi: 10.1093/molehr/gah115

17. Wu M-H, Chuang P-C, Chen H-M, Lin C-C, Tsai S-J. Increased leptin expression in endometriosis cells is associated with endometrial stromal cell proliferation and leptin gene up-regulation. *Mol Hum Reprod* (2002) 8:456–64. doi: 10.1093/molehr/8.5.456

18. Ahn J-H, Choi YS, Choi J-H. Leptin promotes human endometriotic cell migration and invasion by up-regulating MMP-2 through the JAK2/STAT3 signaling pathway. *Mol Hum Reprod* (2015) 21:792–802. doi: 10.1093/molehr/gav039

19. Manjunathan R, Devarajan N, Ragunathan M. Possible Mechanism of Human Recombinant Leptin-Induced VEGFA Synthesis via PI3K/Akt/mTOR/S6 Kinase Signaling Pathway while Inducing Angiogenesis: An Analysis Using Chicken Chorioallantoic Membrane Model. *J Vasc Res* (2021) 58:343–60. doi: 10.1159/000516498

20. Bohlouli S, Rabzia A, Sadeghi E, Chobsaz F, Khazaei M. *in vitro* Anti-Proliferative Effect of Adiponectin on Human Endometriotic Stromal Cells through AdipoR1 and AdipoR2 Gene Receptor Expression. *Iran BioMed J* (2016) 20:12–7. doi: 10.7508/ibj.2016.01.002

21. Bohlouli S, Khazaei M, Teshfam M, Hassanpour H. Adiponectin effect on the viability of human endometrial stromal cells and mRNA expression of adiponectin receptors. *Int J Fertil Steril* (2013) 7:43–8.

22. Choi YS, Oh HK, Choi J-H. Expression of adiponectin, leptin, and their receptors in ovarian endometrioma. *Fertil Steril* (2013) 100:135–141.e2. doi: 10.1016/j.fertnstert.2013.03.019

23. Takemura Y, Osuga Y, Harada M, Hirata T, Koga K, Yoshino O, et al. Concentration of adiponectin in peritoneal fluid is decreased in women with endometriosis. *Am J Reprod Immunol* (2005) 54:217–21. doi: 10.1111/j.1600-0897.2005.00300.x

24. Takemura Y, Osuga Y, Harada M, Hirata T, Koga K, Morimoto C, et al. Serum adiponectin concentrations are decreased in women with endometriosis. *Hum Reprod* (2005) 20:3510–3. doi: 10.1093/humrep/dei233

25. Peng Y, Peng C, Fang Z, Chen G. Bioinformatics analysis identifies molecular markers regulating development and progression of endometriosis and potential therapeutic drugs. *Front Genet* (2021) 12:622683. doi: 10.3389/fgene.2021.622683

26. American Society of Reproductive. Medicine Revised American Society of Reproductive Medicine classification of endometriosis 1996. *Fertil Sterill* (1997) 67:817–21. doi: 10.1016/S0015-0282(97)81391-X

27. Lee JC, Kim SH, Oh YS, Kim JH, Lee SR, Chae HD. Increased expression of retinol-binding protein 4 in ovarian endometrioma and its possible role in the pathogenesis of endometriosi. *Int J Mol Sci* (2021) 22:5827. doi: 10.3390/ijms22115827

28. Wang T, Zhang X, Bheda P, Revollo JR, Imai S, Wolberger C. Structure of Nampt/PBEF/visfatin, a mammalian NAD+ biosynthetic enzyme. *Nat Struct Mol Biol* (2006) 13:661–2. doi: 10.1038/nsmb1114

29. Varma V, Yao-Borengasser A, Rasouli N, Bodles AM, Phanavanh B, Lee MJ, et al. Human visfatin expression: relationship to insulin sensitivity, intramyocellular lipids, and inflammation. *J Clin Endocrinol Metab* (2007) 92:666–72. doi: 10.1210/jc.2006-1303

30. Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to metabolic syndrome. *Endocr Rev* (2000) 21:697–738. doi: 10.1210/edrv.21.6.0415

31. Krasnyi AM, Sadekova AA, Smolnova TY, Chursin VV, Buralkina NA, Chuprynin VD, et al. The levels of ghrelin, glucagon, visfatin and glp-1 are decreased in the peritoneal fluid of women with endometriosis along with the increased expression of the CD10 protease by the macrophages. *Int J Mol Sci* (2022) 23:10361. doi: 10.3390/ijms231810361

32. Sezginer EK, Kırlangıç Ö.F, Tanrıverdi MDE, Topçu HO, Gür S. Analysis of changes in serum levels and gene expression profiles of novel adipocytokines (Omentin, vaspin, irisin and visfatin) and their correlation with serum C-reactive protein levels in women diagnosed with endometriosis. *Turk J Pharm Sci* (2022) 19:48–53. doi: 10.4274/tjps.galenos.2021.52284

33. Codoñer-Franch P, Alonso-Iglesias E. Resistin: insulin resistance to Malignancy. *Clin Chim Acta* (2015) 438:46–54. doi: 10.1016/j.cca.2014.07.043

34. Oh KY, Ha YR, Yi KW, Park HT, Shin J-H, Kim T, et al. Increased expression of resistin in ectopic endometrial tissue of women with endometriosis. *Am J Reprod Immunol* (2017) 78:e12726. doi: 10.1111/aji.12726

35. Yi KW, Shin J-H, Park HT, Kim T, Kim SH, Hur J-Y. Resistin concentration is increased in the peritoneal fluid of women with endometriosis. *Am J Reprod Immunol* (2010) 64:318–23. doi: 10.1111/j.1600-0897.2010.00840.x

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 08 December 2023
DOI 10.3389/fendo.2023.1261781

OPEN ACCESS

EDITED BY
Iveta Yotova,
Medical University of Vienna, Austria

REVIEWED BY
Verima Pereira,
University of California, San Francisco, United States
Chandni Talwar,
Baylor College of Medicine, United States

*CORRESPONDENCE
Prakash Nagarkatti
prakash@mailbox.sc.edu

†PRESENT ADDRESS
Hasan Alghetaa,
Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq
Amira Mohammed,
Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

RECEIVED 19 July 2023
ACCEPTED 17 November 2023
PUBLISHED 08 December 2023

CITATION
Alghetaa H, Mohammed A, Singh NP, Bloomquist RF, Chatzistamou I, Nagarkatti M and Nagarkatti P (2023) Estrobolome dysregulation is associated with altered immunometabolism in a mouse model of endometriosis. *Front. Endocrinol.* 14:1261781. doi: 10.3389/fendo.2023.1261781

Estrobolome dysregulation is associated with altered immunometabolism in a mouse model of endometriosis

Hasan Alghetaa†, Amira Mohammed†, Narendra P. Singh, Ryan F. Bloomquist, Ioulia Chatzistamou, Mitzi Nagarkatti and Prakash Nagarkatti*

Department of Pathology, Microbiology and Immunology, School of Medicine, University of South Carolina, Columbia, SC, United States

Introduction: Endometriosis is a painful disease that affects around 5% of women of reproductive age. In endometriosis, ectopic endometrial cells or seeded endometrial debris grow in abnormal locations including the peritoneal cavity. Common manifestations of endometriosis include dyspareunia, dysmenorrhea, chronic pelvic pain and often infertility and symptomatic relief or surgical removal are mainstays of treatment. Endometriosis both promotes and responds to estrogen imbalance, leading to intestinal bacterial estrobolome dysregulation and a subsequent induction of inflammation.

Methods: In the current study, we investigated the linkage between gut dysbiosis and immune metabolic response in endometriotic mice. Ovariectomized BALB/c mice received intraperitoneal transplantation of endometrial tissue from OVX donors (OVX+END). Control groups included naïve mice (Naïve), naïve mice that received endometrial transplants (Naive+END) and OVX mice that received the vehicle (OVX+VEH). Colonic content was collected 2 weeks post-transplantation for 16s rRNA pyrosequencing and peritoneal fluid was collected to determine the phenotype of inflammatory cells by flow cytometry.

Results: We noted a significant increase in the number of peritoneal fluid cells, specifically, T cells, natural killer (NK) cells, and NKT cells in OVX+END mice. Phylogenetic taxonomy analysis showed significant dysbiosis in OVX+END mice, with an increase in abundance of Phylum Tenericutes, Class Mollicutes, Order Aneroplasmatales, and Genus Aneroplasma, and a decrease in Order Clostridiales, and Genus Dehalobacterium, when compared to OVX+VEH controls. The metabolomic profile showed an increase in some tricarboxylic acid cycle (TCA)-related metabolites accompanied by a reduction in short-chain fatty acids (SCFA) such as butyric acid in OVX+END mice. Additionally, the mitochondrial and ATP production of immune cells was enforced to a maximal rate in OVX+END mice when compared to OVX+VEH mice.

Conclusion: The current study demonstrates that endometriosis alters the gut microbiota and associated immune metabolism.

KEYWORDS

endometriosis, microbiome, short chain fatty acids, T-cell metabolism, estrobolome, metabolome, immunometabolism

Introduction

Endometriosis is a chronic inflammatory condition and one of the most common gynecological disorders in the world, affecting an estimated 5% of women of childbearing age and resulting in significant global morbidity and medical expenditure (1). In endometriosis, ectopic endometrial glandular and stromal tissues are found outside of the uterus, and, like native endometrium, these tissues respond through growth and proliferation to estrogen-dependent signals (2, 3). Depending on the location of the ectopic tissue, endometriosis can result in significant inflammation, pain, and often infertility. While they can occur almost anywhere, almost all cases of ectopia are found somewhere between the uterine tubes and across the peritoneum, giving rise to the prevailing theory that endometriosis arises through the aberrant retrograde flow of shed endometrial lining up the uterine tubes during the menstrual cycle (4).

The pain and poor response to treatment of endometriosis are commonly attributed to the inflammatory response against ectopic tissue encountered in the disease, particularly during peaks of estrogen release at the transition from the proliferative phase to ovulation during the menstrual cycle (5, 6). Because of the biological role of the endometrium, endometriosis is a strongly estrogen-dependent disorder (7–9) and thus estrogen is considered a mitogen for the inflammatory process (10). Steroid hormones like estrogen are major controllers of reproductive capacity and also serve in the interplay of functions of immunocytes during inflammatory responses (10, 11). As the menstrual cycle progresses from ovulation to the luteal phase, estrogen levels drop and progesterone receptors are upregulated by endometrial tissue. Upon binding of this hormone, proliferation of endometrial tissue slows and moves to a glandular secretory function. While endometriosis tissue has been shown to downregulate progesterone receptors, these hormonal switches in function have been proposed as a mechanism of treatment for endometriosis (2, 11, 12). Other therapies have been proposed or attempted to some degree of success including analgesics for pain management (13), epigenetic regulation (14, 15), hormone therapy (16), dietary supplements (17, 18), surgical removal (13) and other symptomatic treatments (2, 19), but there is still not a reliable therapy for addressing this disorder.

The biological importance of estrogen is not limited to reproductive function, as it also plays a major role in microbiome metabolism (11, 20–22), hematological profile rearrangements (23) as well as in the regulation of immunometabolism (24–26). Intriguingly, while systemic levels of estrogen fluctuate through the normal ovulatory cycle, these steroid fluctuations have not been shown to have a significant effect on the gut microbiota (27). However, during prolonged periods of hormonal up or downregulations, such as during pregnancy, both steroid levels and gut microbiota are altered (28–30). The interplay between gut microbiome and estrogen levels has been designated as the "estrobolome" and is controlled by specific genes. Here, β-glucuronidase enzyme encoded by *gus* gene, native to the gut cleaves conjugated estrogen secreted into the intestine through bile and releases estrogen in its biologically active form (31). Furthermore, *gus* gene is expressed by common gut bacteria including *Ruminococcus gnavus*, *Staphylococcus aureus* and Clostridium (32). The normal estrobolome is essential for regulation of the reproductive cycle and it has been reported that in bacteria-free mice, reproductive capacity is impeded (33) but is then normalized to fertile levels when bacterial recolonization occurs (34). Free estradiol (E2) produced from gut β-glucuronidase together with ovarian produced E2 acts in concert to stimulate the immune system in a cyclical manner (24). During these immunological responses, macrophages increase their rate of glycolytic and tricarboxylic acid cycle shortly after being activated (35) and effector T cells accommodate glycolytic pathways in production of ATP molecules (36).

In this study we elucidated the dysregulation of immunometabolism as a response to estrobolome alterations during endometriosis. By seeding stimulated endometrial tissue to the peritoneum in syngeneic mouse transplant experiments, we explored the resulting perturbations of systemic inflammatory cells, gut microbiome, metabolome and immunometabolism encountered in endometriosis to obtain holistic understanding of the nature of changes occurring during this clinical disorder.

Materials and methods

Experimental animals

Female BALB/c mice aged between 6 – 8 weeks utilized in this study were purchased from The Jackson Laboratory and acclimatized for at least one week after delivery. Randomized grouping of animals was performed and every 4 mice were housed in same cage till the end of experiments. All study mice were exposed to same pathogen-free housing conditions with freely accessible food (standard chow) and water, 18 – 25°C temperature, and alternating 12 light/12 dark. Approval from the Institutional Animal Care and Use Committee (IUCAC) and from the University of South Carolina was obtained before performance of any study experiments (AUP2374).

Materials

Surgical instruments including Castroviejo scissors, uniband LA-1 micro point scissors, serrated jaw MF-2 micro forceps, fully curved micro forceps MF-3, straight micro forceps soldering tweezers, insertion/extraction tweezers and anti-wicking tweezers were purchased from Cedarlane labs – Canada. Surgical sutures including 5-0 Perma Hand Silk Black 1X18" PS-3, 5-0 Perma Hand Silk Black 2X60" no needle and 4-0 Perma Hand Silk Black 1X18" G-3 were purchased from the Ethicon – USA. Diethylstilbestrol (DES) was purchased from Sigma – USA and prepared in mineral oil (Sigma-Aldrich, USA). GIMA 2mm diameter biopsy punch

Abbreviations: DES, diethylstilbesterol; E2, estradiol; END, transplanted endometrial tissue; OVX, ovariectomized; SCFAs, short chain fatty acids; VEH, vehicle.

(GIMA, UK) was used to divide the endometrial layer into 2mm pieces. An 18G syringe needle (BD, USA) attached to a tuberculin syringe (BD, USA) was used to deliver endometrial specimens into the peritoneal cavity. Banamine (Merck, USA) solution was used every 12 hours for 2 two days to alleviate the pain during post-surgical care period. DMEM/F12 medium was used for preparation of endometrial transplant tissue (Sigma-Aldrich, USA). Skin incisions were closed with a Reflex Clip Applier (World Precision Instrument, USA) and clips were removed using Reflex Clip Removing Forceps (World Precision Instrument, USA).

Induction of experimental endometriosis

As the endometriosis is an estrogen-dependent disorder, mouse model was designed to mimic this disorder by using ovariectomized mice treated with estradiol (37). Briefly, donor and recipient mice were anaesthetized with inhaled isoflurane before being surgically prepared. The right and left ovaries with attached salpinx were ligated and removed. Muscular layers were sutured with silk, while the skin was closed with metal clips. Systemic analgesia, Benamine, was given every 12 hours post-surgery for 48 hours. Seven days later, the clips were removed and OVX mice were used.

The endometrial tissue was used to induce the experimental endometriosis in mice as described (38, 39). The schematic details are also provided in Figure 1. Briefly, BALB/c female mice were assigned to one of four experimental groups (Figure 1): OVX+END, ovariectomized mice receiving transplanted endometrial tissue; OVX+VEH, ovariectomized mice injected intraperitoneally with PBS but without transplant; Naïve (N) mice, non-ovariectomized mice with no treatment as control group and lastly, Naïve+END (NE), non-ovariectomized mice receiving transplanted endometrial tissue. All DES treatments were performed as 100µg/kg of DES injected subcutaneously. In the OVX+END and OVX+VEH groups (collectively considered ovariectomized-OVX), a dose of DES was delivered and ovariectomy was performed at day 0, while N and NE groups were housed without intervention. On day 5, OVX mice received another injection of DES in order to stimulate endometrial growth while N and NE groups were still under the influence of endogenous estrogen from their ovaries. Here, N group received no further intervention until they were euthanized. On day 7, OVX mice were split into OVX+VEH group, endometrium transplant donor group (END), and the OVX+END group described above to receive the transplanted tissue. One donor endometrium (END) provided enough tissue to seed the peritoneum of two recipient mice.

The donor mice were euthanized via overdose of isoflurane inhaled anesthesia. In a petri dish containing DMEM/F12 medium, the uterine horns of donor mice were longitudinally opened, and the endometrial layer was pealed from the myometrium and serosa layers using micro forceps and then divided into approximately 2mm pieces. This tissue was then transferred to another petri dish containing phosphate buffer saline (PBS). Approximately 35µg (wet weight) of the endometrial tissue samples were loaded into a 1ml syringe via an 18g needle in a total volume of 500 µl PBS. Loaded endometrial tissues were then transplanted to the peritoneal cavity

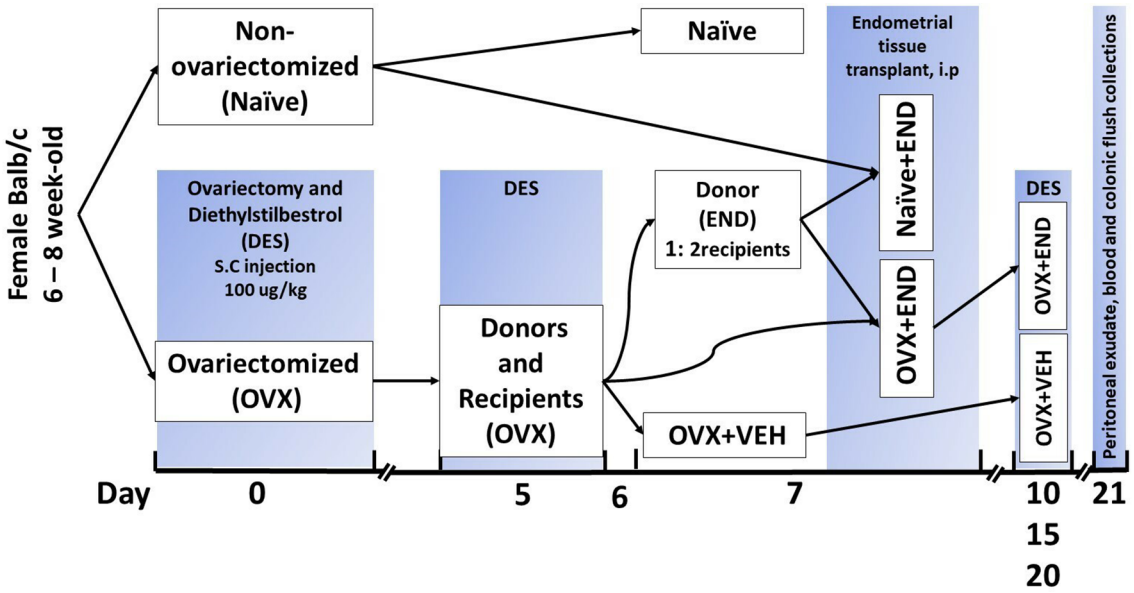


FIGURE 1
Experimental design. Mice were ovariectomized (OVX) and received analgesia after finishing the surgery and then repeated every 12 hours during post-surgical care period up to 48 hours to alleviate the pain. All ovariectomized mice were injected subcutaneously with diethylstilbesterol (DES) for 5 days after removal of ovaries and then repeated every 5 days till end of experiment. Closing clips were removed from all ovariectomized mice at day 7 post-surgical operation. Ovariectomized mice were divided into donors and recipients on day 7 at a ratio of 1:2. Harvested endometrial tissues (END) from OVX mice were transplanted into OVX (OVX+END) or non-ovariectomized (Naïve+END) recipient mice. As controls, we used non-ovariectomized naïve mice (Naïve), Naïve mice that received endometrial tissue (Naïve+END), and ovariectomized mice injected with vehicle (OVX+VEH).

through abdominal wall of either OVX+END or Naïve+END recipients to complete the transplant procedure.

At days 10, 15, and 20, the OVX+END and OVX+VEH groups received injections of DES, post ovariectomy procedure, while Naïve+END and Naïve did not receive DES still under hormonal influence of intact ovaries. The experimental endpoint was 14 days post endometrial transplant, which was 21 days post OVX procedure.

Tissue samplings for downstream analysis

At the endpoint of the experiments (14 days post-transplantation experiments), all study mice were anesthetized and euthanized under isoflurane for tissue sampling and downstream analysis. Blood samples were collected for complete blood count by VetScan analyzer system (Abaxis, USA) and for serological analysis. Single cell suspension of peritoneal fluid (PF) was prepared by using RBCs-lysis buffer (Sigma-Aldrich, USA) and strained with the 70-micron strainer (ThermoFisher, USA) to be utilized in either real-time metabolism analyzer Seahorse (Agilent, USA) or FACS-Celesta flowcytometry sorting system (BD, USA). Colon flushes (CF) were collected from the colon under aseptic conditions in sterile PBS for microbiome analysis. CF for metabolomic and short chain fatty acids analysis were collected in sterile distilled water. Uterine horns were excised for histopathological investigation with hematoxylin and eosin (H&E) staining and to collect uterine horn lavage fluid (ULF).

Inflammatory cell counting of peritoneal fluid and uterine lavage fluid

PF was collected by injecting the mice under deep anesthesia by isoflurane, then the skin of abdominal wall was opened to expose the peritoneal sac. Five milliliters of sterile PBS were intraperitoneally injected and the mouse then rolled thoroughly for 3 – 5 minutes to elute all the peritoneal traces and cells. ULF was collected by slow-passing 2 – 3 ml of PBS and collected in centrifuge tubes. Collected PF and ULF was then transferred to conical tubes for centrifugation and separation of the supernatant and stored at -80°C for analysis. While the pelleted cells were treated with RBC lysis buffer for 30 – 60 seconds and then blocked by using cold 10%-FBS buffer and washed with cold FACS for cell counting. Cells were resuspended in FACS buffer for counting by using Trypan blue staining and TC20 automated cell counter (40).

Mononuclear cell isolation and T cells subset determination from peritoneal fluid

Briefly, peritoneal fluid was collected in cold FACS and processed into single cell suspension by using RBC lysis buffer (Sigma-Aldrich, USA) for about 60 seconds and then washed with cold FACS. Then all samples were filtered with 70µm strainer (ThermoFisher, USA), centrifuged at 4°C, 1000 RPM for 10 minutes and then the cell pellets were resuspended in cold FACS. To determine the T cell and natural killer (NK) subset, isolated cells were stained with anti-CD3-APC and NK1.1-BB515 antibodies (Biolegend, USA), respectively. Finally, the stained samples were analyzed by using BD-FACS Celesta flow cytometry system (BD, USA) and acquired data were visualized by using built-in Diva software (BD, USA) as described before (41).

Peripheral blood count assessment

Whole blood was obtained via retroorbital vein rupture by using heparinized capillary tubes inserted into medial canthus of isoflurane-anesthetized mice. The collected blood samples were kept in heparinized tube before being transferred to be analyzed by blood-autoanalyzer system, Vetscan HM5 hematology analyzer (Abaxis, USA) for complete blood count assessment.

^{3}H-Thymidine incorporation assay

To evaluate the proliferative capacity of PF inflammatory cells, 10^5 cells per well were seeded in DMEM/F12 medium for 12 hours incubated with 1µCui/well of ^{3}H-thymidine isotope at 37°C and 5% CO2, then radioactivity was measured using MicroBeta Trilux liquid-scintillation counter to (36, 42).

Histopathological examination of uterine tissue

Harvested uterine horns were fixed in 4% paraformaldehyde overnight and then stored in PBS at 4°C till the time of sectioning and staining with hematoxylin and eosin (43).

Colonic microbiota analysis

The collected colonic flushes were prepared for 16s rRNA as described by previous publications (44, 45).

Measurement of short chain fatty acids levels in colonic flushes

Briefly, colons of study mice were opened and about 100 mg of luminal solid contents were removed and then suspended with distilled water. Collected flushes were centrifuged to separate the supernatant and stored at -80°C for future use. At time of SCFA analysis, all samples were thawed gradually in ice box and then acidified by hydrochloric acid (HCL) before adding 4-methylvaleric acid as internal standard. Gas chromograph CP-3800 (Varian) and spectrometry mass (GC-MS) system was used to quantify the SCFAs. Varian MS Workstation (version 6.9.2) was used to collect and analyze acquired data. Finally, the linear equation was

used to calculate the concentrations of SCFAs in each sample (41, 46).

Glycolytic and tricarboxylic acid metabolites measurement and metabolome profiling

Serum was separated from study mice and used for metabolic extraction by using liquid chromatograph mass spectrometry (LC-MS) as described in our previous study (36). Briefly, metabolites derived from the TCA and glycolysis were characterized by using 5mM of ammonium acetate (pH9.9) and other buffers in assistance of Luna 3µM NH2 100 A° chromatography column (Phenomenex, CA). All identified metabolites were normalized to internal standard (47).

Real-time metabolism analysis of mitochondrial respiration by XFp Seahorse analyzer

These two metabolic pathways were evaluated by using real-time metabolism analyzer Seahorse (Agilent, USA) according to the manufacturer's protocols. The principle of measurements was based on the calculation of oxygen consumption rate (OCR) and extra cellular acidification rate (ECAR). Cells ($3X10^5$) taken from single cell suspension of PF was seeded into XFp miniplates by assistance of Cell-Tak biological adhesive (Corning, USA). At the end of analyzer run, Hoechst 33342 dye (Invitrogen, USA) was applied to the plate wells to count the remaining live cells in order to normalize the acquired data based on the number of living cells used in this run. Counting of live cells was performed by using automated imaging microscope, Cytation5 Imaging System (BioTek, USA). Generated data by Seahorse were transferred to be interpreted by Seahorse Wave Desktop Software (Agilent, USA) to calculate the kinetic energy (36).

Statistical analysis

All experiments of this study were repeated 3 independent times. Sample sizes varied between 3 – 5 mice per group and the numbers are stated in the figure legend. Metabolomes were normalized and analyzed as Log2 examined by t-test by using R-studio (R Studio Inc, USA) as described previously (36). One-Way ANOVA test was applied whenever there were more than two groups to compare with multiple Tukey's correction. The t-test was applied with Holm-Sidak correction method to compare two groups. $p<0.05$ considered as significant threshold and the levels were depicted as: *$p<0.05$, **$p<0.01$, ***$p<0.001$ and $^{\#}p<0.0001$. When statistical comparison includes more than three groups, different lowercase letters were used to depict significant differences among these groups. For example, groups having the same letters 'a' would be insignificant while groups depicting 'a' and 'b' would be statistically significant.

Results

Endometrial transplants in OVX mice trigger an exacerbated inflammatory response in the peritoneal cavity and peripheral blood

At fourteen days post-receiving endometrial transplantation, local and systemic immunologic responses were evident (Figures 2, 3). Histopathological investigation of uterine horns excised from the four groups of mice using H&E staining showed that in the OVX+END group there was an increase in the inflammatory cells (yellow arrows) to the endometrium as well as myometrium (Figures 2A, B) when compared to the control OVX+Veh or Naive groups. By using ImageJ software, the statistical analysis showed significant increase in the number of inflammatory cells (mostly neutrophils) in the endometrium (endometritis) as well as in myometrium (myometritis) of OVX+END group in comparison with all other study groups (Figure 2C). There was also significant augmentation ($p<0.05$) in the inflammatory cells in the peritoneal cavity of OVX+END group when compared to OVX+VEH or Naïve groups (Figure 3A, left panel). Interestingly, the Naïve +END group also behaved similar to OVX+END group. The cell counts in the uterine lumen were not significantly altered in all groups tested (Figure 2A, right panel). The total WBC counts in the peripheral blood of the OVX+END group was significantly higher ($p<0.05$) than the OVX+VEH and naïve mice (Figure 3B, right panel). The percentage of NK cells were higher in the OVX+END group when compared to other groups (Figure 3C). The total number of $CD3^+$ T cells and $CD3^+NK^+$ cells in the peritoneal cavity were found to be significantly increased ($p<0.05$) in the OVX +END group when compared to Naïve or OVX+VEH groups (Figures 3D, E). The percentage of lymphocytes and neutrophils in the peripheral blood were studied too. When we tested the percentage of lymphocytes in the blood, it was decreased in all groups when compared to the naïve group (Figure 3F). The neutrophil percentage in the peripheral blood of mice transplanted with endometrial tissue (Naïve+END, OVX+END) was significantly higher ($P<0.05$) than in the non-transplanted mice (Figure 3G). ^{3}H-thymidine incorporation assay was performed to estimate the proliferative capacity of the inflammatory cells in the peritoneal fluid and to assess the ability of these cells to proliferate. Interestingly, the results showed that there was significant increase ($p<0.05$) in proliferation capacity of inflammatory cells isolated from OVX+END group when compared to Naïve or OVX+VEH group. The Naïve+END mice also demonstrated a similar response as the OVX+END mice (Figure 3H). Overall, the results indicated that the endometrial transplants in OVX mice were associated with an exacerbated inflammatory response in the peritoneal cavity and peripheral blood. It was interesting to note that naïve mice that received the endometrial transplant (Naïve+END), also exhibited some immunological changes like OVX+END group such as increase in the percentages of neutrophils while with respect to most of the immunological profiles studied, they behaved like the naïve controls.

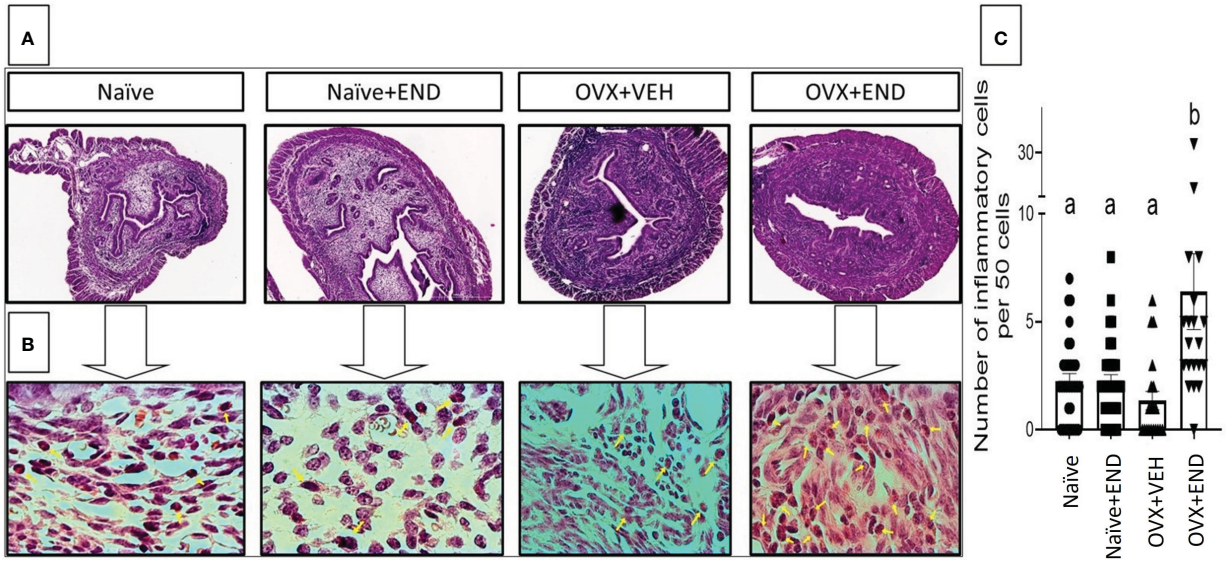


FIGURE 2
Histopathological investigation of uterine horn 14 days post-endometrial tissue transplantation. H&E-stained cross-sections of uterine horn of different experimental groups, (n=4) examined under **(A)** 4X and **(B)** 100X; yellow arrows point the inflammatory cells in the uterine parenchyma. **(C)** Statistical analysis of counted inflammatory cells per 50 cells in every examined field (n= 20–31) calculated by One-way ANOVA test. Different lowercase letters depict significant differences when $p<0.05$. For detailed P-value, please check Supplementary Table S1.

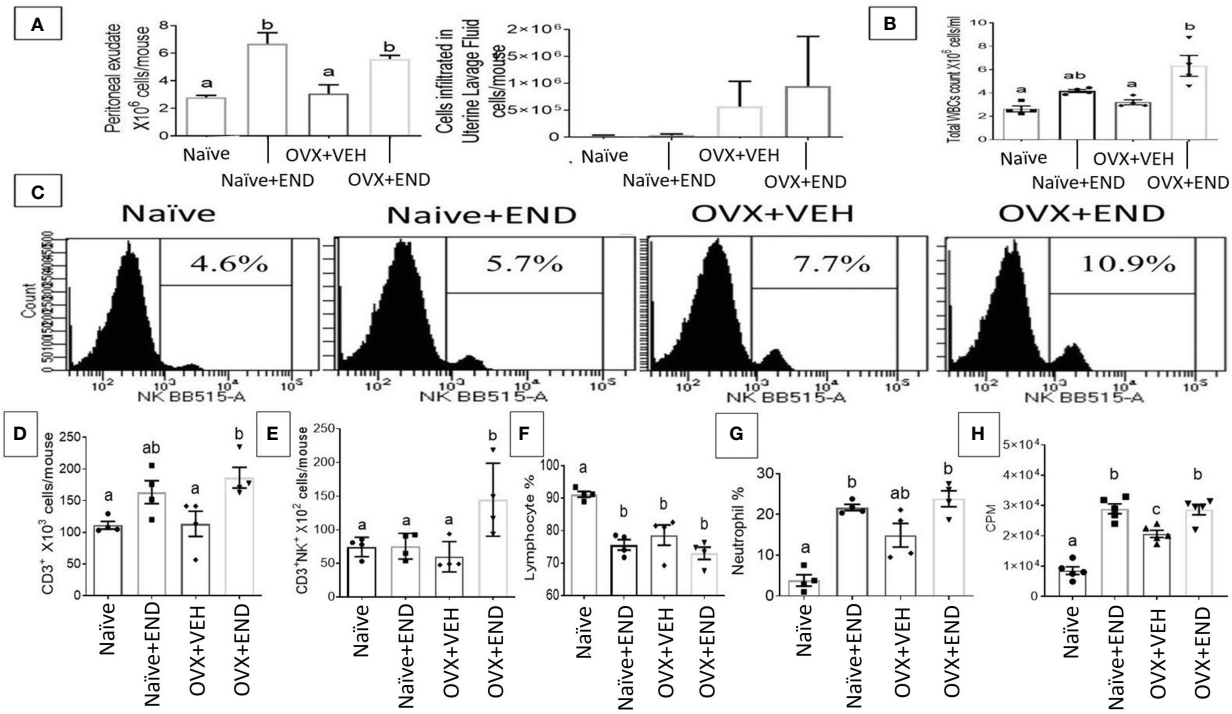


FIGURE 3
Evaluation of endometriosis induction 14 days post-endometrial tissue transplantation. **(A)** Total number of inflammatory cells in the peritoneal cavity (left) and uterine lavage fluid (right) counted by using hemocytometer chamber with trypan blue dye. **(B)** Total white blood cells in peripheral blood measured by Vetscan HM5, (n=4). **(C)** Flow cytometry results of peritoneal fluid (PF) cells stained for natural killer (NK) population, (n=4). **(D)** Total number of CD3+ T-lymphocytes in PF, (n=4). **(E)** Total natural killer T-cells ($CD3^+NK^+$) in PF, (n=4). **(F)** Percentage of lymphocyte population in the peripheral blood, (n=4). **(G)** Percentage of neutrophil population in the peripheral blood, (n=4). **(H)** Proliferation of inflammatory cells in the peritoneal cavity isolated from different study groups detected using ^{3}H-thymidine-incorporation assay, (n=5). One-way ANOVA statistical analysis used for panels **(B, D-H)**. Different lowercase letters depict significant differences when $p<0.05$. CPM, Counts per minute, For detailed P-value, please check Supplementary Table S2.

Endometrial transplantation in Naïve or OVX mice leads to enhanced gus-enriched bacteria like Ruminococcus spp

Investigation of gut microbial communities via sequencing the 16s rRNA V3-V4 regions showed that there was dysbiosis in the microbial environment of the colon in all experimental groups compared to the Naïve group (Figure 4). Bioinformatic analysis of our collected data showed that the commensal bacteria of individual mice were clustered closely within their own group, while the 4 groups tested showed clear segregation (Figure 4A). A cladogram generated from linear discriminant analysis effect size (LEfSe) demonstrated that Clostridiales_order, Tenericutes phylum and its down tree ancestry, Mollicutes class, Anaerplasmatales order, Anaeroplasmaceae family, and Anaeroplasma genus constituted the detectable commensal bacteria in the guts of the Naïve group (Figure 4B). The Naïve+END and OVX+END mice showed simultaneous deviation from Naive and OVX+VEH gut microbiotas and showed similarities between each other with the enrichment of Phylum Tenericutes, Class Mollicutes, Order Aneroplasmatales, and Genus Aneroplasma. Lacnospiraceae family and its Coprococcus genus, Ruminococcus genus, and *Ruminococcus gnavus* were commensal biomarkers in the Naïve +END group that were not present in the Naïve group (Figure 4B). Also, Clostridiales order was the biomarker bacteria in the colons of OVX+VEH (Figure 4B). Statistical analysis of occupational taxonomy units (OTUs) of bacteria showed significant alterations ($p<0.05$) among the study groups (Figure 4C). Furthermore, linear discriminant analysis (LDA) scores set at >2-fold change among the study groups (Naïve, Naive +END, OVX+VEH and OVX+END), using PICRUST-generated level 3 KEGG pathways of 16s rRNA data revealed that there was no biomarker functional pathway of the bacteria for Naïve+END group (Figure 4D). The other groups showed different pathways involved in the bacterial metabolism that are related to the host metabolism (Figure 4D). Together, these data suggested that each treatment group had a distinct population of bacteria (Figure 4A) and that Naïve or OVX mice that received endometrial transplants exhibited enhanced *gus*-enriched bacteria like Ruminococcus spp. which in turn could improve the E2 metabolism as a consequence (Figure 4B).

Short-chain fatty acids produced by gut microbiota exhibit differential expression following endometrial transplants

Next, we studied the bacterial end-product concentrations in the guts represented by the short chain fatty acids (SCFAs) by using Gas Chromatography-Mass Spectrometry (GC-MS) showed in Figure 5A. Upon examination of the 4 groups of mice, the overall trend indicated that significant ($p<0.05$) decreased levels of acetic acid (Figure 5B), propionic acid (Figure 5C), butyric acid

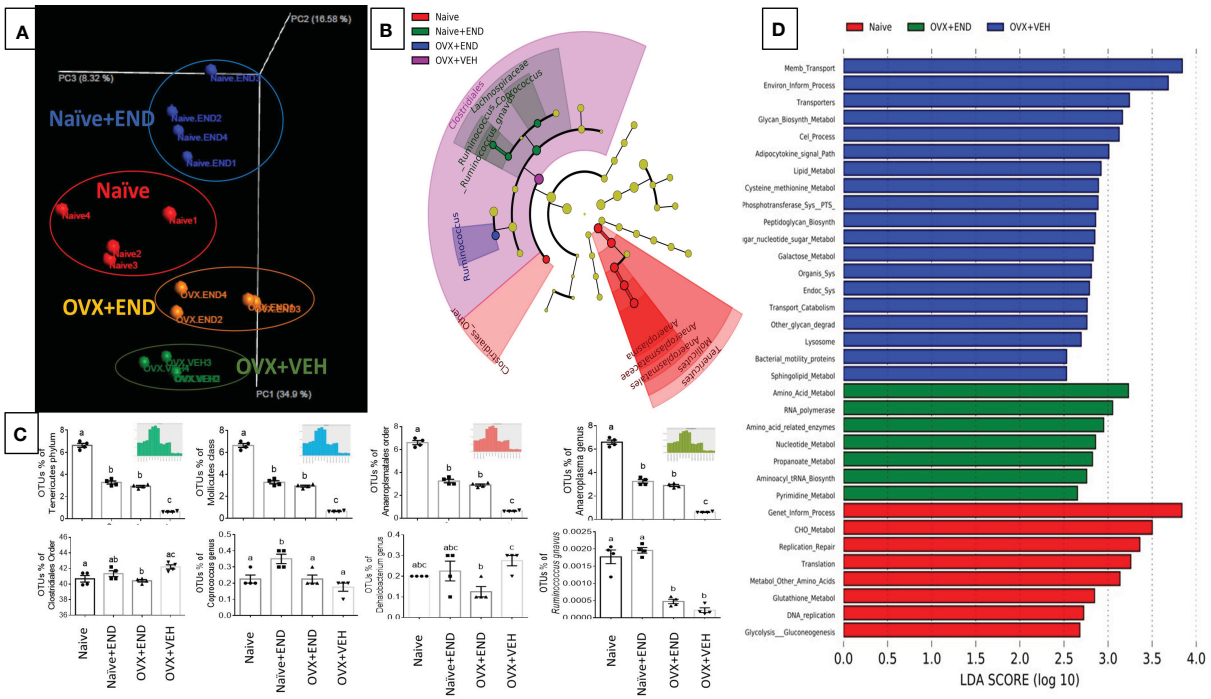


FIGURE 4
Characterization of bacterial communities and their functional metabolism pathways via 16s rRNA metagenomic analysis. **(A)** Unweighted beta-diversity of the commensal gut microbiome. **(B)** Cladogram using results of the linear discriminant analysis (LDA) model of bacterial hierarchy. **(C)** One-way ANOVA statistical comparisons among the experimental groups based on the percentage of occupational taxonomy units (OTUs) of biomarker bacteria. **(D)** LDA analysis of the microbial metabolic pathways via metagenomic functional prediction of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST). Different lowercase letters in **(C)** indicates significant differences when $p<0.05$. (n=4). For detailed P-value, please check Supplementary Table S3.

(Figure 5D) and valeric acid (Figure 5E) in OVX+END group when compared to the Naïve group. Similar changes were also seen in OVX+VEH group suggesting that ovariectomy and DES treatment had significant impact on colonic SCFAs. The Naïve+END group also exhibited both similarities and differences in SCFAs when compared to the Naïve group thereby suggesting that endometrial transplants in naïve mice also induced some changes in SCFAs. Together, these data suggested that bacterial metabolites, SCFAs, exhibit differential expression following endometrial transplants and because SCFAs also regulate immune cells, the SCFAs may play a role in immune response during endometriosis.

Endometrial transplantation enhances the immunometabolism in inflammatory cells and altered plasma metabolome

We next studied the interplay between estrobolome dysregulation of microbiota and their potential influence on the immune cells' metabolomic response in the endometriosis model using cells from the peritoneal cavity. These cells were seeded into specific culture plates of XFp Seahorse real-time metabolism analyzer before being incubated and analyzed based on calculation of oxygen consumption rate and extracellular acidification rate to estimate the ATP production rate (Figures 6, 7). Real-time metabolism analysis revealed that the basal and stressed mitochondrial respiration of immune cells were significantly ($p<0.05$) higher in the Naïve+END (Figures 6A, B) and OVX+END groups (Figures 7A, B) in comparison to non-transplanted mice of Naïve and OVX+VEH groups, respectively. Furthermore, the main source of ATP production in the Naïve group was via mitochondrial respiration (Figures 6C, D). However, transplantation of endometrial tissues led to significantly ($p<0.05$) accelerated mitochondrial ATP production which in turn significantly ($p<0.05$) increased the total ATP molecules in cells from Naïve+END group (Figure 6D). In the OVX groups, endometrial transplantation modified the respiratory pathways of inflammatory cells (Figures 7B, C). Stimulating the inflammatory cells in the peritoneal cavity of OVX+END mice led to further significant ($p<0.05$) increase in the rate of ATP production via mitochondrial and glycolytic processes in comparison with OVX-VEH group (Figure 7D). Together, these data suggested that endometrial transplantation-induced immune cells exhibit accelerated metabolic rate.

TCA-related metabolites levels, such as fumarate, glutamate, malate and succinate were significantly ($p<0.05$) higher in Naïve +END group than in Naïve mice (Figure 6E). Interestingly, glycolysis-related metabolites such as 3-phosphoglycerate and 2-phosphglycerate (3PG and 2PG), glucose-6-phosphate and fructose-6-phosphate (G6P/F6P) and phosphoenolpyruvate (PEP) were significantly ($p<0.05$) elevated in the plasma of Naïve+END mice in comparison to Naïve group (Figure 6E). Interestingly, when such metabolites were studied in OVX+END mice, some of the metabolites showed opposite results. For example, the levels of 3PG, 2PG, lactate, malate, PEP were decreased while some such as glucose/fructose, glyceraldehyde, ketoglutarate, oxalate, and

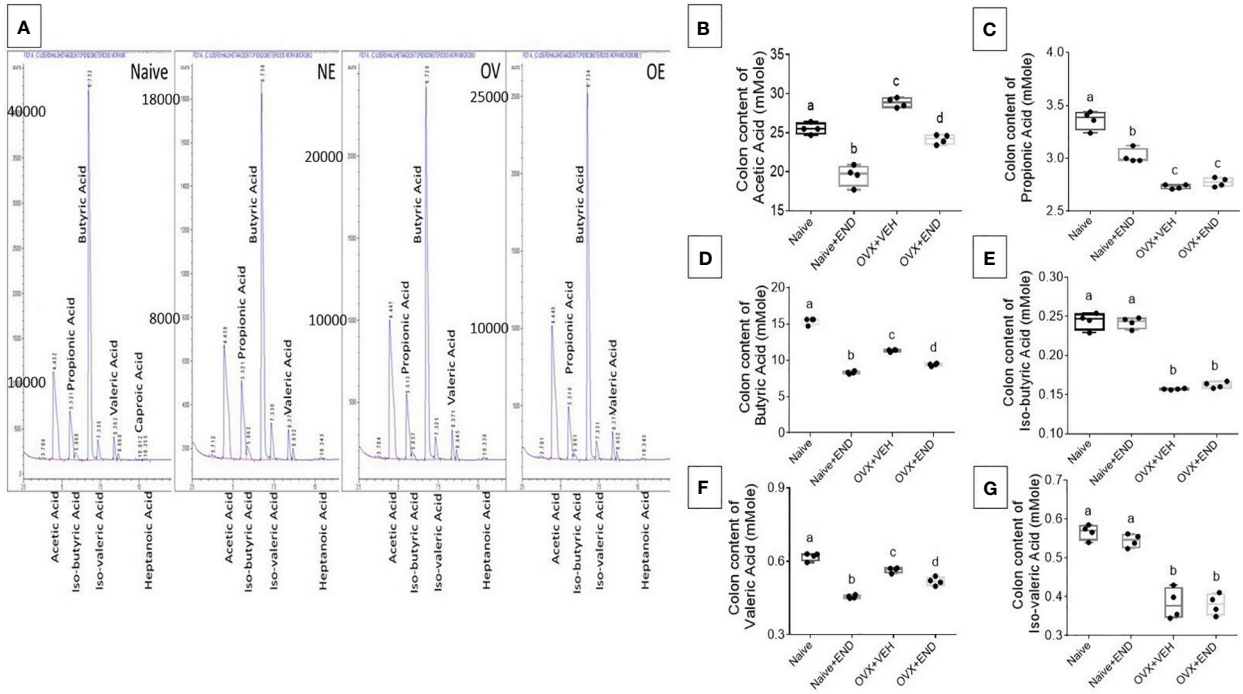


FIGURE 5
Colonic enrichment of short chain fatty acids (SCFAs). **(A)** GC-MS panels showing peaks of SCFAs. **(B–G)** One-way ANOVA statistical analysis of the colonic content of SCFAs, (n=4). **(B)** Acetic acid. **(C)** Propionic acid. **(D)** Butyric acid. **(E)** Iso-butyric acid. **(F)** Valeric acid. **(G)** Iso-Valeric acid. Different lowercase letters in **(C)** indicates significant differences when $p<0.05$. For detailed P-value, please check Supplementary Table S4.

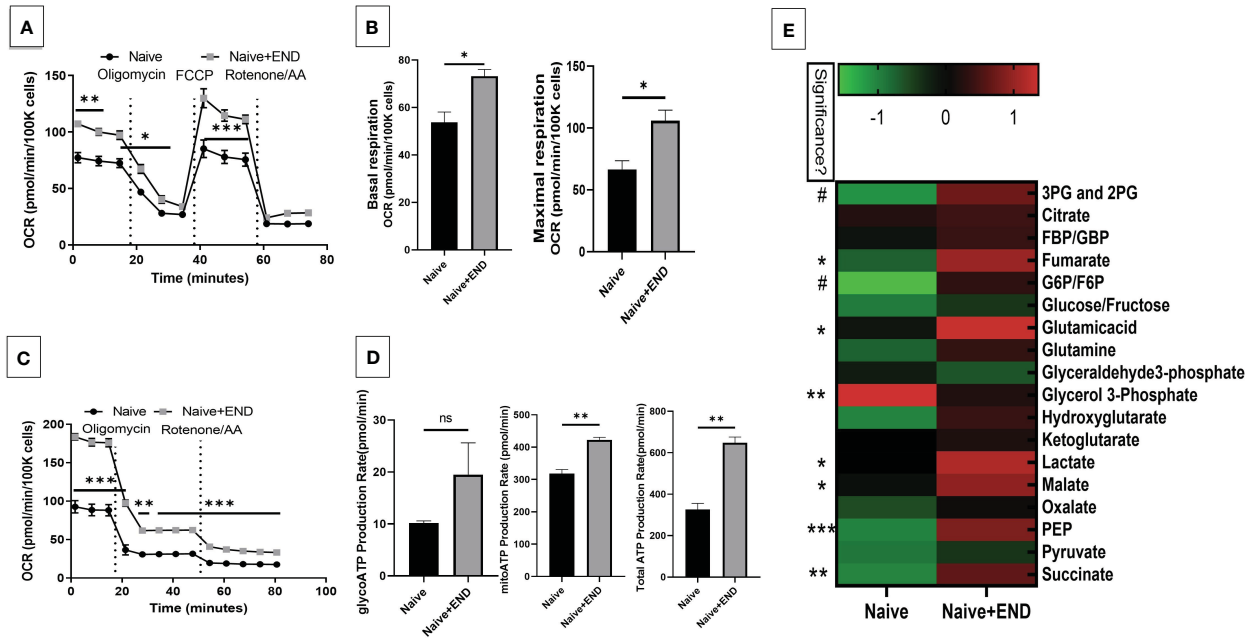


FIGURE 6
Effect of metabolic pathways of PF inflammatory cells in non-ovariectomized mice. **(A)** Cell Mito Stress test detected by real-time metabolism analyzer Seahorse (n=3). FCCP: carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone. AA: antimycin **(A. B)** Kinetic calculation of basal (left) and maximal (right) mitochondrial respiration. **(C)** ATP production rate test detected by real-time metabolism analyzer Seahorse (n=3). **(D)** Kinetic calculations of glycolytic ATP production rate (left), mitochondrial ATP production rate (middle) and total ATP production rate (right). **(E)** LC-MS showing the plasma metabolomic profile regarding TCA and glycolysis pathways, (n=4). ns, not significant; *p<0.05, **p<0.01, ***p<0.001, #p<0.0001.

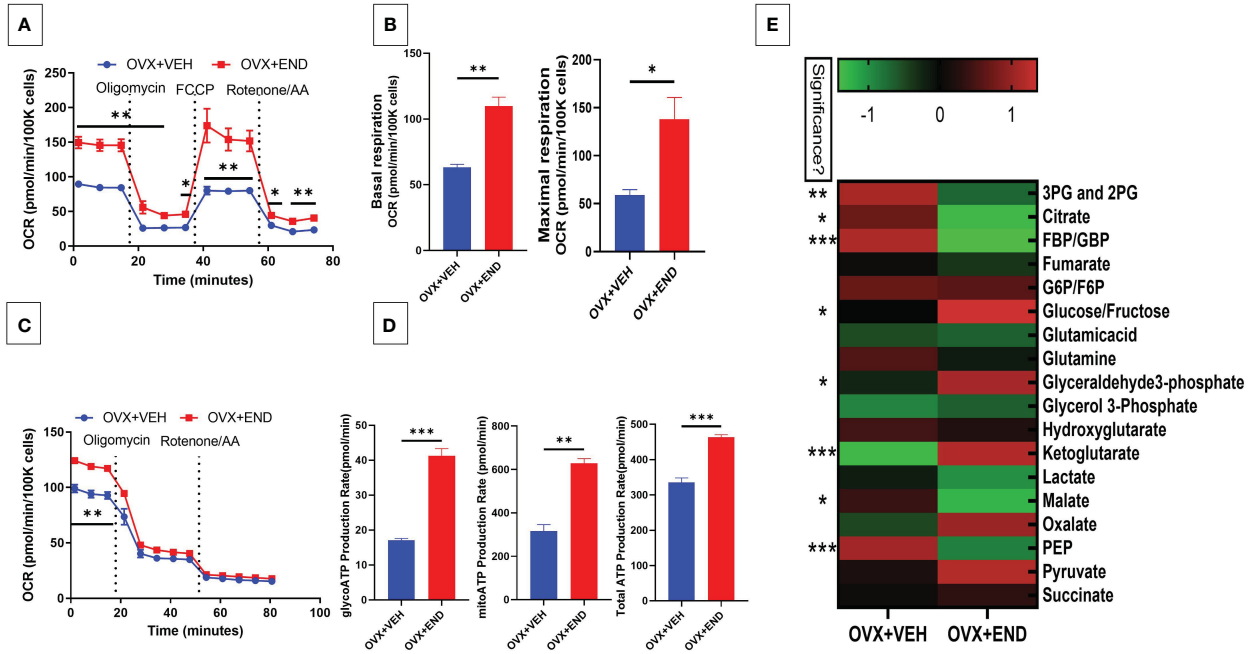


FIGURE 7
Effect of metabolic pathways of PF inflammatory cells in ovariectomized mice. **(A)** Cell Mito Stress test detected by real-time metabolism analyzer Seahorse (n=3). FCCP: carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone. AA: antimycin **(A. B)** Kinetic calculation of basal (left) and maximal (right) mitochondrial respiration. **(C)** ATP production rate test detected by real-time metabolism analyzer Seahorse (n=3). **(D)** Kinetic calculations of glycolytic ATP production rate (left), mitochondrial ATP production rate (middle) and total ATP production rate (right). **(E)** LC-MS showing the plasma metabolomic profile regarding the TCA and glycolysis pathways, (n=4). *p<0.05, **p0.01, ***p<0.001.

pyruvate were increased in OVX+END when compared to the OVX +VEH group (Figure 7E). It was noteworthy that several of the metabolite expression profile was different between OVX+END vs Naïve+END group (Figure 6E vs Figure 7E), thereby suggesting that ovariectomy did affect the metabolomic profile in addition to endometrial transplant.

Discussion

For decades, researchers have investigated the interface between the metabolism and immune response in various physiological and pathological conditions, such as inflammatory bowel diseases (48), cardiovascular disease (49), and in a myriad of cellular models (36, 41, 50–55). The development of endometriosis is regulated by estrogen metabolism and host inflammation, which in turn are influenced by several factors such as the microbiome and the estrobolome (56). However, to date, few estrobolome studies have been performed to investigate the estrogen-microbiome axis across disease (20, 57, 58). While the study of the relationship between the estrobolome-endometriosis-gut microbiome-axis is ripe for discovery, the multifactorial causes of endometriosis add challenges to understanding its pathogenesis (12).

Many studies have been performed that associate endometriosis with changes in gut microbiome (59). It is also well established that endometriosis is a chronic estrogen-dependent inflammatory disease with endometrial stroma and glands outside the uterine cavity (60). However, whether there is cross-talk between gut dysbiosis and inflammation is not clear. Moreover, how the metabolites produced by the endometriosis-induced gut microbiota alter the immune metabolism in the inflammatory cells has not been previously studied. In the current study, we tried to connect all such events induced during endometriosis by using both naïve and OVX mice to get a better understanding of the cross-talk between endometriosis, gut microbiota and immune functions in the host.

There are several murine models of endometriosis (61). In the present study, we used the well-established mouse model of endometriosis in which the endometrial tissue is injected intraperitoneally into the recipient OVX mice (39). Such models have also been compared to models in which the recipient mice are intact that have not undergone ovariectomy (62), similar to our Naïve+END group. Both models develop endometrial lesions although the progression of the disease varies to some extent. In the current study, we noted that OVX+END group demonstrated similarities to the Naïve+END group with respect to the induction of some changes in the microbiota and immune profile while differing in some respects. For example, both OVX+END and Naïve+END groups showed similar induction of peritoneal inflammatory cells and neutrophils in the blood. Also, both groups exhibited increased presence of Ruminococcus genus. However, the OVX+END and Naïve+END groups showed significant difference in their expression of certain microbes, SCFA, and metabolite profile. The main difference between OVX +END and Naïve+END mice is that the former lacked the ovarian hormones but received exogenous administration of estrogen, while the Naïve+END mice had intact hypothalamic-pituitary-ovarian axis which controls several hormones and regulate female reproduction. Thus, our studies suggested that such hormones may play a role in regulating the microbial dysbiosis and immune metabolism during endometriosis.

In the present study, we investigated the microbial dysbiosis and the immunocyte metabolism triggered following endometrial transplants. Endometriosis is best hypothesized to arise from abnormal growth of endometrium-like tissue derived from escaped uterus that get implanted in the pelvic and abdominal cavity wall (13). This triggers an inflammatory response consistent with our observation of an excessive number of immune cells in the parenchyma of uterus (Figures 2A-C), peritoneal fluid, and an increase in total WBC counts in peripheral blood (Figures 3A-G). Accumulations of such cells is hypothesized to contribute to the decline in fertility rate and pain with menstruation in endometriosis (2, 13, 19). The immune cells that infiltrate is heterogeneous. Fukui et al. showed the infiltration of NK cells to the peritoneal cavity leading to inflammation during endometriosis (63). In addition, plasma cells in the uterine tissue could also serve as a biomarker of non-invasive diagnosis of endometriosis (3). Other cells reported to infiltrate include dendritic cells and neutrophils (3, 5, 64). In the current study, we noted an increase in $CD3^{+}$T cells, NK cells, and NKT cells in the uterine cavity as well as increases in lymphocytes and neutrophils in the blood.

The aggregation of different immunocytes in the peritoneum seen in endometriosis has the potential to increase levels of reactive oxygen species present, which in turn could enhance inflammatory cell proliferation (Figure 3H) and leading to tissue damage (6). Clinical trials have been performed to treat endometriosis via suppression of the inflammatory response from oxidative stress using herbal-derivative anti-oxidants (13, 17, 18), miRNA regulation (14), or hormonal manipulation (11, 14). It has been hypothesized that there is an integral interplay between the gastrointestinal microbiome and estrogen and other hormones (16, 22, 31). We therefore examined perturbations of the gut-microbiota to determine if endometrial transplantation altered the gastrointestinal homeostasis and microbiome profile (Figure 4). Interestingly, our results showed that there was a distinct separation between the microbial communities of each study group (Figure 4A). Moreover, we found that there was a biomarker bacteria for each group of Naïve (Tenericutes, Mollicutes, Anaeroplasmatales, Anaeroplasmataceae and Anaeroplasma), Naïve+END (Lachnospiraceae family includes Coprocuccus and [Ruminococcus] genera), OVX+VEH (Clostrediales order), and OVX+END (*Ruminococcus* spp) (Figure 4B). Interestingly, most of biomarker bacteria in all study groups except Naïve mice shared one gene involvement, the *gus*-gene, that is common in Firmicutes (65) and responsible for encoding β-glucuronidase enzyme (32, 66), which in turn removes the glucuronic acid from conjugated substrates such as steroid hormones and other xenobiotics secreted to the intestinal lumen through the bile duct after being processed by hepatic glucuronidation to promote reabsorption of them as aglycone steroid hormones or xenobiotics via enterohepatic circulation again (67–69). This aligns with our PICRUST findings (Figure 4D) that predicted an increase amino acids and pyrimidine

metabolism in OVX+END mice, as compared to the lipid metabolism predicted in OVX+VEH and the carbohydrate metabolism predicted in the Naïve group.

Several mechanisms have been attributed to the beneficial effects of microbiome in health maintenance including the production of short chain fatty acids (SCFAs) (70). Enrichments of *Ruminococcus gnavus* in Naïve and Naïve+END groups (Figure 4C) and Lachnospiraceae in Naïve+END groups are all major producers of short chain fatty acids (SCFAs) and may explain the elevated levels of propionic (Figure 5C) and Iso-butyric (Figure 5E) acids in the colonic flushes of respective groups (32, 70). However, butyric acid, which is the most beneficial SCFA for intestinal homeostasis, energy metabolism, and anti-inflammatory effects (71–74) was significantly higher in the Naïve group in comparison with all other groups (Figure 5D), which could be due to the abundance of *Ruminococcus gnavus* (Figure 4C). These data also suggested that decreased butyric acid production in Naïve +END and OVX+END groups may be related to increased inflammation seen in these groups.

Another major role of the microbiome is to metabolize estrogen hormone under the regulation of *gus* gene (32, 66). Any deviation in the steroid hormone production in the host circulation could lead to initiation of disease through microbiome dysregulation (16, 20, 22, 31, 57). Nevertheless, the balance of steroid production during the sexual cycle is necessary for reproduction (22). Long-term changes in reproductive hormones lead to shifts in gut microbiome (28–30) and the increase in progesterone levels during pregnancy is associated with an increase in beneficial bacteria like Bifidobacterium (29). Gut dysbiosis certainly influences immune system function (41, 52, 75) as well as reproductive physiology through the modulation of reproductive hormones function and vice versa (22, 28, 29, 57, 66). In our study, we found through metabolic evaluation of the immune cell in the peritoneum of endometriotic and non-endometriotic mice (Figures 6, 7) that the immunometabolism of these cells shifted, likely due to the microbiota and estrobolome dysbiosis (20, 22, 58). Estrogen plays important regulatory role in the maintenance of the immune system functions and to counteract inflammations and oxidative stress, thus, the reproductive system-gut axis and its related metabolism and metabolites are important regulators to stabilize the health and homeostasis (9, 76).

In summary, the current study suggests that reversing the endometriotic gut microbiota by, for example, through fecal transplants from healthy donors or increasing diet enrichment with prebiotics and/or probiotics could help in suppressing the endometriosis pathogenesis as well as severity which may result in minimizing its driven complications.

Data availability statement

The datasets presented in this study can be found in online repositories, https://www.ncbi.nlm.nih.gov/geo, under accession number GSE248213.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee (IUCAC) in the University of South Carolina before performance of any study experiments (AUP2374). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HA: Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. AM: Data curation, Formal analysis, Methodology, Writing – review & editing. NS: Formal analysis, Methodology, Writing – review & editing. RB: Writing – review & editing. IC: Formal analysis and Methodology. MN: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. PN: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported in part by NIH R01ES030144, R01AI123947, R01AI160896, R01AI129788, P20GM103641, and P01AT003961 to PN and MN.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1261781/full#supplementary-material

References

1. Sarria-Santamera A, Orazumbekova B, Terzic M, Issanov A, Chaowen C, Asúnsolo-del-Barco A. Systematic review and meta-analysis of incidence and prevalence of endometriosis. *Healthcare* (2020) 9(1):29. doi: 10.3390/healthcare9010029

2. Vercellini P, Vigano P, Somigliana E, Fedele L. Endometriosis: pathogenesis and treatment. *Nat Rev Endocrinol* (2014) 10(5):261–75. doi: 10.1038/nrendo.2013.255

3. Freitag N, Pour SJ, Fehm TN, Toth B, Markert UR, Weber M, et al. Are uterine natural killer and plasma cells in infertility patients associated with endometriosis, repeated implantation failure, or recurrent pregnancy loss? *Arch Gynecol Obstet* (2020) 302(6):1487–94. doi: 10.1007/s00404-020-05679-z

4. Van der Linden PJ. Theories on the pathogenesis of endometriosis. *Hum Reprod* (1996) 11(suppl_3):53–65. doi: 10.1093/humrep/11.suppl_3.53

5. Gardella B, Dominoni M, Gritti A, Arrigo A, Antonucci S, Carletti GV, et al. Endometriosis pain and epithelial neutrophil activating peptide-78 levels. *Sci Rep* (2022) 12(1):3227. doi: 10.1038/s41598-022-07349-3

6. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal* (2014) 20(7):1126–67. doi: 10.1089/ars.2012.5149

7. Chantalat E, Valera M-C, Vaysse C, Noirrit E, Rusidze M, Weyl A, et al. Estrogen receptors and endometriosis. *Int J Mol Sci* (2020) 21(8):2815. doi: 10.3390/ijms21082815

8. Abbas HK. Study the pathological effects of the combination of estrogen and progesterone hormones on some organs experimentally induced in mice. *Iraqi J Veterinary Med* (2012) 36(1):128–36. doi: 10.30539/Iraqijvm.v36i1.556

9. Naji TS, Al- Rubae'I SHN, Turki KM. The association between hormonal variation, antioxidant status and oxidative stress in Iraqi women with endometriosis. *J Faculty Med Baghdad* (2016) 58(2):192–6. doi: 10.32007/jfacmedbagdad.582242

10. Bulun SE, Cheng Y-H, Yin P, Imir G, Utsunomiya H, Attar E, et al. Progesterone resistance in endometriosis: link to failure to metabolize estradiol. *Mol Cell Endocrinol* (2006) 248(1-2):94–103. doi: 10.1016/j.mce.2005.11.041

11. Szukiewicz D. Aberrant epigenetic regulation of estrogen and progesterone signaling at the level of endometrial/endometriotic tissue in the pathomechanism of endometriosis. *Hormones Epigenetics Vitamins Hormones* (2023) . p:193–235. doi: 10.1016/bs.vh.2022.11.005

12. Yong PJ, Bedaiwy MA, Alotaibi F, Anglesio MS. Pathogenesis of bowel endometriosis. *Best Pract Res Clin Obstetrics Gynaecology* (2021) 71:2–13. doi: 10.1016/j.bpobgyn.2020.05.009

13. Franca PRC, Lontra ACP, Fernandes PD. Endometriosis: A disease with few direct treatment options. *Molecules* (2022) 27(13):4034. doi: 10.3390/molecules27134034

14. Rezk NA, Lashin MB, Sabbah NA. Mirna 34-a regulate sirt-1 and foxo-1 expression in endometriosis. *Non-coding RNA Res* (2021) 6(1):35–41. doi: 10.1016/j.ncrna.2021.02.002

15. Assaf L, Eid AA, Nassif J. Role of ampk/mtor, mitochondria, and ros in the pathogenesis of endometriosis. *Life Sci* (2022) 306. doi: 10.1016/j.lfs.2022.120805

16. Chlebowski RT, Rohan TE, Manson JE, Aragaki AK, Kaunitz A, Stefanick ML, et al. Breast cancer after use of estrogen plus progestin and estrogen alone: analyses of data from 2 women's health initiative randomized clinical trials. *JAMA Oncol* (2015) 1 (3):296–305. doi: 10.1001/jamaoncol.2015.0494

17. Golabek A, Kowalska K, Olejnik A. Polyphenols as a diet therapy concept for endometriosis-current opinion and future perspectives. *Nutrients* (2021) 13(4):1347. doi: 10.3390/nu13041347

18. Madanes D, Meresman G, Valla SA, Hassan N, Kiesel L, Greve B, et al. Resveratrol impairs cellular mechanisms associated with the pathogenesis of endometriosis. *Reprod BioMedicine Online* (2022) 44(6):976–90. doi: 10.1016/j.rbmo.2022.02.008

19. Bonavina G, Taylor HS. Endometriosis-associated infertility: from pathophysiology to tailored treatment. *Front Endocrinol (Lausanne)* (2022) 13:1020827. doi: 10.3389/fendo.2022.1020827

20. Baker JM, Al-Nakkash L, Herbst-Kralovetz MM. Estrogen–gut microbiome axis: physiological and clinical implications. *Maturitas* (2017) 103:45–53. doi: 10.1016/j.maturitas.2017.06.025

21. Chen J, Douglass J, Habibi M. Microbiota and estrogen metabolism. *Compr Gut Microbiota* (2022), 27–34. doi: 10.1016/B978-0-12-819265-8.00069-3

22. Hussain T, Murtaza G, Kalhoro DH, Kalhoro MS, Metwally E, Chughtai MI, et al. Relationship between gut microbiota and host-metabolism: emphasis on hormones related to reproductive function. *Anim Nutr* (2021) 7(1):1–10. doi: 10.1016/j.aninu.2020.11.005

23. Khazaal NM, Alghetaa H, Al-Shuhaib MB. Hematological parameters as indicators for litter size and pregnancy stage in awassi Ewes. *Iraqi J Veterinary Med* (2023) 47(1):68–73. doi: 10.30539/ijvm.v47i1.1504

24. Eaton SA, Sethi JK. Immunometabolic links between estrogen, adipose tissue and female reproductive metabolism. *Biology* (2019) 8(1):8. doi: 10.3390/biology8010008

25. Kovats S. Estrogen receptors regulate innate immune cells and signaling pathways. *Cell Immunol* (2015) 294(2):63–9. doi: 10.1016/j.cellimm.2015.01.018

26. Patel S, Homaei A, Raju AB, Meher BR. Estrogen: the necessary evil for human health, and ways to tame it. *BioMed Pharmacother* (2018) 102:403–11. doi: 10.1016/j.biopha.2018.03.078

27. Wallace JG, Potts RH, Szamosi JC, Surette MG, Sloboda DM. The murine female intestinal microbiota does not shift throughout the estrous cycle. *PloS One* (2018) 13(7): e0200729. doi: 10.1371/journal.pone.0200729

28. Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, Backhed HK, et al. Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell* (2012) 150(3):470–80. doi: 10.1016/j.cell.2012.07.008

29. Nuriel-Ohayon M, Neuman H, Ziv O, Belogolovski A, Barsheshet Y, Bloch N, et al. Progesterone increases bifidobacterium relative abundance during late pregnancy. *Cell Rep* (2019) 27(3):730–6 e3. doi: 10.1016/j.celrep.2019.03.075

30. Mallott EK, Borries C, Koenig A, Amato KR, Lu A. Reproductive hormones mediate changes in the gut microbiome during pregnancy and lactation in phayre's leaf monkeys. *Sci Rep* (2020) 10(1):9961. doi: 10.1038/s41598-020-66865-2

31. McIntosh FM, Maison N, Holtrop G, Young P, Stevens VJ, Ince J, et al. Phylogenetic distribution of genes encoding beta-glucuronidase activity in human colonic bacteria and the impact of diet on faecal glycosidase activities. *Environ Microbiol* (2012) 14(8):1876–87. doi: 10.1111/j.1462-2920.2012.02711.x

32. Beaud D, Tailliez P, Anba-Mondoloni J. Genetic characterization of the beta-glucuronidase enzyme from a human intestinal bacterium, ruminococcus gnavus. *Microbiol (Reading)* (2005) 151(Pt 7):2323–30. doi: 10.1099/mic.0.27712-0

33. Eriksson H, Gustafsson JA, Sjovall J. Steroids in germfree and conventional rats. Free steroids in faeces from conventional rats. *Eur J Biochem* (1969) 9(2):286–90. doi: 10.1111/j.1432-1033.1969.tb00607.x

34. Shimizu K, Muranaka Y, Fujimura R, Ishida H, Tazume S, Shimamura T. Normalization of reproductive function in germfree mice following bacterial contamination. *Exp Anim* (1998) 47(3):151–8. doi: 10.1538/expanim.47.151

35. Lauterbach MA, Hanke JE, Serefidou M, Mangan MSJ, Kolbe C-C, Hess T, et al. Toll-like receptor signaling rewires macrophage metabolism and promotes histone acetylation via atp-citrate lyase. *Immunity* (2019) 51(6):997–1011.e7. doi: 10.1016/j.immuni.2019.11.009

36. Alghetaa H, Mohammed A, Singh N, Wilson K, Cai G, Putluri N, et al. Resveratrol attenuates staphylococcal enterotoxin B-activated immune cell metabolism via upregulation of mir-100 and suppression of mtor signaling pathway. *Front Pharmacol* (2023) 14:1106733. doi: 10.3389/fphar.2023.1106733

37. Greaves E, Cousins FL, Murray A, Esnal-Zufiaurre A, Fassbender A, Horne AW, et al. A novel mouse model of endometriosis mimics human phenotype and reveals insights into the inflammatory contribution of shed endometrium. *Am J Pathol* (2014) 184(7):1930–9. doi: 10.1016/j.ajpath.2014.03.011

38. Hirata T, Osuga Y, Yoshino O, Hirota Y, Harada M, Takemura Y, et al. Development of an experimental model of endometriosis using mice that ubiquitously express green fluorescent protein. *Hum Reprod* (2005) 20(8):2092–6. doi: 10.1093/humrep/dei012

39. Zhang T, Zhou J, Man GCW, Leung KT, Liang B, Xiao B, et al. Mdscs drive the process of endometriosis by enhancing angiogenesis and are a new potential therapeutic target. *Eur J Immunol* (2018) 48(6):1059–73. doi: 10.1002/eji.201747417

40. Mohammed A, Alghetaa H, Sultan M, Singh NP, Nagarkatti P, Nagarkatti M. Administration of Δ9-tetrahydrocannabinol (Thc) post-staphylococcal enterotoxin B exposure protects mice from acute respiratory distress syndrome and toxicity. *Front Pharmacol* (2020) 11:893. doi: 10.3389/fphar.2020.00893

41. Alghetaa H, Mohammed A, Zhou J, Singh N, Nagarkatti M, Nagarkatti P. Resveratrol-mediated attenuation of superantigen-driven acute respiratory distress syndrome is mediated by microbiota in the lungs and gut. *Pharmacol Res* (2021) 167:105548. doi: 10.1016/j.phrs.2021.105548

42. Neamah WH, Busbee PB, Alghetaa H, Abdulla OA, Nagarkatti M, Nagarkatti P. Ahr activation leads to alterations in the gut microbiome with consequent effect on induction of myeloid derived suppressor cells in a cxcr2-dependent manner. *Int J Mol Sci* (2020) 21(24):9613. doi: 10.3390/ijms21249613

43. Alghetaa H, Mohammed A, Sultan M, Busbee P, Murphy A, Chatterjee S, et al. Resveratrol protects mice against seb-induced acute lung injury and mortality by mir-193a modulation that targets tgf-B Signalling. *J Cell Mol Med* (2018) 22(5):2644–55. doi: 10.1111/jcmm.13542

44. Sarkar S, Kimono D, Albadrani M, Seth RK, Busbee P, Alghetaa H, et al. Environmental microcystin targets the microbiome and increases the risk of intestinal inflammatory pathology via nox2 in underlying murine model of nonalcoholic fatty liver disease. *Sci Rep* (2019) 9(1):8742. doi: 10.1038/s41598-019-45009-1

45. Weber N, Liou D, Dommer J, MacMenamin P, Quiñones M, Misner I, et al. Nephele: A cloud platform for simplified, standardized and reproducible microbiome data analysis. *Bioinformatics* (2018) 34(8):1411–3. doi: 10.1093/bioinformatics/btx617

46. Al-Ghezi ZZ, Busbee PB, Alghetaa H, Nagarkatti PS, Nagarkatti M. Combination of cannabinoids, delta-9-tetrahydrocannabinol (Thc) and cannabidiol (Cbd), mitigates

experimental autoimmune encephalomyelitis (Eae) by altering the gut microbiome. *Brain Behavior Immun* (2019) 82:25–35. doi: 10.1016/j.bbi.2019.07.028

47. Mohammed A, Alghetaa HFK, Miranda K, Wilson K, Singh N P, Cai G, et al. Δ9-tetrahydrocannabinol prevents mortality from acute respiratory distress syndrome through the induction of apoptosis in immune cells, leading to cytokine storm suppression. *Int J Mol Sci* (2020) 21(17):6244. doi: 10.3390/ijms21176244

48. Pan X, Zhu Q, Pan LL, Sun J. Macrophage immunometabolism in inflammatory bowel diseases: from pathogenesis to therapy. *Pharmacol Ther* (2022) 238:108176. doi: 10.1016/j.pharmthera.2022.108176

49. Tabas I, Bornfeldt KE. Intracellular and intercellular aspects of macrophage immunometabolism in atherosclerosis. *Circ Res* (2020) 126(9):1209–27. doi: 10.1161/CIRCRESAHA.119.315939

50. Deretic V. Autophagy in inflammation, infection, and immunometabolism. *Immunity* (2021) 54(3):437–53. doi: 10.1016/j.immuni.2021.01.018

51. Makowski L, Chaib M, Rathmell JC. Immunometabolism: from basic mechanisms to translation. *Immunol Rev* (2020) 295(1):5–14. doi: 10.1111/imr.12858

52. Mohammed A, Alghetaa HK, Zhou J, Chatterjee S, Nagarkatti P, Nagarkatti M. Protective effects of delta(9) -tetrahydrocannabinol against enterotoxin-induced acute respiratory distress syndrome are mediated by modulation of microbiota. *Br J Pharmacol* (2020) 177(22):5078–95. doi: 10.1111/bph.15226

53. Ryan DG, O'Neill LAJ. Krebs cycle reborn in macrophage immunometabolism. *Annu Rev Immunol* (2020) 38:289–313. doi: 10.1146/annurev-immunol-081619-104850

54. Saravia J, Raynor JL, Chapman NM, Lim SA, Chi H. Signaling networks in immunometabolism. *Cell Res* (2020) 30(4):328–42. doi: 10.1038/s41422-020-0301-1

55. Wu R, Chen F, Wang N, Tang D, Kang R. Acod1 in immunometabolism and disease. *Cell Mol Immunol* (2020) 17(8):822–33. doi: 10.1038/s41423-020-0489-5

56. Salliss ME, Farland LV, Mahnert ND, Herbst-Kralovetz MM. The role of gut and genital microbiota and the estrobolome in endometriosis, infertility and chronic pelvic pain. *Hum Reprod Update* (2021) 28(1):92–131. doi: 10.1093/humupd/dmab035

57. Neugent ML, Kumar A, Hulyalkar NV, Lutz KC, Nguyen VH, Fuentes JL, et al. Recurrent urinary tract infection and estrogen shape the taxonomic ecology and function of the postmenopausal urogenital microbiome. *Cell Rep Med* (2022) 3 (10):1–18. doi: 10.1016/j.xcrm.2022.100753

58. Flores R, Shi J, Fuhrman B, Xu X, Veenstra TD, Gail MH, et al. Fecal microbial determinants of fecal and systemic estrogens and estrogen metabolites: A cross-sectional study. *J Trans Med* (2012) 10(1). doi: 10.1186/1479-5876-10-253

59. Ser H-L, Au Yong S-J, Shafiee MN, Mokhtar NM, Ali RAR. Current updates on the role of microbiome in endometriosis: A narrative review. *Microorganisms* (2023) 11 (2):360. doi: 10.3390/microorganisms11020360

60. Lin Y-H, Chen Y-H, Chang H-Y, Au H-K, Tzeng C-R, Huang Y-H. Chronic niche inflammation in endometriosis-associated infertility: current understanding and future therapeutic strategies. *Int J Mol Sci* (2018) 19(8):2385. doi: 10.3390/ijms19082385

61. Burns KA, Pearson AM, Slack JL, Por ED, Scribner AN, Eti NA, et al. Endometriosis in the mouse: challenges and progress toward a 'Best fit' Murine model. *Front Physiol* (2021) 12:806574. doi: 10.3389/fphys.2021.806574

62. Dorning A, Dhami P, Panir K, Hogg C, Park E, Ferguson GD, et al. Bioluminescent imaging in induced mouse models of endometriosis reveals differences in four model variations. *Dis Model Mech* (2021) 14(8). doi: 10.1242/dmm.049070

63. Fukui A, Mai C, Saeki S, Yamamoto M, Takeyama R, Kato T, et al. Pelvic endometriosis and natural killer cell immunity. *Am J Reprod Immunol* (2020) 85(4). doi: 10.1111/aji.13342

64. Abramiuk M, Grywalska E, Malkowska P, Sierawska O, Hrynkiewicz R, Niedzwiedzka-Rystwej P. The role of the immune system in the development of endometriosis. *Cells* (2022) 11(13):2028. doi: 10.3390/cells11132028

65. McIntosh FM, Maison N, Holtrop G, Young P, Stevens VJ, Ince J, et al. Phylogenetic distribution of genes encoding B-glucuronidase activity in human colonic bacteria and the impact of diet on faecal glycosidase activities. *Environ Microbiol* (2012) 14(8):1876–87. doi: 10.1111/j.1462-2920.2012.02711.x

66. Kwa M, Plottel CS, Blaser MJ, Adams S. The intestinal microbiome and estrogen receptor-positive female breast cancer. *J Natl Cancer Inst* (2016) 108(8):1–10. doi: 10.1093/jnci/djw029

67. Beaud D, Tailliez P, Anba-Mondoloni J. Genetic characterization of the B-glucuronidase enzyme from a human intestinal bacterium, ruminococcus gnavus. *Microbiology* (2005) 151(7):2323–30. doi: 10.1099/mic.0.27712-0

68. Dabek M, McCrae SI, Stevens VJ, Duncan SH, Louis P. Distribution of Î²-glucosidase and Î²-glucuronidase activity and of Î²-glucuronidase gene gus in human colonic bacteria. *FEMS Microbiol Ecol* (2008) 66(3):487–95. doi: 10.1111/j.1574-6941.2008.00520.x

69. Gloux K, Berteau O, El oumami H, Béguet F, Leclerc M, Doré J. A metagenomic B-glucuronidase uncovers a core adaptive function of the human intestinal microbiome. *Proc Natl Acad Sci* (2010) 108(supplement_1):4539–46. doi: 10.1073/pnas.1000066107

70. Igudesman D, Crandell J, Corbin KD, Muntis F, Zaharieva DP, Casu A, et al. The intestinal microbiota and short-chain fatty acids in association with advanced metrics of glycemia and adiposity among young adults with type 1 diabetes and overweight or obesity. *Curr Dev Nutr* (2022) 6(10):nzac107. doi: 10.1093/cdn/nzac107

71. Fu X, Liu Z, Zhu C, Mou H, Kong Q. Nondigestible carbohydrates, butyrate, and butyrate-producing bacteria. *Crit Rev Food Sci Nutr* (2019) 59(sup1):S130–S52. doi: 10.1080/10408398.2018.1542587

72. Liu H, Wang J, He T, Becker S, Zhang G, Li D, et al. Butyrate: A double-edged sword for health? *Adv Nutr* (2018) 9(1):21–9. doi: 10.1093/advances/nmx009

73. Ahmed RM, Mohammed AK. Role of sodium butyrate supplement on reducing hepatotoxicity induced by lead acetate in rats. *Iraqi J Veterinary Med* (2022) 46(2):29–35. doi: 10.30539/ijvm.v46i2.1408

74. Ahmed RM, Mohammed AK. Amelioration of hepatotoxicity by sodium butyrate administration in rats. *World Vet J* (2022), 12(3):323–9. doi: 10.54203/scil.2022.wvj41

75. Alharris E, Mohammed A, Alghetaa H, Zhou J, Nagarkatti M, Nagarkatti P. The ability of resveratrol to attenuate ovalbumin-mediated allergic asthma is associated with changes in microbiota involving the gut-lung axis, enhanced barrier function and decreased inflammation in the lungs. *Front Immunol* (2022) 13:805770. doi: 10.3389/fimmu.2022.805770

76. Hassan Al-Tai TH, Al-Hadithi HS, Abdulsalam HS. The role of serum il-6 changes in evaluation of endometriosis. *J Faculty Med Baghdad* (2013) 55(2):158–61. doi: 10.32007/jfacmedbagdad.552646

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 29 February 2024
DOI 10.3389/fendo.2024.1271351

OPEN ACCESS

EDITED BY
Achmad Kemal Harzif,
University of Indonesia, Indonesia

REVIEWED BY
Zhen Tan,
Peking University, China
Marcos Edgar Herkenhoff,
University of São Paulo, Brazil

*CORRESPONDENCE
Shaojie Dong
dongshaojie@mail.xjtu.edu.cn
Lin Niu
niulin@xjtu.edu.cn

RECEIVED 02 August 2023
ACCEPTED 06 February 2024
PUBLISHED 29 February 2024

CITATION
Jin B, Wang P, Liu P, Wang Y, Guo Y, Wang C, Jia Y, Zou R, Dong S and Niu L (2024) Association between periodontitis and endometriosis: a bidirectional Mendelian randomization study.
Front. Endocrinol. 15:1271351.
doi: 10.3389/fendo.2024.1271351

Association between periodontitis and endometriosis: a bidirectional Mendelian randomization study

Bilun Jin[1,2,3], Pengfei Wang[4], Peiqi Liu[1,2,3], Yijie Wang[1,2,3], Yi Guo[1,2,3], Chenxu Wang[1,2,3], Yue Jia[1,2,3], Rui Zou[1,2,3], Shaojie Dong[1,2,3]* and Lin Niu[1,2,3,5]*

[1]Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Research, College of Stomatology, Xi'an Jiaotong University, Xi'an, China, [2]Clinical Research Center of Shaanxi Province for Dental and Maxillofacial Diseases, College of Stomatology, Xi'an Jiaotong University, Xi'an, China, [3]College of Stomatology, Xi'an Jiaotong University, Xi'an, China, [4]Centre of Stomatology, West China Xiamen Hospital of Sichuan University, Xiamen, China, [5]Bioinspired Engineering and Biomechanics Center (BEBC), Xi'an Jiaotong University, Xi'an, China

Introduction: A potential association between periodontitis and endometriosis has been indicated in previous observational studies. Nevertheless, the causal link between these two disorders has not been clarified.

Methods: Based on publicly available genome-wide association study (GWAS) summary datasets, we conducted a bidirectional Mendelian randomization (MR) study to investigate the relationship between periodontitis and endometriosis and its subtypes. Single nucleotide polymorphisms (SNPs) strongly associated with candidate exposures at the genome-wide significance level ($P < 5 \times 10^{-8}$) were selected as instrumental variables (IVs). The inverse variance-weighted regression (IVW) was performed to estimate the causal effect of periodontitis on endometriosis. We further conducted two sensitivity analyses, MR-Egger and weighted median, to test the validity of our findings. The main results were replicated via data from the UK Biobank. Finally, a reverse MR analysis was performed to evaluate the possibility of reverse causality.

Results: The IVW method suggested that periodontitis was positively associated with endometriosis of the pelvic peritoneum (OR = 1.079, 95% CI = 1.016 to 1.146, $P = 0.014$). No causal association was indicated between periodontitis and other subtypes of endometriosis. In reversed analyses, no causal association between endometriosis or its subtypes and periodontitis was found.

Conclusions: Our study provided genetic evidence on the causal relationship between periodontitis and endometriosis of the pelvic peritoneum. More studies are necessary to explore the underlying mechanisms.

KEYWORDS

causal associations, inflammation, bacterial spread, infertility, periodontal diseases

1 Introduction

Periodontitis is a chronic inflammation characterized by loss of periodontal tissue support and alveolar bone, clinical attachment loss, and bleeding gums (1). An updated study verified that 46% of adults from the United States had periodontitis (2). The oral microbial communities detuning in periodontitis could elicit immune subversion and contribute to diseases in distance (3). Therefore, periodontitis not only affects oral functions but is also associated with some systemic diseases (4). Diseases of the cardiovascular, neurodegenerative, and even reproductive systems have been confirmed to be associated with periodontitis in various observational studies until now (5). However, limited research explored the causal effects between those diseases and periodontitis. Further study of the causality between periodontitis and associated comorbidities will provide novel insights into systemic disease treatment.

Endometriosis, similar to periodontitis, is a chronic inflammatory disease in the reproductive system. It affects 5%–10% of women of reproductive age worldwide and has been defined as a major reason for infertility (6). Endometriosis is divided into three phenotypes based on extrauterine sites where endometrial glands and stroma abnormally present 1) superficial peritoneal lesions (SUPs), 2) ovarian endometriomas (OMAs), and 3) deep infiltrating endometriosis (DIE) (7). SUP penetration is limited to 5 mm under the peritoneal surface layer (8). SUP is the least severe endometriosis with the mildest symptoms. OMA refers to chocolate cysts in the ovary, which contain a dark brown fluid (9). DIE is deemed to be the most terrible subtype with an erosive extent deeper than 5 mm under the peritoneum. Patients with DIE always suffer from severe pain (10). These phenotypes also differ at the cellular level. Single-cell profiling suggested more disorders of hormonal, inflammatory, and immunological signatures in DIE, compared with SUP and OMA (8). Because of the heterogeneity of clinical features and invasive diagnostic methods, there is an average of 6.7 years before definitive diagnosis (11, 12).

When focusing on the relationship between endometriosis and periodontitis, an earlier cross-sectional study (N = 2,664) based on the National Health and Nutrition Examination Survey demonstrated that women with endometriosis had a 57% higher risk of gingivitis and periodontitis than those without [adjusted odds ratio (OR) = 1.57, 95% CI = 1.06, 2.33] (13). A case–control study (N = 50) showed that participants with endometriosis had a higher gingival index than normal, and moderate and severe periodontitis was more common in women with endometriosis compared with normal groups (14). In a word, some observational studies have revealed a potential correlation between periodontitis and endometriosis. However, the issue of whether there exists a causal relationship between periodontitis and endometriosis or its subtypes remains unknown.

Mendelian randomization (MR) is an economical and time-saving option to explore the causal effects. Single-nucleotide polymorphisms (SNPs) are assigned randomly during the formation of a sperm cell. They could be used to reduce reverse causation or confounders and then assess the causal relationship between exposures and outcomes (15, 16). Bidirectional MR is one of the developed MR, which is performed in both ways to reduce misleading estimates from elementary MR (17). Some studies have used bidirectional MR to explore the association direction between periodontitis and different diseases, such as arthritis (18), depression (19), and psoriasis (20).

Here, we performed a bidirectional two-sample MR study between periodontitis and endometriosis. SNPs as instrumental variables (IVs) and their associations with outcomes were selected from relevant genome-wide association studies (GWASs). In addition, we selected three endometriosis subgroup datasets in order to represent SUP, OMA, and DIE, respectively. We aim to provide more genetic evidence to define the relationship between periodontitis and endometriosis.

2 Materials and methods

2.1 Study design

A bidirectional two-sample Mendelian randomization study was utilized to explore the causal association between periodontitis and endometriosis. We also performed a two-sample MR analysis using the dataset from the UK Biobank (UKB) to validate the causality of periodontitis on endometriosis. As shown in Figure 1, the study is based on three vital assumptions: firstly, there are strong associations between exposure and IVs. Secondly, IVs are not associated with the confounders. Thirdly, IVs should totally affect outcomes through exposure.

The present study was based on publicly available GWAS summary data, and ethical consent had been obtained in the original studies (21–25). All the data can be used without any restrictions.

2.2 GWAS datasets of periodontitis on the risk of endometriosis

We selected the IVs associated with periodontitis from three published GWAS datasets (23–25). All the participants were Europeans, including Dutch, German, and European-American. SNPs associated with the exposure at the genome-wide significance level ($P < 5 \times 10^{-8}$) were selected.

The datasets of endometriosis and its subgroups were obtained from FinnGen. The FinnGen study was initiated in 2017, which included more than half a million participants (age >18 years) who lived in Finland (26). The FinnGen GWAS summary data were extracted from the MRC-IEU database for endometriosis overall (8,828 cases, 68,969 controls), endometriosis of the ovary (3,231 cases, 68,969 controls), endometriosis of the pelvic peritoneum (2,953 cases, 68,969 controls), and also endometriosis of the rectovaginal septum and vagina (1,360 cases, 68,969 controls). Then, we also chose another dataset of endometriosis from UKB as the validation. UKB is a large prospective study, which aims to investigate the role of genetics, environment, and lifestyle in the causes of leading diseases. Its data come from 500,000 volunteers aged 40–69 in the United Kingdom (27). More details of exposure and outcome datasets are shown in Supplementary Table 1.

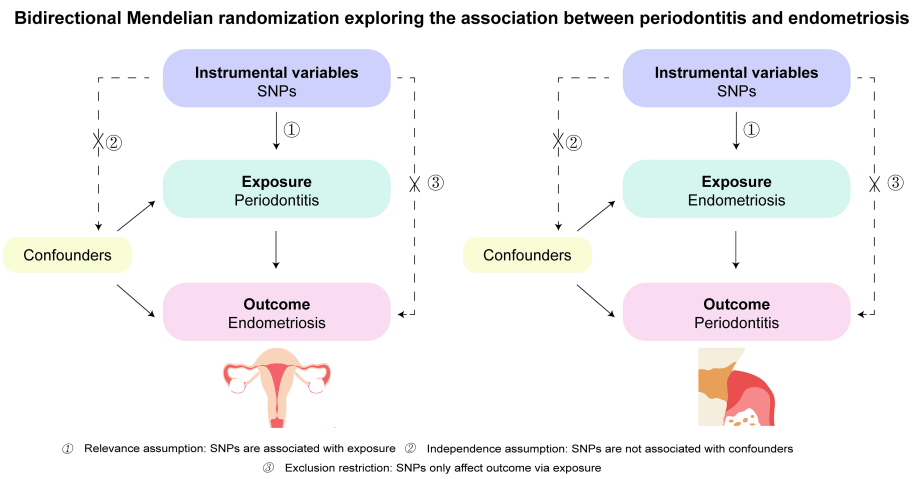


FIGURE 1
Schematics for the bidirectional Mendelian randomization design. Mendelian randomization requires valid genetic instrumental variants satisfying three assumptions. SNPs, single-nucleotide polymorphisms.

2.3 GWAS datasets of endometriosis on the risk of periodontitis

The exposure data of endometriosis and its phenotypes were obtained from the same FinnGen study as mentioned above. The outcome data for the risk of periodontitis were obtained from a GWAS meta-analysis conducted by the Gene Lifestyle Interactions in Dental Endpoints (GLIDE) on Europeans (22). Seven primary studies were included in the analysis. Periodontitis was recognized via the Centers for Disease Control and Prevention/American Academy of Periodontology definitions (28), probing depth (29), or even self-reported (30). The details of the primary studies are shown in Supplementary Table 2. In our study, we excluded the data of people with Hispanic/Latino ancestry. Data from European ancestry were collected from the original analysis.

2.4 Selection of instrumental variables

SNPs that were strongly associated with exposure at the genome-wide significance level ($P < 5 \times 10^{-8}$) were selected as IVs. We conducted several quality-control measures to select qualified IVs. First, the independence of SNPs was assessed based on stringent criteria ($r^2 > 0.001$; clumping window < 10,000 kb). Second, we used the PhenoScanner tool to check whether any of the selected SNPs were associated with potential confounders at the outcome. We set the threshold at genome-wide significance ($P < 5 \times 10^{-8}$) when using the PhenoScanner tool. Third, proxy SNPs were not used as IVs if SNPs were not in the 1000G reference panel. In addition, SNPs with a minor allele frequency of less than 0.01 should be excluded to avoid potential bias from the original GWAS due to the low confidence. The R^2 and F statistics were also calculated to avoid bias from weak instruments. SNPs were excluded if the F statistics were less than 10.

For the analyses of periodontitis as exposure, we selected five SNPs as valid IVs (Supplementary Table 3). In reversed analyses, during the selection of IVs associated with endometriosis, rs58502716 was not in the 1000G reference panel, and it was removed from the instruments. In addition, SNPs associated with endometriosis of the rectovaginal septum and vagina at a genome-wide significance level of $P < 5 \times 10^{-6}$ were selected in order to obtain enough IVs. rs76109112, rs139869063, rs200290589, and rs117783935 were eliminated as their F statistics were no more than 10. Finally, 10, 10, 5, and 13 SNPs were recognized as IVs associated with endometriosis overall, endometriosis of the ovary, endometriosis of the pelvic peritoneum, and endometriosis of the rectovaginal septum and vagina, respectively (Supplementary Tables 4–7).

2.5 Statistical analysis

The inverse variance-weighted (IVW) method in the random-effects model was applied as a dominating approach to analyze the bidirectional causal relationship between periodontitis and endometriosis. Weighted median, MR-Egger, and simple median were also added as complementary approaches to reduce potential horizontal pleiotropy and bias from the IVW method (31). The weighted median method could generate an unbiased estimate if more than half of the weight from effective IVs (32). MR-Egger is more suitable if the $P_{intercept} < 0.05$ because it can provide the estimates after pleiotropy is corrected (33).

To test the validity of our findings, sensitivity analyses were conducted using weighted median and MR-Egger regression. Q-tests were performed in both IVW and MR-Egger regression to assess potential heterogeneity. The MR-Egger intercept was used to assess whether the included SNPs had potential horizontal pleiotropy. Weighted median provides consistent estimates when at least 50% of the information is from valid instrumental variables. Leave-one-out analyses were also utilized to estimate the causality and heterogeneity of the study (34). As for the pleiotropy analysis, the P-value of the MR-Egger intercept less than 0.05 is considered to suggest the pleiotropy of the study (35).

Additionally, the statistical power of each analysis was calculated via mRnd, an online calculator (https://shiny.cnsgenomics.com/

mRnd/). All data were analyzed by R (version 3.1.5), combined with the R package "TwoSampleMR." A *P*-value < 0.05 was considered to be statistically significant. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guideline (Supplementary Table 8).

3 Results

3.1 The causal effect of periodontitis on endometriosis

The results of MR analyses on the association between periodontitis and the risk of endometriosis are shown in Figure 2. In the subgroup analyses, we observed that periodontitis was positively associated with endometriosis of the pelvic peritoneum (OR = 1.079, 95% CI = 1.016 to 1.146, *P* = 0.014). The replication of the main results using UKB data supported the null causality of periodontitis on endometriosis (Supplementary Figure 1). No causal association was indicated between periodontitis and other subtypes of endometriosis. Suggestive estimates were observed in weight median, MR-Egger, and simple median methods similarly.

Although other robust MR methods did not manifest a significant association between periodontitis and endometriosis of the pelvic peritoneum, the sensitivity analysis showed no potential horizontal pleiotropy (Table 1). Meanwhile, no heterogeneity existed in the IVW analyses, and none of the SNPs had a distinctive effect on estimates in the leave-one-out analyses (Supplementary Figures 2–10).

3.2 The causal effect of endometriosis on periodontitis

The results of the MR analyses on the association between endometriosis and the risk of periodontitis are shown in Figure 3. There was no causal effect of endometriosis or its subtypes on periodontitis. Consistent estimates were provided through other robust methods. As for the sensitivity analysis, no heterogeneity was found among SNPs, except for endometriosis of the rectovaginal septum and vagina (*P* = 0.048) (Table 1). In view of this excess heterogeneity, a random-effects analysis was performed as the main method in this study (36). Furthermore, no evidence for pleiotropy was observed via MR-Egger analyses. No pivotal difference emerged in estimates after we singly removed SNP and repeated the MR analysis.

Our study had a power of > 80% to detect an effect of OR = 1.255 of periodontitis on the risk of endometriosis and a power of > 80% to detect an effect of OR = 1.394 of endometriosis on the risk of periodontitis, on a 5% significance level.

4 Discussion

Periodontitis has been a momentous public health problem. It not only damages oral health but also becomes a potential cause of some systemic conditions (37). Endometriosis is a common chronic disease with several symptoms, including painful menstrual cramps, abdominal pain, and so on (38). The etiology of endometriosis is unclear, so there are still difficulties in treatment. Some observational studies reported the correlation between periodontitis and endometriosis, but the causal effect cannot be affirmed (13, 14, 39). To our knowledge, this was an innovative study to investigate the bidirectional causal effect between periodontitis and endometriosis. Our research showed a potential cause effect of periodontitis on endometriosis of the pelvic peritoneum. In reversed analyses, there was no causal association of endometriosis or its subtypes on the risk of periodontitis.

A promising finding in this study is the causal effect of periodontitis on endometriosis of the pelvic peritoneum, which cast new light on the pathogenesis of endometriosis. Previous research surrounding the etiology of endometriosis of the pelvic

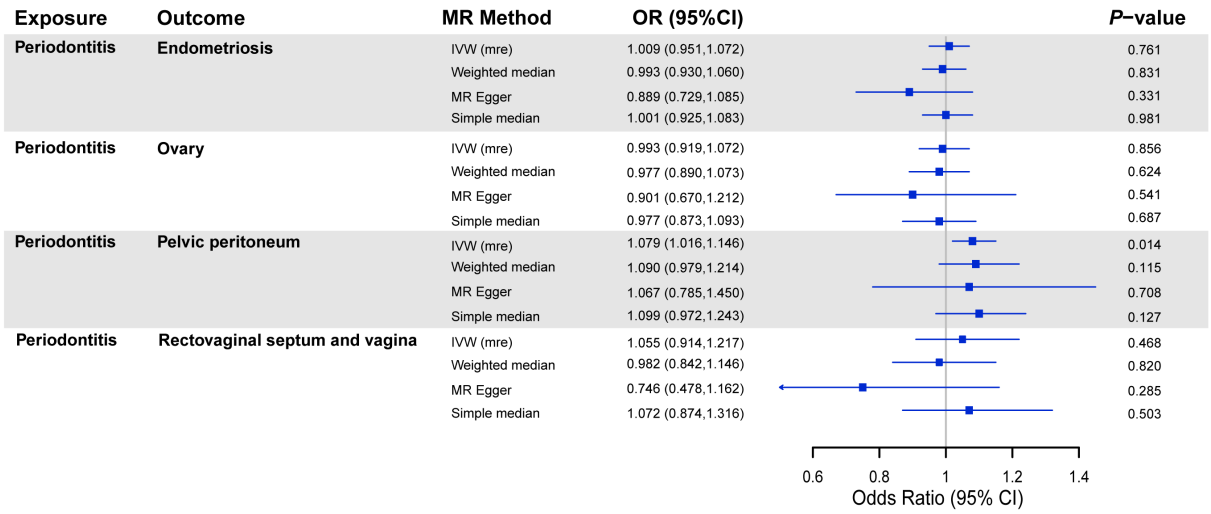


FIGURE 2
MR analyses on the association between periodontitis and risk of endometriosis. OR, odds ratio; CI, confidence interval; IVW (mre), multiplicative random effect inverse-variance weighted; SNPs, single-nucleotide polymorphisms.

10.3389/fendo.2024.1271351

TABLE 1 Results of potential pleiotropy and heterogeneity assessments using different MR analyses.

Exposure	Outcome	Heterogeneity		Pleiotropy
		P-value for Cochran's *Q*	Cochran's *Q* statistic	*P*-value for MR-Egger intercept
Periodontitis	Endometriosis	0.307	4.813	0.284
Periodontitis	Endometriosis (validation)	0.434	3.799	0.232
Periodontitis	Ovary	0.476	3.510	0.553
Periodontitis	Pelvic peritoneum	0.738	1.989	0.944
Periodontitis	Rectovaginal septum and vagina	0.254	5.347	0.209
Endometriosis	Periodontitis	0.744	5.958	0.400
Ovary	Periodontitis	0.833	5.018	0.806
Pelvic peritoneum	Periodontitis	0.279	5.082	0.999
Rectovaginal septum and vagina	Periodontitis	0.048	21.144	0.763

peritoneum manifested that cells from the endometrium evade immune surveillance in the peritoneum and contribute to the disease, but other mechanisms are uncertain (40). The cause effect of periodontitis on endometriosis of the pelvic peritoneum might be explained by the mechanisms provided below (and these mechanisms are also summarized in Figure 4):

4.1 Periodontal pathogenic bacteria

Oral bacteria such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum* are prone to transmit into the placenta and induce intrauterine infection (41–43). Similarly, periodontal pathogens might locate in the pelvic peritoneum and then exacerbate endometriosis *in situ*. *Porphyromonas gingivalis* could activate peritoneal macrophages and trigger the production of interleukin-1 (IL-1) (44). Subsequent inflammatory cascades, together with increasing IL-1 expression, are pivotal throughout the development of endometriosis in the pelvic peritoneum (45). The frequency of *Fusobacterium* in endometriosis and endometrial tissues was significantly higher in endometriosis patients than in normal women. Animal studies also demonstrated that *Fusobacterium* from the oral cavity was a possible source of infecting the uterus through hematogenous transmission (46).

4.2 Inflammatory response

Periodontitis patients usually have a higher count of leukocytes, elevated levels of circulating C-reactive protein, as well as lower

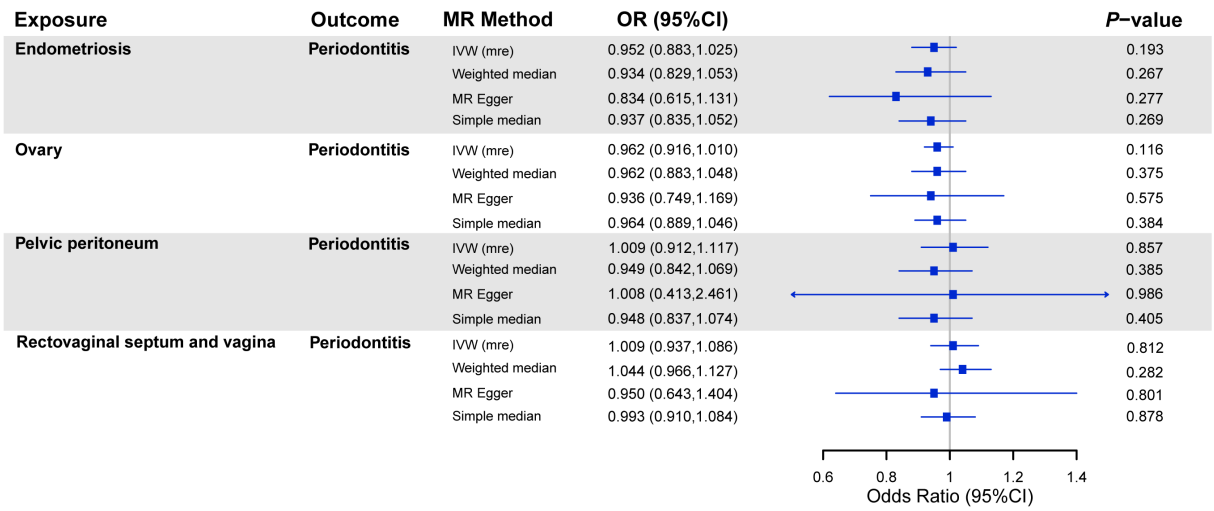


FIGURE 3
MR analyses on the association between endometriosis and risk of periodontitis. *Note*: OR, odds ratio; CI, confidence interval; IVW (mre), multiplicative random effect inverse-variance weighted; SNPs, single-nucleotide polymorphisms.

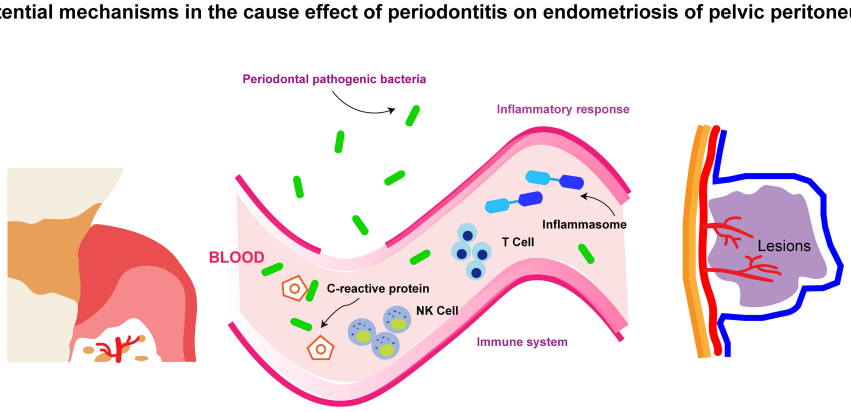


FIGURE 4
The schematic diagram for the potential mechanisms in the cause effect of periodontitis on endometriosis of pelvic peritoneum.

levels of hemoglobin and reduced numbers of red blood cells (47). Inflammasome, a multiprotein complex, is also considered as an intermedia. Periodontitis might increase Nlrp7 (a kind of inflammasome) and promote the progression of endometriosis (48). In a word, systemic inflammatory response is conjectured as a mechanism by which periodontitis causes systemic diseases.

4.3 Immune system

Immune dysfunction has been certified as a key cause of endometriosis of the peritoneum. For example, a genomics single-cell RNA-sequencing study first reported that T-cell receptor-positive macrophages, increasing macrophages, and natural killer (NK) dendritic cells existed in the human peritoneal fluid of endometriosis (49). NK cell therapies are also a promising treatment for endometriosis (50). In addition, reducing the immune response induced by *P. gingivalis* is helpful in alleviating some metabolic diseases associated with periodontitis (51). There is reason to suspect that periodontitis causes endometriosis through autoimmune dysregulation.

Up to now, accepted treatments for endometriosis patients include continuous medical therapies and surgeries to remove pathological tissue (52). However, persistent medications suppress hormone levels and produce side effects, such as hair loss, acne, and mood changes, and operations would lead to inevitable relapse (53). This MR study revealed periodontitis as a novel point to manage endometriosis patients. Targeting a lower risk of periodontitis may therefore improve the prognosis of people with endometriosis of the pelvic peritoneum. In other words, supportive periodontal therapies and routine oral hygiene interventions may produce beneficial effects in treating women with endometriosis of the pelvic peritoneum. In the future, some clinical randomized controlled trials with more samples should be performed to verify the causal relationship. More research is necessary not only to clarify the underlying biological pathways but also to confirm therapeutic targets for endometriosis of the pelvic peritoneum.

The bidirectional MR study has some strengths. Firstly, genetic liability plays a crucial role in the course of periodontitis and endometriosis (18, 38). GWASs allowed larger sample sizes and less bias from population structure. Prevalence rates in the database were almost consistent with those reported in the literature. Hence, MR analysis is suitable for the inference of causality between both (54). Meanwhile, the MR analyses on both sides clarified the direction of causality. Secondly, endometriosis was further subdivided into three subgroups. An interesting finding is that periodontitis might be a cause of endometriosis of the pelvic peritoneum, which has not been realized before.

Some limitations should also be considered. Firstly, IVs had a deficient association with endometriosis of the rectovaginal septum and vagina, since the threshold of *P*-value was set to 5×10^{-6}. Therefore, more convincing evidence is needed to prove the causality between endometriosis of the rectovaginal septum and vagina and periodontitis. Secondly, a weak power was found in each analysis, due to OR close to 1, so more participants were required to detect the causal effects in the future (55).

5 Conclusions

In summary, this bidirectional MR study indicated a positive causal relationship between periodontitis and endometriosis of the pelvic peritoneum in the European population. More epidemiological investigations are indispensable to explore the causality between those two diseases. Further research on the mechanism is also needed in the future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical approval had been obtained in all original published studies. The OpenGWAS Database is a publicly available dataset, and GWAS of oral diseases complied with all relevant ethical regulations, including the Declaration of Helsinki, and ethical approval for data collection and analysis was obtained by each study from local boards.

Author contributions

BJ: Conceptualization, Data curation, Formal analysis, Writing – original draft. PW: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – review & editing. PL: Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. YW: Data curation, Formal analysis, Methodology, Software, Visualization, Writing – review & editing. YG: Investigation, Methodology, Software, Writing – review & editing. CW: Investigation, Software, Visualization, Writing – review & editing. YJ: Data curation, Investigation, Methodology, Writing – review & editing. RZ: Funding acquisition, Supervision, Visualization, Writing – review & editing. SD: Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Writing – review & editing. LN: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research work was supported by the National Natural Science Foundation of China (No.81970981, No.82102221), the Key Research and Development Project of Shaanxi Province (No.2023-YBSF-389), Fundamental Research Funds of Xi'an Jiaotong University for Free Exploration and Innovation-Project for Teacher (XZY012021069), and Project to Enhance the Base of Innovation Ability of Xi'an City-Medical Research (21YXYJ0123).

Acknowledgments

We thank FinnGen, UKB, and MRC-IEU for making the summary data publicly available. We are thankful to the investigators and participants who contributed to those studies.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1271351/full#supplementary-material

References

1. Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, et al. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Clin Periodontol.* (2018) 45 Suppl 20:S162–s70. doi: 10.1111/jcpe.12946

2. Eke PI, Dye BA, Wei L, Slade GD, Thornton-Evans GO, Borgnakke WS, et al. Update on prevalence of periodontitis in adults in the United States: NHANES 2009 to 2012. *J Periodontol.* (2015) 86:611–22. doi: 10.1902/jop.2015.140520

3. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol.* (2015) 15:30–44. doi: 10.1038/nri3785

4. Bui FQ, Almeida-da-Silva CLC, Huynh B, Trinh A, Liu J, Woodward J, et al. Association between periodontal pathogens and systemic disease. *BioMed J.* (2019) 42:27–35. doi: 10.1016/j.bj.2018.12.001

5. Xu W, Zhou W, Wang H, Liang S. Roles of Porphyromonas gingivalis and its virulence factors in periodontitis. *Adv Protein Chem Struct Biol.* (2020) 120:45–84. doi: 10.1016/bs.apcsb.2019.12.001

6. Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. *Fertil Steril.* (2012) 98:511–9. doi: 10.1016/j.fertnstert.2012.06.029

7. Chapron C, Marcellin L, Borghese B, Santulli P. Rethinking mechanisms, diagnosis and management of endometriosis. *Nat Rev Endocrinol.* (2019) 15:666–82. doi: 10.1038/s41574-019-0245-z

8. Shin S, Chung YJ, Moon SW, Choi EJ, Kim MR, Chung YJ, et al. Single-cell profiling identifies distinct hormonal, immunologic, and inflammatory signatures of endometriosis-constituting cells. *J Pathol.* (2023) 261:323–34. doi: 10.1002/path.6178

9. Hoyle AT, Puckett Y. Endometrioma. *StatPearls*. Treasure Island (FL): StatPearls Publishing LLC (2023).

10. Chapron C, Pietin-Vialle C, Borghese B, Davy C, Foulot H, Chopin N. Associated ovarian endometrioma is a marker for greater severity of deeply infiltrating endometriosis. *Fertil Steril.* (2009) 92:453–7. doi: 10.1016/j.fertnstert.2008.06.003

11. Nnoaham KE, Hummelshoj L, Webster P, d'Hooghe T, de Cicco Nardone F, de Cicco Nardone C, et al. Impact of endometriosis on quality of life and work productivity: a multicenter study across ten countries. *Fertil Steril.* (2011) 96:366–73.e8. doi: 10.1016/j.fertnstert.2011.05.090

12. Koninckx PR, Fernandes R, Ussia A, Schindler L, Wattiez A, Al-Suwaidi S, et al. Pathogenesis based diagnosis and treatment of endometriosis. *Front Endocrinol (Lausanne)*. (2021) 12:745548. doi: 10.3389/fendo.2021.745548

13. Kavoussi SK, West BT, Taylor GW, Lebovic DI. Periodontal disease and endometriosis: analysis of the National Health and Nutrition Examination Survey. *Fertil Steril*. (2009) 91:335–42. doi: 10.1016/j.fertnstert.2007.12.075

14. Thomas V, Uppoor AS, Pralhad S, Naik DG, Kushtagi P. Towards a common etiopathogenesis: periodontal disease and endometriosis. *J Hum Reprod Sci*. (2018) 11:269–73. doi: 10.4103/jhrs.JHRS_8_18

15. Sekula P, Del Greco MF, Pattaro C, Köttgen A. Mendelian randomization as an approach to assess causality using observational data. *J Am Soc Nephrol*. (2016) 27:3253–65. doi: 10.1681/asn.2016010098

16. Ference BA, Holmes MV, Smith GD. Using mendelian randomization to improve the design of randomized trials. *Cold Spring Harb Perspect Med*. (2021) 11 (7):a040980. doi: 10.1101/cshperspect.a040980

17. Davey Smith G, Hemani G. Mendelian randomization: genetic anchors for causal inference in epidemiological studies. *Hum Mol Genet*. (2014) 23:R89–98. doi: 10.1093/hmg/ddu328

18. Yin KJ, Huang JX, Wang P, Yang XK, Tao SS, Li HM, et al. No genetic causal association between periodontitis and arthritis: A bidirectional two-sample mendelian randomization analysis. *Front Immunol*. (2022) 13:808832. doi: 10.3389/fimmu.2022.808832

19. Nolde M, Holtfreter B, Kocher T, Alayash Z, Reckelkamm SL, Ehmke B, et al. No bidirectional relationship between depression and periodontitis: A genetic correlation and Mendelian randomization study. *Front Immunol*. (2022) 13:918404. doi: 10.3389/fimmu.2022.918404

20. Baurecht H, Freuer D, Welker C, Tsoi LC, Elder JT, Ehmke B, et al. Relationship between periodontitis and psoriasis: A two-sample Mendelian randomization study. *J Clin Periodontol*. (2022) 49:573–9. doi: 10.1111/jcpe.13620

21. Si S, Li J, Tewara MA, Xue F. Genetically determined chronic low-grade inflammation and hundreds of health outcomes in the UK biobank and the finnGen population: A phenome-wide mendelian randomization study. *Front Immunol*. (2021) 12:720876. doi: 10.3389/fimmu.2021.720876

22. Shungin D, Haworth S, Divaris K, Agler CS, Kamatani Y, Keun Lee M, et al. Genome-wide analysis of dental caries and periodontitis combining clinical and self-reported data. *Nat Commun*. (2019) 10:2773. doi: 10.1038/s41467-019-10630-1

23. Schaefer AS, Richter GM, Nothnagel M, Manke T, Dommisch H, Jacobs G, et al. A genome-wide association study identifies GLT6D1 as a susceptibility locus for periodontitis. *Hum Mol Genet*. (2010) 19:553–62. doi: 10.1093/hmg/ddp508

24. Munz M, Willenborg C, Richter GM, Jockel-Schneider Y, Graetz C, Staufenbiel I, et al. A genome-wide association study identifies nucleotide variants at SIGLEC5 and DEFA1A3 as risk loci for periodontitis. *Hum Mol Genet*. (2017) 26:2577–88. doi: 10.1093/hmg/ddx151

25. Munz M, Richter GM, Loos BG, Jepsen S, Divaris K, Offenbacher S, et al. Meta-analysis of genome-wide association studies of aggressive and chronic periodontitis identifies two novel risk loci. *Eur J Hum Genet*. (2019) 27:102–13. doi: 10.1038/s41431-018-0265-5

26. Yang F, Hu T, Cui H. Serum urate and heart failure: a bidirectional Mendelian randomization study. *Eur J Prev Cardiol*. (2022) 29:1570–8. doi: 10.1093/eurjpc/zwac100

27. Peakman TC, Elliott P. The UK Biobank sample handling and storage validation studies. *Int J Epidemiol*. (2008) 37 Suppl 1:i2–6. doi: 10.1093/ije/dyn019

28. Page RC, Eke PI. Case definitions for use in population-based surveillance of periodontitis. *J Periodontol*. (2007) 78:1387–99. doi: 10.1902/jop.2007.060264

29. Polk DE, Weyant RJ, Crout RJ, McNeil DW, Tarter RE, Thomas JG, et al. Study protocol of the Center for Oral Health Research in Appalachia (COHRA) etiology study. *BMC Oral Health*. (2008) 8:18. doi: 10.1186/1472-6831-8-18

30. Yu YH, Steffensen B, Ridker PM, Buring JE, Chasman DI. Candidate loci shared among periodontal disease, diabetes and bone density. *Front Endocrinol (Lausanne)*. (2022) 13:1016373. doi: 10.3389/fendo.2022.1016373

31. Huang D, Lin S, He J, Wang Q, Zhan Y. Association between COVID-19 and telomere length: A bidirectional Mendelian randomization study. *J Med Virol*. (2022) 94:5345–53. doi: 10.1002/jmv.28008

32. Bowden J, Davey Smith G, Haycock PC, Burgess S. Consistent estimation in mendelian randomization with some invalid instruments using a weighted median estimator. *Genet Epidemiol*. (2016) 40:304–14. doi: 10.1002/gepi.21965

33. Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. *Int J Epidemiol*. (2015) 44:512–25. doi: 10.1093/ije/dyv080

34. Hemani G, Zheng J, Elsworth B, Wade KH, Haberland V, Baird D, et al. The MR-Base platform supports systematic causal inference across the human phenome. *Elife*. (2018) 7:e34408. doi: 10.7554/eLife.34408

35. Li Y, Liu H, Ye S, Zhang B, Li X, Yuan J, et al. The effects of coagulation factors on the risk of endometriosis: a Mendelian randomization study. *BMC Med*. (2023) 21:195. doi: 10.1186/s12916-023-02881-z

36. Papadimitriou N, Dimou N, Tsilidis KK, Banbury B, Martin RM, Lewis SJ, et al. Physical activity and risks of breast and colorectal cancer: a Mendelian randomisation analysis. *Nat Commun*. (2020) 11:597. doi: 10.1038/s41467-020-14389-8

37. D'Aiuto F, Gkranias N, Bhowruth D, Khan T, Orlandi M, Suvan J, et al. Systemic effects of periodontitis treatment in patients with type 2 diabetes: a 12 month, single-centre, investigator-masked, randomised trial. *Lancet Diabetes Endocrinol*. (2018) 6:954–65. doi: 10.1016/S2213-8587(18)30038-X

38. Peiris AN, Chaljub E, Medlock D. Endometriosis. *Jama*. (2018) 320:2608. doi: 10.1001/jama.2018.17953

39. MaChado V, Lopes J, Patrão M, Botelho J, Proença L, Mendes JJ. Validity of the association between periodontitis and female infertility conditions: a concise review. *Reproduction*. (2020) 160:R41–r54. doi: 10.1530/rep-20-0176

40. Saunders PTK, Horne AW. Endometriosis: Etiology, pathobiology, and therapeutic prospects. *Cell*. (2021) 184:2807–24. doi: 10.1016/j.cell.2021.04.041

41. Fardini Y, Chung P, Dumm R, Joshi N, Han YW. Transmission of diverse oral bacteria to murine placenta: evidence for the oral microbiome as a potential source of intrauterine infection. *Infect Immun*. (2010) 78:1789–96. doi: 10.1128/iai.01395-09

42. Thomas C, Timofeeva I, Bouchoucha E, Canceill T, Champion C, Groussolles M, et al. Oral and periodontal assessment at the first trimester of pregnancy: The PERISCOPE longitudinal study. *Acta Obstet Gynecol Scand*. (2023) 102:669–80. doi: 10.1111/aogs.14529

43. Katz J, Chegini N, Shiverick KT, Lamont RJ. Localization of P. gingivalis in preterm delivery placenta. *J Dent Res*. (2009) 88:575–8. doi: 10.1177/0022034509338032

44. Hanazawa S, Murakami Y, Hirose K, Amano S, Ohmori Y, Higuchi H, et al. Bacteroides (Porphyromonas) gingivalis fimbriae activate mouse peritoneal macrophages and induce gene expression and production of interleukin-1. *Infect Immun*. (1991) 59:1972–7. doi: 10.1128/iai.59.6.1972-1977.1991

45. Young VJ, Brown JK, Saunders PT, Horne AW. The role of the peritoneum in the pathogenesis of endometriosis. *Hum Reprod Update*. (2013) 19:558–69. doi: 10.1093/humupd/dmt024

46. Muraoka A, Suzuki M, Hamaguchi T, Watanabe S, Iijima K, Murofushi Y, et al. Fusobacterium infection facilitates the development of endometriosis through the phenotypic transition of endometrial fibroblasts. *Sci Transl Med*. (2023) 15:eadd1531. doi: 10.1126/scitranslmed.add1531

47. Loos BG. Systemic markers of inflammation in periodontitis. *J Periodontol*. (2005) 76:2106–15. doi: 10.1902/jop.2005.76.11-S.2106

48. Bullon P, Navarro JM. Inflammasome as a key pathogenic mechanism in endometriosis. *Curr Drug Targets*. (2017) 18:997–1002. doi: 10.2174/1389450117666160709013850

49. Zou G, Wang J, Xu X, Xu P, Zhu L, Yu Q, et al. Cell subtypes and immune dysfunction in peritoneal fluid of endometriosis revealed by single-cell RNA-sequencing. *Cell Biosci*. (2021) 11:98. doi: 10.1186/s13578-021-00613-5

50. Hoogstad-van Evert J, Paap R, Nap A, van der Molen R. The promises of natural killer cell therapy in endometriosis. *Int J Mol Sci*. (2022) 23(10):5539. doi: 10.3390/ijms23105539

51. Blasco-Baque V, Garidou L, Pomié C, Escoula Q, Loubieres P, Le Gall-David S, et al. Periodontitis induced by Porphyromonas gingivalis drives periodontal microbiota dysbiosis and insulin resistance via an impaired adaptive immune response. *Gut* (2017) 66(5):872–85. doi: 10.1136/gutjnl-2015-309897

52. Falcone T, Flyckt R. Clinical management of endometriosis. *Obstet Gynecol*. (2018) 131:557–71. doi: 10.1097/aog.0000000000002469

53. Guo SW. Recurrence of endometriosis and its control. *Hum Reprod Update*. (2009) 15:441–61. doi: 10.1093/humupd/dmp007

54. Skrivankova VW, Richmond RC, Woolf BAR, Yarmolinsky J, Davies NM, Swanson SA, et al. Strengthening the reporting of observational studies in epidemiology using mendelian randomization: the STROBE-MR statement. *Jama*. (2021) 326:1614–21. doi: 10.1001/jama.2021.18236

55. Burgess S. Sample size and power calculations in Mendelian randomization with a single instrumental variable and a binary outcome. *Int J Epidemiol*. (2014) 43:922–9. doi: 10.1093/ije/dyu005

frontiers | Frontiers in Endocrinology

TYPE Study Protocol
PUBLISHED 15 March 2024
DOI 10.3389/fendo.2024.1336496

OPEN ACCESS

EDITED BY
Iveta Yotova,
Medical University of Vienna, Austria

REVIEWED BY
Valentina Caputi,
University College Cork, Ireland
Stacy McAllister,
Emory University, United States
Zhexin Ni,
Second Military Medical University, China

*CORRESPONDENCE
Kanako Hayashi
k.hayashi@wsu.edu

†These authors contributed equally to this work

RECEIVED 10 November 2023
ACCEPTED 28 February 2024
PUBLISHED 15 March 2024

CITATION
Herup-Wheeler T, Shi M, Harvey ME, Talwar C, Kommagani R, MacLean JA II and Hayashi K (2024) High-fat diets promote peritoneal inflammation and augment endometriosis-associated abdominal hyperalgesia.
Front. Endocrinol. 15:1336496.
doi: 10.3389/fendo.2024.1336496

High-fat diets promote peritoneal inflammation and augment endometriosis-associated abdominal hyperalgesia

Tristin Herup-Wheeler[1†], Mingxin Shi[1†], Madeleine E. Harvey[1], Chandni Talwar[2,3], Ramakrishna Kommagani[2,3], James A. MacLean II[1] and Kanako Hayashi[1*]

[1]School of Molecular Bioscience, Center for Reproductive Biology, Washington State University, Pullman, WA, United States, [2]Department of Pathology & Immunology, Baylor College of Medicine, Houston, TX, United States, [3]Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, United States

Immune dysfunction is one of the central components in the development and progression of endometriosis by establishing a chronic inflammatory environment. Western-style high-fat diets (HFD) have been linked to greater systemic inflammation to cause metabolic and chronic inflammatory diseases, and are also considered an environmental risk factor for gynecologic diseases. Here, we aimed to examine how HFD cause an inflammatory environment in endometriosis and discern their contribution to endometriotic-associated hyperalgesia. Our results showed that HFD-induced obesity enhanced abdominal hyperalgesia that was induced by endometriotic lesions. Peritoneal inflammatory macrophages and cytokine levels increased by lesion induction were elevated by chronic exposure to HFD. Increased expression of pain-related mediators in the dorsal root ganglia was observed after lesion induction under the HFD condition. Although HFD did not affect inflammatory macrophages in the peritoneal cavity without lesion induction, the diversity and composition of the gut microbiota were clearly altered by HFD as a sign of low-grade systemic inflammation. Thus, HFD alone might not establish a local inflammatory environment in the pelvic cavity, but it can contribute to further enhancing chronic inflammation, leading to the exacerbation of endometriosis-associated abdominal hyperalgesia following the establishment and progression of the disease.

KEYWORDS

endometriosis, pain, high-fat diets, inflammation, macrophages, microbiota

1 Introduction

Endometriosis is a chronic and incurable inflammatory disorder and affects approximately 10% of reproductive-aged women (1, 2). It is associated with debilitating chronic pelvic pain and infertility, which substantially reduce the quality of life of women and their families (3, 4). Because endometriosis is estrogen-dependent, current treatments focus on inhibiting estrogen production and function. However, hormonal treatments and surgical excision of lesions are often of limited efficacy with high recurrence rates, frequent side effects, additional costs, and potential morbidity (5). As nearly 70% of patients suffer unsolved chronic pain and other related conditions (6), the direct costs of endometriosis were estimated at $12,118 per patient per year in the US, and indirect costs were $15,737 (7). The pathogenesis of endometriosis is a complex process and remains to be fully understood. Retrograde menstruation has been widely accepted as the origin of endometriotic tissues (8). However, as retrograde menstruation occurs in more than 90% of menstruating women (9), the pathogenesis of the disease is not well understood, and other factors must contribute to establishing endometriotic lesions and disease progression (1, 4, 10).

Obesity is an epidemic health burden affecting nearly 40% of adults and 18% of children in the United States (11). Being overweight and obese are considered critical risk factors for chronic diseases, as fat accumulation causes low-grade chronic inflammation (12) characterized by immune cell infiltration into adipose tissues and elevated proinflammatory factors (13). Moreover, excessive fat consumption and accumulation in the body alter gut microbiota, resulting in dysbiosis to induce low-grade systemic inflammation (14). Obesity-induced inflammation is associated with metabolic and autoimmune disorders in women, causing reproductive dysfunctions such as polycystic ovary syndrome (PCOS), implantation and pregnancy failure, and pregnancy complications, including miscarriages (15–18). While endometriosis is a chronic inflammatory disease, several epidemiological studies have reported an inverse correlation between endometriosis and body mass index (BMI) (19). However, obesity does not protect against endometriosis (19), and BMI is correlated with the severity but not the frequency of disease diagnoses (20). Thus, BMI does not provide a simple risk factor for a heterogeneous endometriotic disease as it does not consider different components of excess weight, such as adipose deposit location and interaction with neighboring tissues (20, 21). Additionally, the correlation between diet-induced obesity and endometriosis-associated pain or hypersensitivity, one of the significant endometriosis symptoms, has not been addressed.

Rigorous prior research suggests that aberrant inflammation contributes to the onset and progression of endometriosis (22–27). Macrophages (MΦ) are considered to be key players in promoting disease progression (25, 28, 29), as abundant MΦ are present in ectopic lesions (30) and elevated in the pelvic cavity (31). These MΦ populations establish an inflammatory environment in the pelvic cavity by secreting cytokines and chemokines, which encourage lesion growth and progression (24, 28, 29, 32, 33) and contribute to endometriosis-associated pelvic pain (32, 34, 35). Diet-induced obesity dysregulates immune cells to induce cytokine secretion (13, 36, 37), increasing the risks of chronic pain. Therefore, the present study seeks to understand whether high-fat diets (HFD) affect the progression of endometriosis disease and immune dysfunctions and how HFD influence endometriosis-associated hyperalgesia.

The present results highlight that endometriosis-associated abdominal hyperalgesia was escalated in lesion-induced HFD mice according to the results of the behavior study and elevated pain-related mediators in the dorsal root ganglion (DRG). Increased proinflammatory MΦ (Ly6C+ MΦ) and cytokines by lesion induction were further enhanced by exposure to HFD. The results also indicate that gut microbiota dysbiosis under the HFD condition contributed to an aberrant inflammatory environment and sensitized endometriosis-associated hypersensitivity.

2 Results

2.1 Diet-induced obesity on endometriosis in mice

To examine the effect of diet-induced obesity on endometriosis, female mice were fed HFD containing 45% fat by calories or standard diets (SD) from the age of 5 weeks (defined as Week 0 of the 12-week as a baseline study or 18-week as an endometriosis study, Figure 1A). We chose to start the study at the age of 5 weeks, as this is the adolescent age of mice, corresponding to the teenage period for humans (38). Moreover, approximately 20% of this population is obese in humans (39). Mice on the 45% fat diets become obese and are considered physiologically similar to the typical Western diets that contain 36-40% fat by energy (40, 41). A standard rodent diet contains approximately 10% fat (40, 42). We assessed body weight (BW) increase, glucose, and insulin levels at 12 weeks after SD or HFD feeding as a baseline study and 18 weeks (6 weeks after endometriosis-like lesions (ELL) induction) as an endometriosis study (Figures 1A–D). BW, blood glucose, and plasma insulin levels were significantly increased in the group of HFD at 12 or 18 weeks, whereas they were not affected by lesion induction. BW, glucose, and insulin levels in the HFD group were similar to the previously reported levels (43, 44).

We next assessed lesion numbers in the endometriosis study at 18 weeks. Lesion numbers were not altered by HFD compared with SD (Figure 2A). We have previously reported that peritoneal MΦ or monocytes are infiltrated into the ELL (28). We thus addressed MΦ infiltration in the lesion staining with CD68, a macrophage marker. CD68+ MΦ were significantly increased within lesions from mice in the HFD group (Figures 2B, C), indicating MΦ infiltration was accelerated in the ELL-HFD mice, although this did not appear to affect lesion development.

2.2 HFD accelerated endometriosis-associated abdominal hyperalgesia

Since HFD can induce chronic pain (12, 13, 45), we performed the von Frey test to examine the abdominal and hind paw retraction threshold to determine whether HFD affects endometriosis-

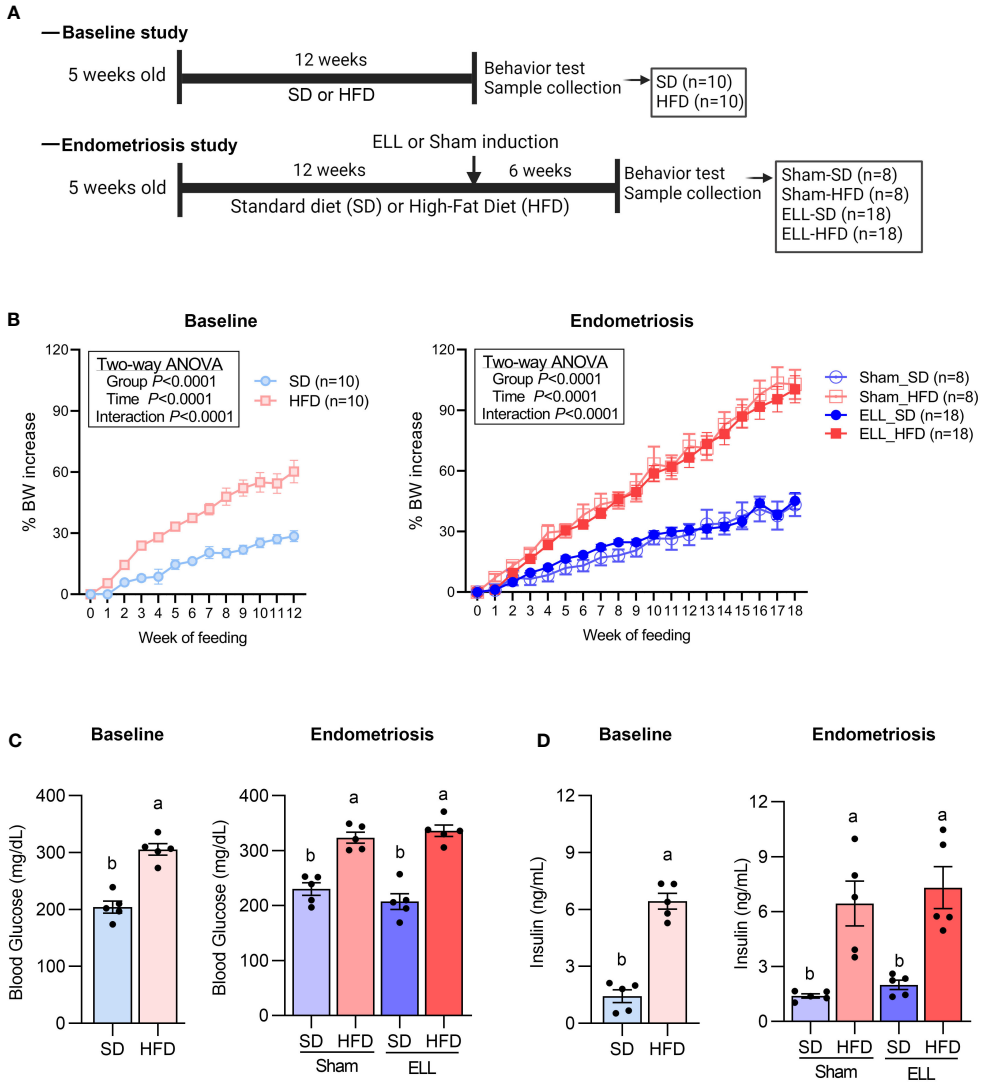


FIGURE 1
Diet-induced obesity in the mouse model of endometriosis. **(A)** Experimental study design as described in Material and Methods. **(B)** Body weight (BW) changes in mice during the feeding of standard diets (SD) or 45% high-fat diets (HFD) for the baseline study or the endometriosis study. Female mice were fed either SD or HFD starting at the age of 5 weeks (defined as Week 0 of the 12-week as a baseline study or 18-week as an endometriosis study). Two-way ANOVA was used to determine the significance between times and groups. **(C)** Blood glucose levels by cardiac puncture were measured by Contour Next (n=5 in each group). **(D)** Plasma insulin levels were quantified by ELISA (n=5 in each group). Data at 12 weeks were analyzed by two-tailed Student's t-test comparing SD and HFD. Data at 18 weeks were analyzed through one-way ANOVA and Tukey's *post hoc* test. Values in graphs are expressed as the mean ± SEM. Different letters indicated significant differences among the groups ($P<0.05$). ELL: endometrial-like lesion.

associated hyperalgesia. We first assessed the abdominal and hind paw retraction threshold at 12 weeks after SD or HFD feeding. The abdominal and hind paw retraction threshold showed no differences under the SD or HFD diets for 12 weeks (Figures 3A, C).

Before examining the effect of HFD on endometriosis-associated hyperalgesia, we examined how lesion induction time-dependently alters endometriosis-associated hyperalgesia in the mouse model. Three days after ELL induction, mice withdrew both abdominal and hind paw retraction thresholds with significantly lighter stimuli compared with those before ELL induction on Day -1 (Supplementary Figures S1A, B). The abdomen and hind paw retraction sensitivity continued until 3 weeks after ELL induction. By Day 42, 6 weeks after ELL induction, the hind paw retraction threshold was no longer significantly different from Day -1, indicating that systemic peripheral hyperalgesia gradually recovered, whereas the local abdomen was still sensitive. Since we examined the effect of chronic HFD exposure on endometriosis-associated hyperalgesia, we chose a chronic stage, 6 weeks after ELL induction, for further analysis, as endometriosis is a chronic disease, and most patients suffer chronic pelvic pain. Furthermore, the timing of disease onset in endometriosis is currently impossible to determine in patients, and the disease diagnosis typically relies on the woman noticing chronic symptoms.

The abdominal and hind paw sensitivity with SD or HFD were evaluated 6 weeks after lesion induction (18 weeks of SD or HFD

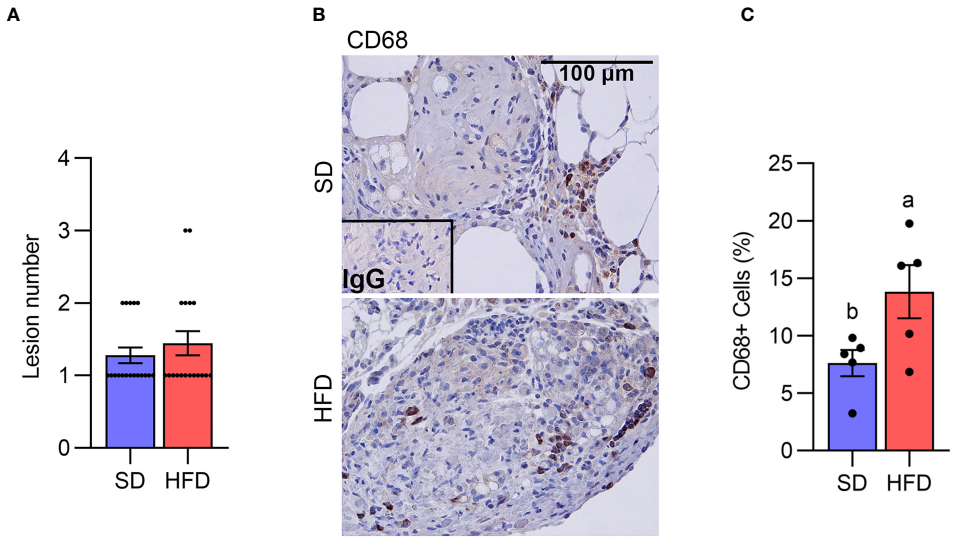


FIGURE 2
Diet-induced obesity increases macrophage infiltration in the lesion. **(A)** Lesion number (n=18 per group). **(B)** CD68 was stained to determine macrophage infiltration in the lesion. **(C)** The quantification of the percentage of CD68+ cells per total cells (n=5). Data were analyzed with the student t-test and are shown as mean ± SEM. Different letters indicated significant differences among the groups ($P<0.05$). SD: standard diets, and HFD: high-fat diets.

feeding). As expected, a significant difference was observed in the abdominal retraction threshold between Sham (vehicle, PBS, control) and ELL, with ELL mice withdrawing from lighter stimuli than Sham mice (Figure 3B). Importantly, ELL-HFD mice were more sensitive than ELL-SD mice (Figure 3B). On the other hand, we did not observe any differences in hind paw retraction threshold among the post-induction groups (Figure 3D). Thus, HFD-induced obesity enhanced abdominal hyperalgesia that was induced by endometriotic lesions.

2.3 HFD increased Ly6C+ MΦ in the peritoneal fluid of ELL mice

As we observed increased MΦ infiltration in the lesions of the HFD group (Figures 2B, C) and ELL-HFD mice have increased hypersensitivity in the abdomen (Figure 3B) in the endometriosis study, we expected to observe differences in the inflammatory environment that is established in the peritoneal cavity. Therefore, we assessed immune cell profiles, MΦ, B- and T-cells, in the peritoneal cavity (Figure 4, Supplementary Figure S2). CD11b + MΦ, CD3+ T-cells, and CD19+ B-cells were not altered by either HFD feeding or lesion induction (Figures 4A, B). We have previously reported that the presence of ELL enhanced the differentiation of recruited (=proinflammatory Ly6C+) MΦ and increased the ablation of embryo-derived resident MΦ (TIM4+ MΦ) (29). We thus examined Ly6C+ cells (monocytes and MΦ), Ly6C+ MΦ, and TIM4+ MΦ. High levels of Ly6C+ cells and Ly6C+ MΦ were observed in the ELL-HFD mice (Figures 4A–C). In particular, Ly6C+ MΦ were further increased in the ELL-HFD mice than those in ELL-SD mice (Figure 4C). In agreement with our previous study (29), TIM4+ MΦ were reduced in ELL-SD and ELL-HFD mice (Figure 4C). Ly6C+ cells, Ly6C+ MΦ and TIM4+ MΦ, as well as CD11b+ MΦ, CD19+ B-cells, and CD3+ T-cells were not affected by HFD feeding at 12 weeks in the baseline study (Supplementary Figure S2). These results suggest that ELL induction under the HFD condition further increases proinflammatory Ly6C+ MΦ in the peritoneal cavity.

2.4 HFD altered peritoneal cytokines in the ELL mice

Proinflammatory MΦ secrete cytokines, chemokines, and growth factors that establish the inflammatory environment (27, 46). Abundant cytokines and chemokines have been observed in the pelvic cavity of endometriosis patients (24, 27). Specifically, the levels of TNFα, IL1β, and IL6 are increased in pelvic MΦ isolated from endometriosis patients (47). Thus, we next examined the secretion of proinflammatory factors, TNFα, IL1β, and IL6, as well as IL10, which is known to possess immunoregulatory function and anti-inflammatory properties (Figures 5A–D). In support of previous reports, TNFα and IL1β levels were elevated in the ELL groups compared with those in the Sham group, while TNFα was further increased in ELL-HFD mice. IL6 tended to be increased by lesion induction in both SD and HFD groups, though we did not see significant differences. IL10 levels were not significantly altered among the groups of Sham-SD, Sham-HFD, and ELL-SD mice, whereas it was significantly lower in the ELL-HFD mice compared with that of ELL-SD mice.

10.3389/fendo.2024.1336496

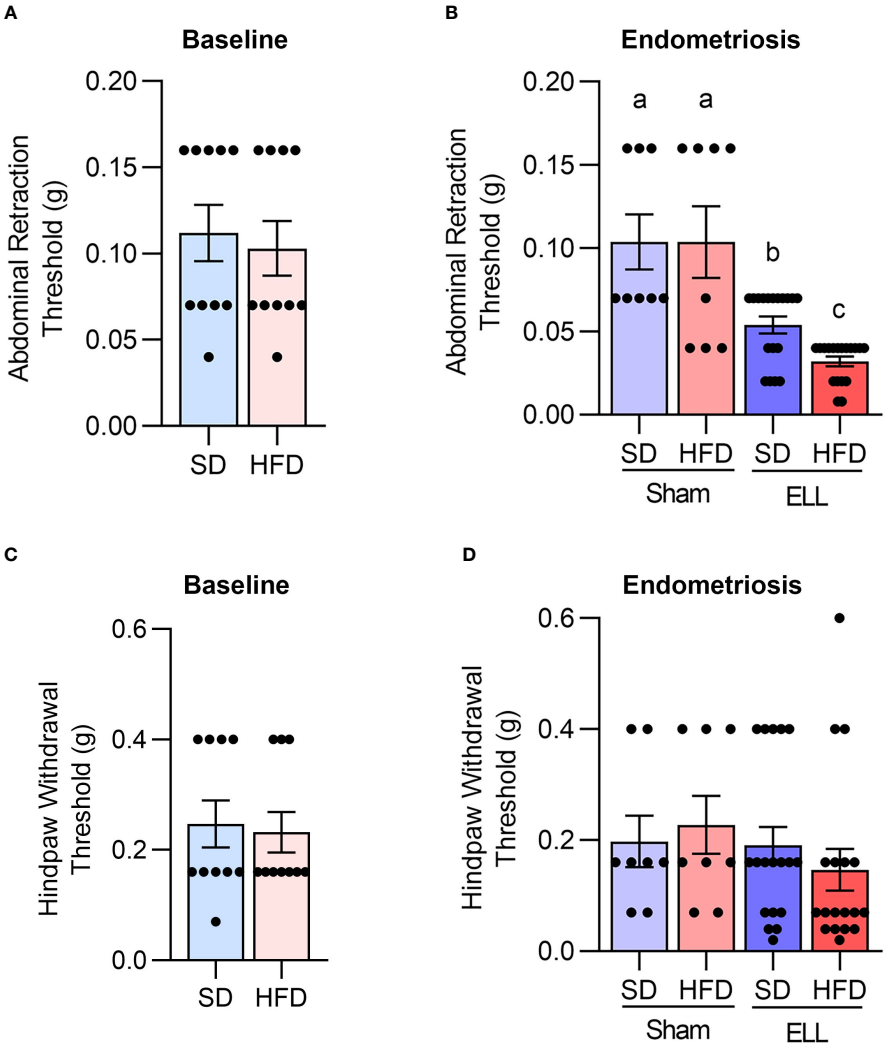


FIGURE 3
HFD accelerates endometriosis-associated abdominal hyperalgesia. Von Frey tests were performed on mice to the lower abdomen and hind paw in the bseline study after 12 weeks of SD or HFD feeding (A and C, n=10), or 6 weeks post-lesion induction in the endometriosis study (**B, D**, a total of 18 weeks of SD or HFD feeding, n=8 for Sham and n=18 for ELL groups). Data are shown as mean ± SEM. Statistical significance was determined by student t-test **(A, C)**, or one-way ANOVA followed by Tukey's *post hoc* test **(B, D)**. Different letters indicated significant differences among the groups ($P<0.05$). ELL: endometrial-like lesion, SD: standard diets, and HFD: high-fat diets.

2.5 HFD stimulated pain-related mediators in the DRG of ELL mice

Aberrant accumulation of inflammatory factors can stimulate peripheral nerve terminals of nociceptor neurons innervating different tissues in peripheral organs (48), resulting in an increase in the expression of transient receptor potential channels e.g., TRPV1. Activation of peripheral nerves is also associated with the increased release of neurotransmitters and neuromodulators such as SP, CGRP, and BDNF. BDNF is known to regulate both initiation and maintenance of chronic endometriosis-associated pain (49, 50) involving neuroangiogenesis (51) and innervation in the pelvic organs (48). We thus examined the inflammatory mediators, neurotransmitters, and neuromodulators in the L4-6 DRG, which are the primary spinal ganglia receiving sensory input from pelvic organs (Figures 6A, B, Supplementary Figure S3). Significantly more BDNF+ neurons were observed in mice fed HFD. BDNF+ neurons were higher in mice when ELL were present and most abundant in the HFD-ELL group. In contrast, CGRP+ neurons were only significantly elevated in the ELL-HFD mice. SP+ neurons were elevated by lesion induction, while HFD further increased SP+ neurons after ELL induction. Although the numbers of TRPV1+ neurons were relatively consistent between Sham- and ELL-mice, there was a significant difference between ELL-HFD mice and ELL-SD mice. These results suggest that lesion induction and/or HFD feeding stimulate endometriosis-associated peripheral pain mediators.

2.6 HFD altered the composition of the gut microbiota

As increased fat accumulation alters gut microbiota and causes low-grade systemic inflammation (14), we next examined 16S

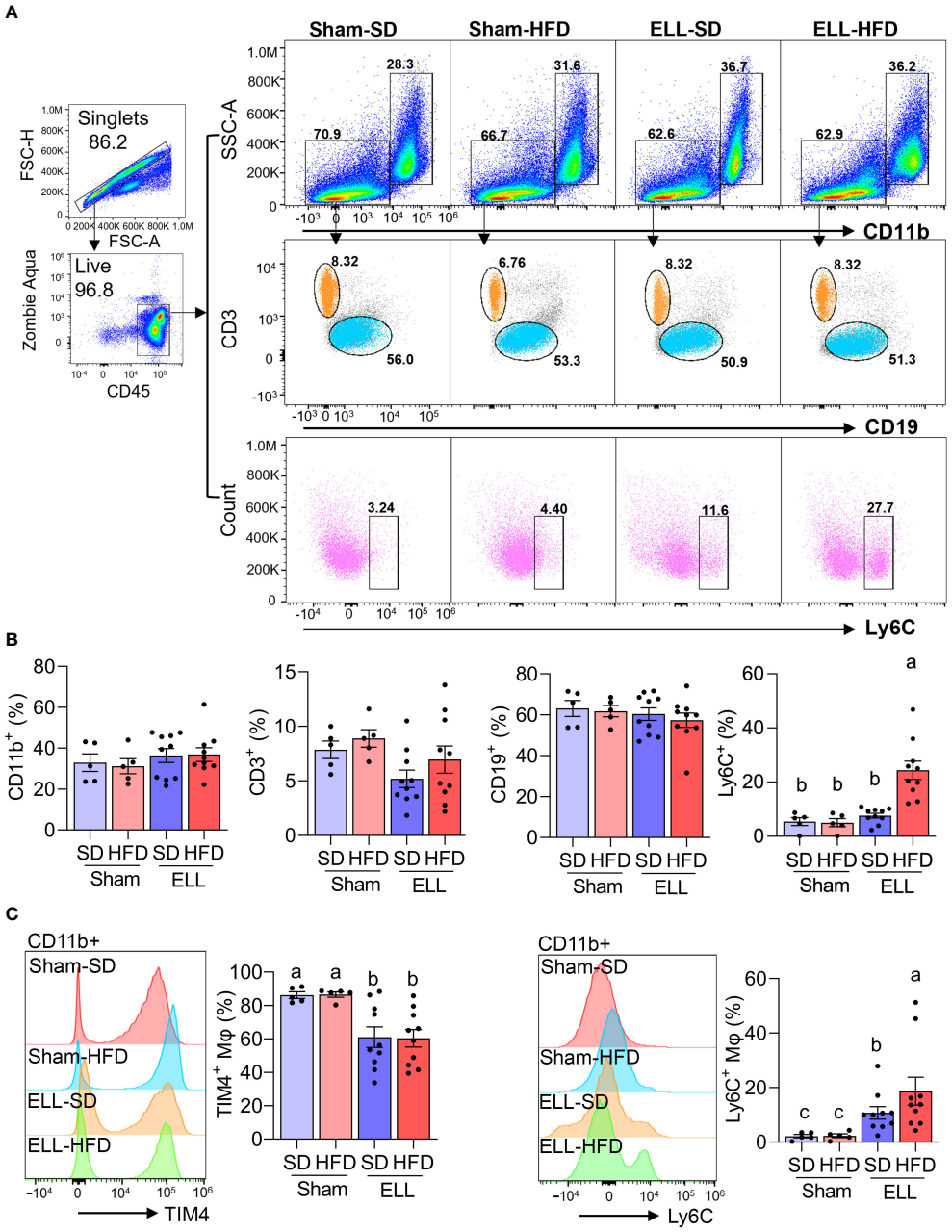


FIGURE 4
HFD increases Ly6C+ macrophages (MΦ) in the peritoneal fluid (PF) of ELL mice. **(A)** Flow cytometer analysis for CD11b+ (MΦ), CD3+ (T-cells), CD19 + (B-cells), and Ly6C+ (monocytes and MΦ) cells in the PF. FSC-H: Forward Scatter-Height; FSC-A: Forward Scatter-Area; SSC-A: Side Scatter-Area. **(B)** Quantification of CD11b+, CD3+, CD19+, and Ly6C+ cells in the groups of Sham-SD (n=5), Sham-HFD (n=5), ELL-SD (n=10) and ELL-HFD (n=10). **(C)** TIM4+ and Ly6C+ MΦ were quantified in the PF. Data were analyzed through One-way ANOVA followed by Tukey's *post hoc* test and expressed as the mean ± SEM. Different letters indicated significant differences among the groups ($P<0.05$). ELL: endometrial-like lesion, SD: standard diets, and HFD: high-fat diets.

rRNA gene sequencing of DNA isolated from fecal samples in SD or HFD with/without ELL-induced mice (Figure 7). Microbial alpha diversity was lower in the feces of HFD-fed mice than in SD-fed mice (Figure 7A). Principal coordinates analysis (PCoA) showed uniquely clustered microbial variance induced by HFD (Figure 7B). However, ELL induction did not alter microbial diversity or variance, indicating that long-term systemic alterations induced by HFD affect the composition of the gut microbiota more than lesion induction in mice.

To assess whether the unique enteric bacterial profiles were attributed to specific taxa, the phyla among samples in the group were profiled (Figure 7C). The proportions of Proteobacteria and Cyanobacteria were reduced under the HFD condition, while feces in ELL-SD mice contained a higher abundance of Proteobacteria than those in Sham-SD mice. However, increased Proteobacteria were not observed in ELL-HFD mice compared to Sham-HFD mice, suggesting that the effect of HFD on Proteobacteria was stronger than lesion induction. Firmicutes and Bacteroidetes, which constitute the majority

10.3389/fendo.2024.1336496

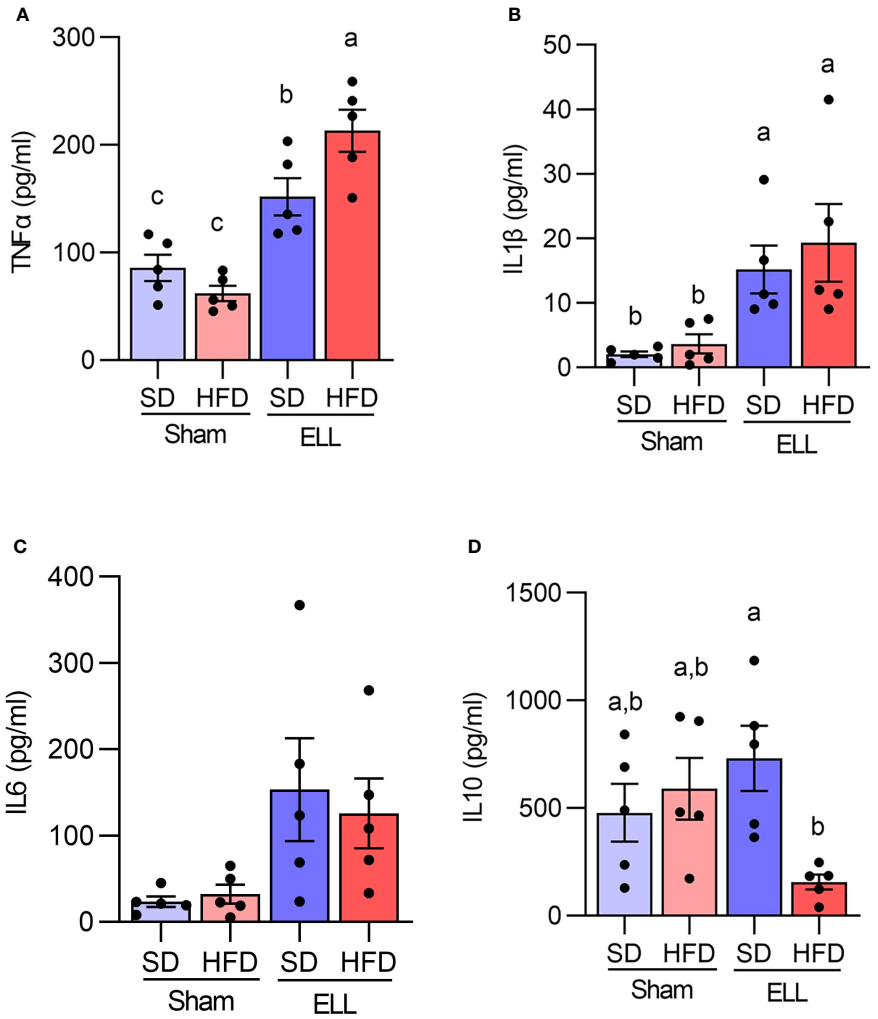


FIGURE 5
Quantification of TNFα, IL1β, IL6, and IL10 in the peritoneal fluid (PF). Peritoneal **(A)** TNFα, **(B)** IL1β, **(C)** IL6, and **(D)** IL10 were measured with IQELISA and analyzed with ANOVA followed by Tukey's *post hoc* test. Values in graphs are expressed as the mean ± SEM (n=5). Different letters indicated significant differences among the groups ($P<0.05$). ELL: endometrial-like lesion, SD: standard diets, and HFD: high-fat diets.

of the gut microbiota, are known to be affected by obesity, as obesity induces a reduction in the abundance of Bacteroidetes and an increase of Firmicutes proportion (52). Although the increase of Firmicutes was minor under the HFD condition, the Bacteroidetes proportion was clearly reduced in Sham-HFD mice. The Bacteroidetes population was retained in ELL-HFD mice, similar to its abundance in Sham-SD and ELL-SD mice, indicating that lesion induction increased Bacteroidetes even though mice were under the HFD condition. This result supports the study from Chadchan et al. that lesion induction increases the abundance of Bacteroidetes (53), while lesion induction with SD did not show a noticeable increase of Bacteroidetes in our study (Sham-SD vs ELL-SD). When we examined bacteria at the genus level, HFD clearly altered several genera among the groups (Figure 7D). In agreement with previous studies (54, 55), HFD strongly elevated *Lactococcus* and *Blautia* genera (red lines). HFD slightly increased *Ligilactobacillus* and *Intestinimonas* genera (red lines), whereas HFD mice contained negligible abundances of *Allobaculum*, *Lactobacillus*, *Dubosiella*, and *Ruminococcus* genera (blue lines). *Odoribacter*, *Turicibacter*, and *Rikenella* genera (green lines) were increased in ELL mice, and the *Bilophila* genus (orange line) was only higher in ELL-HFD mice. These data suggest that HFD or ELL alter the bacteria diversity and composition associated with endometriosis.

3 Discussion

Endometriosis is generally classified into four stages according to the revised criteria from the American Society of Reproductive Medicine (rASRM) and the American Fertility Society (AFS) based on lesion size, location, and the extent of adhesions (4, 56). However, disease symptoms, such as endometriosis-associated pain, are not correlated with the staging system (4, 57). Patients with stage I disease can have severe pain, while stage IV patients can be asymptomatic (1, 58), indicating that several other factors contribute to disease symptoms. Due to the chronic inflammatory nature of endometriosis, the disease progression and symptoms can be affected by environmental factors. In the present study, our results highlight that Western-style HFD-induced obesity did not

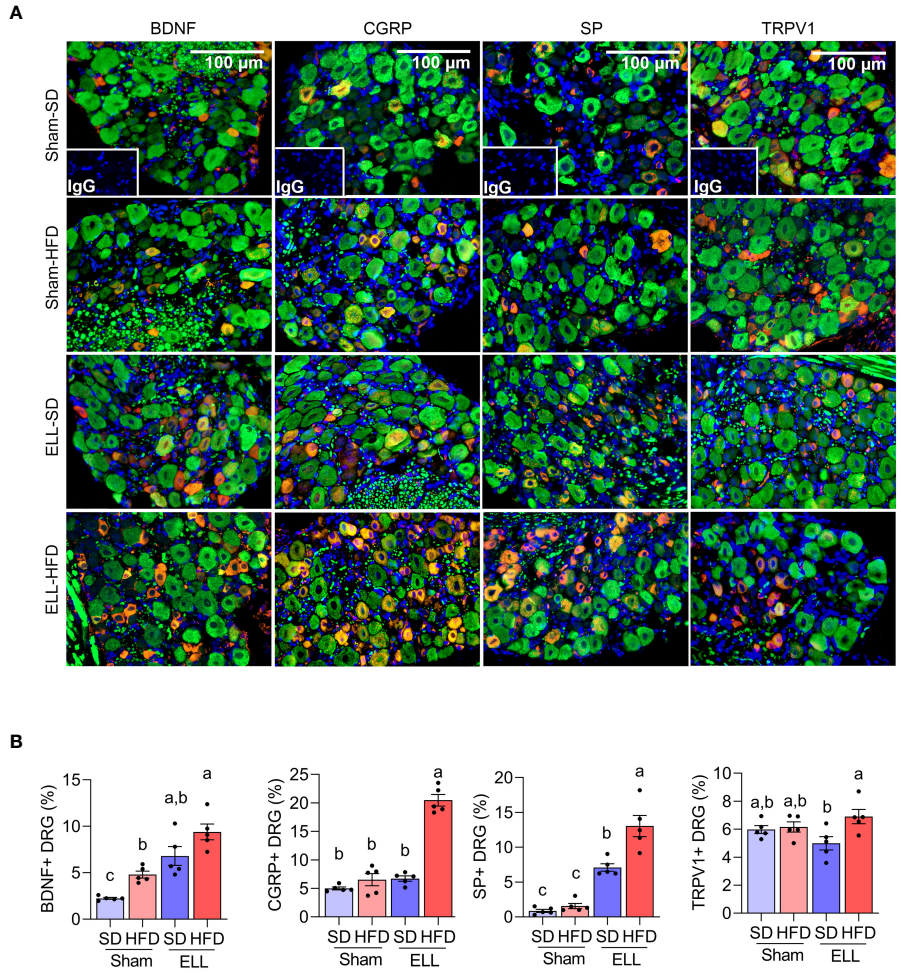


FIGURE 6
HFD stimulates pain-related mediators in the DRG of ELL mice. **(A)** Immunofluorescence results of BDNF, CGRP, SP, TRPV1, and neurofilament (NF, green) in DRG. NF was used as a marker of DRG cell body and was co-stained with BDNF, CGRP, SP, or TRPV1. **(B)** BDNF, CGRP, SP, or TRPV1 positive DRG per NF positive DRG was counted and quantified (n=5 per group). One-way ANOVA followed by Tukey's *post hoc* test was used for statistical analysis. Data were shown as mean ± SEM. Different letters indicated significant differences among the groups ($P<0.05$). ELL: endometrial-like lesion, SD: standard diets, and HFD: high-fat diets. DRG: dorsal root ganglia.

alter endometriotic lesion numbers (=disease progression) but enhanced disease-related hyperalgesia (=endometriosis-associated pain). The important findings are: 1) Peritoneal inflammatory (Ly6C+) MΦ and cytokine levels, especially TNFα, increased by lesion induction were elevated by chronic exposure to HFD. 2) Pain-related mediators, such as neurotransmitters CGRP and SP, in the DRG were further stimulated after lesion induction under the HFD condition. 3) Although HFD alone did not affect peritoneal Ly6C+ MΦ without lesion induction, the diversity and composition of the gut microbiota were clearly altered by HFD as a sign of low-grade systemic inflammation (14). Thus, HFD might not be able to establish solely a local inflammatory environment in the pelvic cavity but can contribute to further enhancing chronic inflammation associated with disease symptoms after the disease is established.

In non-human primates, rhesus macaque females exposed to testosterone (T) and/or consumed Western-style diets (WSD) at the time of menarche for 7 years developed endometriosis, especially T+WSD resulted in earlier onset of disease with high stages and large chocolate cysts (59). In a mouse model of endometriosis, HFD-induced obese mice increased lesion number and weight, which depended on leptin or leptin receptor (60). Another mouse study of endometriosis showed that HFD increased lesion number and MΦ infiltration and proinflammatory and prooxidative stress-related genes in the lesion when *Klf9* null donor endometrial fragments were inoculated as a donor tissue (61). This group further reported reduced lesion number and weight when wild-type donor tissues were used, whereas enhanced signs of inflammation were not observed in this study, indicating variability of distinct genetic dysfunctions and lesion environment for endometriosis progression (62).

One of the hallmarks of diet-induced obesity is low-grade chronic inflammation (12). Chronic consumption of HFD leads to the accumulation of MΦ and T-cells in adipose tissues to secrete proinflammatory cytokines (13). We have previously reported that lesion induction enhances the process of differentiation and

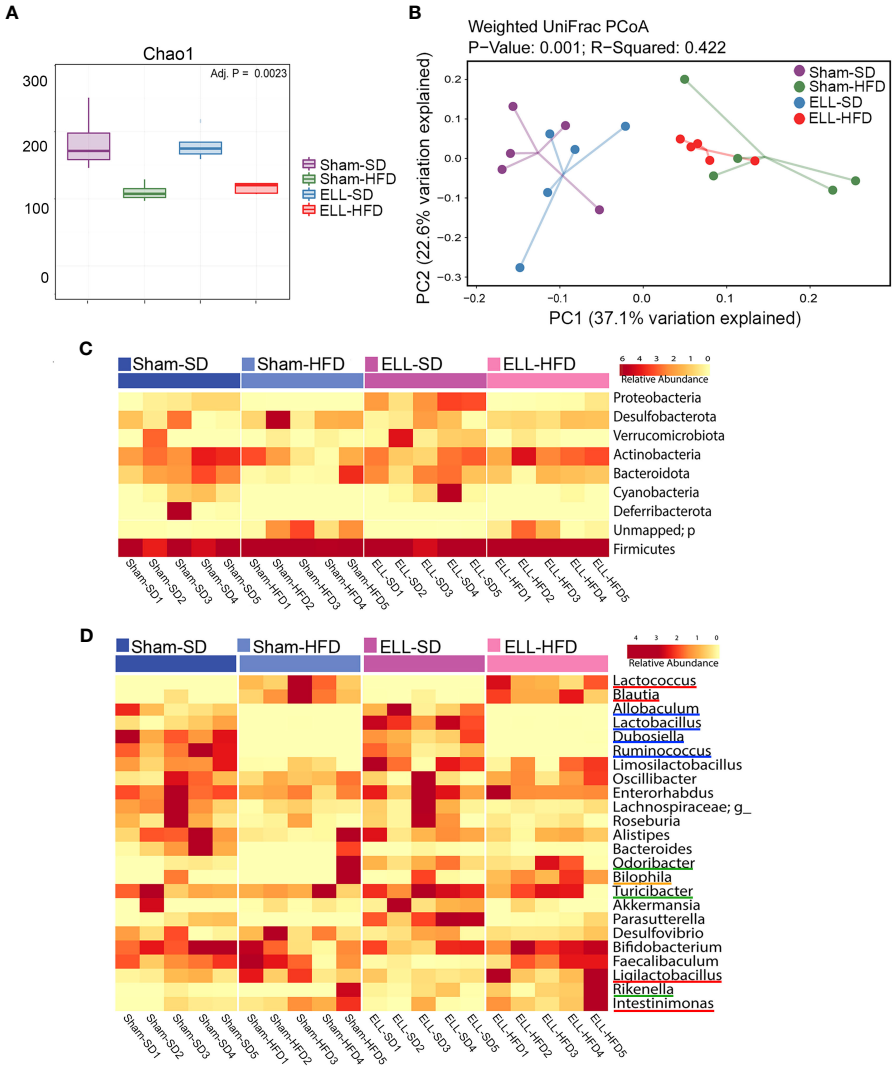


FIGURE 7
HFD altered the composition of the gut microbiota. **(A)** Box plots corresponding to the Chao1 diversity index (alpha diversity). **(B)** Principal Coordinates Analysis (PCoA) of beta-diversity based on weighted Unifrac dissimilarities in fecal samples. P = 0.001, R=0.422. (n=5 per group). **(C)** Heatmap representation of relative abundances of the phyla in feces. **(D)** Heatmap depiction of the relative abundances of the genera in feces (n=5 per group). ELL: endometrial-like lesion, SD: standard diets, and HFD: high-fat diets.

maturation of monocyte-derived MΦ and increases Ly6C+ proinflammatory MΦ in the peritoneal cavity while reducing the maintenance of embryo-derived resident TIM4+ MΦ (29). The present study showed that Ly6C+ MΦ were higher in ELL mice and further increased in mice exposed to HFD, indicating the impact of HFD contribution to peritoneal inflammation after disease onset. In support of our findings, an HFD-induced proinflammatory environment promotes the differentiation of Ly6C+ monocyte into inflammatory MΦ, which migrate to the lung and worsen its pathophysiology (63). TIM4+ residential MΦ were reduced in both ELL-SD and ELL-HFD mice, whereas HFD did not further alter TIM4+ MΦ. Peritoneal inflammation can induce the macrophage disappearance reaction (MDR), by which the reduction of residential MΦ occurs. We have previously shown that extreme MDR of TIM4+ MΦ was induced 3 days after lesion induction, and it gradually recovered. However, it remains slightly diminished 6 weeks after disease onset (29). Thus, the recovery of residential TIM4+ MΦ from MDR, which includes replenishment and proliferation, is less likely affected by exposure to HFD. On the other hand, an alteration in the distribution of peritoneal T-cells by lesion induction and HFD was not observed in the study, suggesting aberrant MΦ functions might be a crucial event for establishing the chronic inflammatory state of endometriosis, as increased MΦ infiltration was also observed in the lesions under the HFD condition. However, heterogeneous T-cell functions and interaction between T-cells and MΦ remain to be studied.

In the present study, abdominal endometriosis-associated hyperalgesia was induced by lesion induction and further sensitized in ELL-HFD mice. This result was supported by the signs of sensitization of peripheral DRG, which was mediated by increased proinflammatory cytokines, TNFα, IL1β, and IL6, that are known to be increased pelvic MΦ in endometriosis patients (47)

and have been targeted for pathological pain (64). Our previous studies show that PF from ELL mice stimulated DRG outgrowth, which was reduced by inhibiting cytokine and chemokine secretion in the peritoneal cavity (28). Thus, the inflammatory environment established in the pelvic cavity is critical for chronic endometriosis-associated hypersensitivity. The elevated sensitivity is not systemic, as our results showed only signs of abdominal hyperalgesia but not hind paw sensitivity by either lesion induction or HFD. Thus, it remains to study how chronic abdominal pain stimulus is delivered and maintained to the central nervous system. As endometriosis-associated pain is one of the significant problems in this disease, its mechanisms with the pathophysiology of endometriosis need to be further studied to enhance the quality of life in patients.

Our study showed that gut microbiota dysbiosis was induced by chronic exposure to HFD. HFD have been known to reduce the diversity of gut microbiota (65). The phyla Firmicutes increase while Bacteroidetes decrease, though there are variations depending on the differences in diet compositions and exposure duration (66, 67). Interestingly, our results showed a lower abundance of Proteobacteria in HFD mice, whereas increased Proteobacteria abundance with HFD consumption has been reported (68). Increased *Allobaculum* abundance has been shown under the HFD condition (69), though the abundance of *Allobaculum* was reduced in our HFD mice. However, this inconsistency is likely due to different types of diet, fat, and other environmental factors in the various studies (14). Despite having variable alterations of gut microbiota, HFD-induced dysbiosis increases gut permeability and creates chronic inflammation, affecting inflammatory diseases directly or indirectly (70).

The present study showed that endometriosis-associated abdominal hyperalgesia was escalated under exposure to HFD. These results include increased proinflammatory MΦ and cytokine levels in the peritoneal cavity, neuromodulators in the DRG, and dysbiosis of gut microbiota. There was no significant difference in mean lesion numbers between control and HFD mice, suggesting that the low-grade pre-induction inflammatory state of HFD mice may not significantly alter the mechanism that allows tissue adherence and survival. However, it is clear that once the ELL is established, the HFD lesions exhibit more MΦ infiltration with a more severe pain phenotype. Retrograde menstruation causes massive inflammatory responses in the pelvic cavity, which involves the recruitment of monocytes that differentiate into proinflammatory MΦ and secrete cytokines and chemokines (27). However, the acute inflammation associated with retrograde menstruation typically resolves by the next menstrual cycle. If women are under systemic low-grade inflammation induced by environmental factors like HFD, it is expected to be hard to solve this acute incidence. As menstrual cycles repeatedly occur in women, each retrograde menstruation induces composite inflammation in the pelvic cavity, and unsolved inflammation can worsen to develop chronic conditions further. Thus, the present results suggest that diet-induced obesity could be a risk factor for establishing a chronic inflammatory environment and severe endometriosis-associated pain, which can be independent of disease progression.

4 Materials and methods

4.1 Animals

All animal experiments were performed at Washington State University according to the NIH guidelines for the care and use of laboratory animals (protocol #6751). C57BL/6 (JAX: 000664) breeder pairs were obtained from the Jackson Laboratory, bred in-house, and maintained in the vivarium with a 12:12 light-dark (LD) cycle under ad libitum conditions of food and water. Female C57BL/6 mice at the age of 5 weeks were used for the studies.

4.2 Mouse model of endometriosis

An experimental mouse model of endometriosis was established by adopting procedures described previously (28, 29, 51, 71–73) and Supplementary Method. Briefly, a 'menses-like' event was induced in ovariectomized estradiol-17β (E_2)- and progesterone-primed donor mice following an established protocol (74). Then, mouse menses-like endometrium scraped from myometrium and cut into fragments (1-2 mm per side) were introduced as the source of syngeneic mouse endometrium (donor) via injection (in 0.2 mL PBS) into the peritoneal cavity of untreated naive mice (recipient) under anesthesia via inhaled isoflurane.

4.3 Study design

To induce diet-dependent obese mice, female mice were fed Teklad Rodent Diet (#2019, Envigo) as SD (Washington State University regular diet) that contain 9-10% of total calories from fat or HFD (D12451, Research Diets) that contain 45% of total calories from fat starting at the age of 5 weeks (defined as Week 0 of the 12-week as a baseline study or 18-week as an endometriosis study, Figure 1A). BW was recorded once a week. In the baseline study, mice were fed with SD (n=10) or HFD (n=10) for 12 weeks. After 12 weeks of feeding, a von Frey behavior test was performed, and peripheral blood and peritoneal lavage were collected. In the endometriosis study, mice were further assigned to sham control without lesion induction or ELL-induced groups twelve weeks after SD or HFD feeding. Thus, there were a total of 4 groups with Sham (vehicle, PBS, control)-SD (n=8), Sham-HFD (n=8), ELL-SD (n=18) and ELL-HFD (n=18). Six weeks after induction (a total of 18 weeks), a behavior test was performed, and fresh feces were collected and immediately frozen at -80C. Mice were then euthanized for sample collections: blood was collected via cardiac puncture, PF was recovered by lavage (4 mL x 2 of ice-cold PBS), and ELL and bilateral lumbar (L4-6) DRG were collected for further analysis. Blood glucose levels (n=5) were measured by Contour Next (Ascensia Diabetes Care), and plasma insulin (n=5) was analyzed by ELISA (EZRMI-13K, Sigma Aldrich), according to the manufacturer's instructions.

4.4 Von Frey test

A behavioral (mechanical sensitivity) test was performed before sample collection (34, 73). Mice were allowed to acclimate in the testing room for 30 min, and then the von Frey test was performed using von Frey Filaments (BIO-VF-M, Bioseb). Filaments were applied 10 times to the skin perpendicular to the lower abdomen and bilateral hind paws. The force in grams (g) of the filament evoking a withdrawal response (50% response count as sensitive) was recorded. Three behaviors were considered positive responses to filament stimulation: 1) sharp retraction of the abdomen, 2) immediate licking and/or scratching of the area of filament stimulation, or 3) jumping. All behavioral tests were performed without describing the identity and details of treatment groups to investigators. The data were analyzed by another investigator. Mice without ELL or sham induction after 12 weeks of SD or HFD feeding were included as a baseline result.

4.5 Flow cytometry

Peritoneal lavages were centrifuged to collect peritoneal exudate cells. After lysing red blood cells by 1x RBC Lysis Buffer (BioLegend), cells were incubated at room temperature for 20 minutes with Zombie Aqua™ Fixable Viability dye (BioLegend) and blocked on ice for 20 minutes with Fc Block anti-CD16/CD32 (Thermo Fisher). Then, cells were stained with fluorochrome-conjugated monoclonal antibodies (Supplementary Table S1) for 1 hour. Samples were acquired with the Attune NxT Acoustic Focusing Cytometer using Attune NxT software (Thermo Fisher), and data were analyzed with FlowJo v10.9. For analysis, only singlets (determined by forward scatter height vs. area) and live cells (Zombie Aqua negative) were used.

4.6 Immunofluorescence

Immunostaining of BDNF, CGRP, SP, TRPV1, neurofliment (NF), and CD68 was performed with cross-sections (5 µm) of paraffin-embedded tissues using specific commercially available primary antibodies (Supplementary Table S1) and AlexaFluor 488 and 568-conjugated F(ab') secondary antibody (Molecular Probe) or VECTASTAIN ABC kit (Vector lab). Immunostaining images were acquired by Leica DM4 B. Cell-specific CD68 positive and total cell numbers were counted by Image J in the area of 0.07244 mm^2, and the percentage of CD68+ cells was shown. NF was used as a pan-neuronal marker and was co-stained with BDNF, CGRP, SP, or TRPV1. BDNF, CGRP, SP, or TRPV1 positive cells in the DRG were counted by Image J in the area of 0.07244 mm^2. The percentages of BDNF, SP, CGRP, or TRPV1 positive cells per NF-positive DRG were shown.

4.7 IQELISA

Protein yield from PF was quantitated by BCA assay (Pierce), and TNFα (IQM-TNFA-1), IL1β (IQM-IL1b-1), IL6 (IQM-IL6-1), and IL10 (IQM-IL10-1) were further quantified by IQELISA kits (Ray Biotech) according to the manufacturer's instructions.

4.8 16S rRNA gene sequencing and analysis

DNA was extracted from fecal pellets (100 mg, n=5 per group) by the QIAmp Power Fecal DNA kit (12850-50, Qiagen). The V4 region of 16S rRNA gene was amplified, and sequencing was performed on an Illumina platform by the Alkek Center for Metagenomics and Microbiome Research at Baylor College of Medicine. Demultiplexed reads were quality filtered after initial trimming, and taxonomic information was retrieved by mapping against SILVA version 138.1 (75) using an identity threshold of 70% in Quantitative Insights Into Microbial Ecology (76). Raw data in FASTQ format were uploaded to the NCBI Sequence Read Archive (PRJNA1007658). This dataset was used for downstream alpha and beta diversity analysis, and top taxa were identified using a mean abundance threshold of ≥ 0.05, as described previously (53). The alpha diversity was measured using Chao1 distances, while the beta diversity was estimated using weighted UniFrac measures (77).

4.9 Statistical analysis

Data at 18 weeks were subjected to one-way ANOVA and Tukey's *post hoc* test to identify differences among the groups using Prism software (Ver. 9.1.0, GraphPad). Data at 12 weeks and lesions at 18 weeks were analyzed by two-tailed Student's t-test comparing SD and HFD. Two-way ANOVA was used to determine the significance between times and groups. All experimental data are presented as mean with standard error of the mean (SEM). Unless otherwise indicated, a P value less than 0.05 was considered to be statistically significant. Different letters indicated significant differences among the groups ($P<0.05$).

Ethics statement

The animal study was approved by Institutional animal care and use committee (IACUC) at Washington State University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

TH-W: Formal analysis, Investigation, Methodology, Writing – review & editing. MS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review &

editing. MH: Investigation, Methodology, Writing – review & editing. CT: Data curation, Formal analysis, Investigation, Writing – review & editing. RK: Funding acquisition, Resources, Supervision, Writing – review & editing. JM: Data curation, Supervision, Writing – review & editing. KH: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by NIH/NICHD, R01HD104619 (to KH) and R01HD102680 (to RK).

Acknowledgments

We thank Logan C. Butler for feeding diets and caring for mice.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1336496/full#supplementary-material

References

1. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *New Engl J Med*. (2020) 382:1244–56. doi: 10.1056/NEJMra1810764

2. Shafrir AL, Farland LV, Shah DK, Harris HR, Kvaskoff M, Zondervan K, et al. Risk for and consequences of endometriosis: A critical epidemiologic review. *Best Pract Res Clin Obstet Gynaecol*. (2018) 51:1–15. doi: 10.1016/j.bpobgyn.2018.06.001

3. Nnoaham KE, Hummelshoj L, Webster P, d'Hooghe T, de Cicco Nardone F, de Cicco Nardone C, et al. Impact of endometriosis on quality of life and work productivity: a multicenter study across ten countries. *Fertility sterility*. (2011) 96:366–373 e8. doi: 10.1016/j.fertnstert.2011.05.090

4. Zondervan KT, Becker CM, Koga K, Missmer SA, Taylor RN, Vigano P. Endometriosis. *Nat Rev Dis Primers*. (2018) 4:9. doi: 10.1038/s41572-018-0008-5

5. As-Sanie S, Black R, Giudice LC, Gray Valbrun T, Gupta J, Jones B, et al. Assessing research gaps and unmet needs in endometriosis. *Am J Obstet Gynecol*. (2019) 221:86–94. doi: 10.1016/j.ajog.2019.02.033

6. Agarwal SK, Foster WG, Groessl EJ. Rethinking endometriosis care: applying the chronic care model via a multidisciplinary program for the care of women with endometriosis. *Int J Womens Health*. (2019) 11:405–10. doi: 10.2147/IJWH.S207373

7. Soliman AM, Yang H, Du EX, Kelley C, Winkel C. The direct and indirect costs associated with endometriosis: a systematic literature review. *Hum Reprod*. (2016) 31:712–22. doi: 10.1093/humrep/dev335

8. Sampson JA. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. *Am J Pathol*. (1927) 3:93–110 43.

9. Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. Retrograde menstruation in healthy women and in patients with endometriosis. *Obstetrics gynecology*. (1984) 64:151–4.

10. Bulun SE, Yilmaz BD, Sison C, Miyazaki K, Bernardi L, Liu S, et al. Endometriosis. *Endocrine Rev*. (2019) 40:1048–79. doi: 10.1210/er.2018-00242

11. Hales CM, Fryar CD, Carroll MD, Freedman DS, Aoki Y, Ogden CL. Differences in obesity prevalence by demographic characteristics and urbanization level among adults in the United States, 2013-2016. *JAMA*. (2018) 319:2419–29. doi: 10.1001/jama.2018.7270

12. Zatterale F, Longo M, Naderi J, Raciti GA, Desiderio A, Miele C, et al. Chronic adipose tissue inflammation linking obesity to insulin resistance and type 2 diabetes. *Front Physiol*. (2019) 10:1607. doi: 10.3389/fphys.2019.01607

13. Artemniak-Wojtowicz D, Kucharska AM, Pyrzak B. Obesity and chronic inflammation crosslinking. *Cent Eur J Immunol*. (2020) 45:461–8. doi: 10.5114/ceji.2020.103418

14. Malesza IJ, Malesza M, Walkowiak J, Mussin N, Walkowiak D, Aringazina R, et al. High-fat, western-style diet, systemic inflammation, and gut microbiota: A narrative review. *Cells 10*. (2021) 10(11), 3164. doi: 10.3390/cells10113164

15. Broughton DE, Moley KH. Obesity and female infertility: potential mediators of obesity's impact. *Fertility sterility*. (2017) 107:840–7. doi: 10.1016/j.fertnstert.2017.01.017

16. McPherson NO, Bell VG, Zander-Fox DL, Fullston T, Wu LL, Robker RL, et al. When two obese parents are worse than one! Impacts on embryo and fetal development. *Am J Physiol Endocrinol Metab*. (2015) 309:E568–81. doi: 10.1152/ajpendo.00230.2015

17. Cardozo ER, Karmon AE, Gold J, Petrozza JC, Styer AK. Reproductive outcomes in oocyte donation cycles are associated with donor BMI. *Hum Reprod*. (2016) 31:385–92. doi: 10.1093/humrep/dev298

18. Bodnar LM, Parks WT, Perkins K, Pugh SJ, Platt RW, Feghali M, et al. Maternal prepregnancy obesity and cause-specific stillbirth. *Am J Clin Nutr*. (2015) 102:858–64. doi: 10.3945/ajcn.115.112250

19. Pantelis A, Machairiotis N, Lapatsanis DP. The formidable yet unresolved interplay between endometriosis and obesity. *ScientificWorldJournal*. (2021) 2021:6653677. doi: 10.1155/2021/6653677

20. Holdsworth-Carson SJ, Dior UP, Colgrave EM, Healey M, Montgomery GW, Rogers PAW, et al. The association of body mass index with endometriosis and disease severity in women with pain. *J Endometr Pelvic Pain Disord*. (2018) 10:79–87. doi: 10.1177/2284026518773939

21. Gutierrez-Rojas CA, Cruz-Soto R, Sanchez-Munoz V, Romero A, Mosti-Molina M, Sanchez-Aguilar HA, et al. Does FMI correlate better than BMI with the occurrence of metabolic changes in obese patients? Study Based on 2007 Consecutive Mexican Patients. *Obes Surg*. (2020) 30:1324–31. doi: 10.1007/s11695-019-04289-2

22. Berkkanoglu M, Arici A. Immunology and endometriosis. *Am J Reprod Immunol*. (2003) 50:48–59. doi: 10.1034/j.1600-0897.2003.00042.x

23. Capobianco A, Monno A, Cottone L, Venneri MA, Biziato D, Di Puppo F, et al. Proangiogenic Tie2(+) macrophages infiltrate human and murine endometriotic lesions and dictate their growth in a mouse model of the disease. *Am J Pathol*. (2011) 179:2651–9. doi: 10.1016/j.ajpath.2011.07.029

24. Rana N, Braun DP, House R, Gebel H, Rotman C, Dmowski WP. Basal and stimulated secretion of cytokines by peritoneal macrophages in women with endometriosis. *Fertility sterility*. (1996) 65:925–30. doi: 10.1016/S0015-0282(16)58262-4

25. Hogg C, Horne AW, Greaves E. Endometriosis-associated macrophages: origin, phenotype, and function. *Front Endocrinol (Lausanne)*. (2020) 11:7. doi: 10.3389/fendo.2020.00007

26. Hogg C, Panir K, Dhami P, Rosser M, Mack M, Soong D, et al. Macrophages inhibit and enhance endometriosis depending on their origin. *Proc Natl Acad Sci U.S.A*. (2021) 118 (6):e2013776118. doi: 10.1073/pnas.2013776118

27. Symons LK, Miller JE, Kay VR, Marks RM, Liblik K, Koti M, et al. The immunopathophysiology of endometriosis. *Trends Mol Med*. (2018) 24:748–62. doi: 10.1016/j.molmed.2018.07.004

28. Shi M, Sekulovski N, Whorton AE, MacLean JA 2nd, Greaves E, Hayashi K. Efficacy of niclosamide on the intra-abdominal inflammatory environment in endometriosis. *FASEB J*. (2021) 35:e21584. doi: 10.1096/fj.202002541RRR

29. Zhao L, Shi M, Winuthayanon S, MacLean JA 2nd, Hayashi K. Niclosamide targets the dynamic progression of macrophages for the resolution of endometriosis in a mouse model. *Commun Biol*. (2022) 5:1225. doi: 10.1038/s42003-022-04211-0

30. Capobianco A, Rovere-Querini P. Endometriosis, a disease of the macrophage. *Front Immunol*. (2013) 4:9. doi: 10.3389/fimmu.2013.00009

31. Hill JA, Faris HM, Schiff I, Anderson DJ. Characterization of leukocyte subpopulations in the peritoneal fluid of women with endometriosis. *Fertility sterility*. (1988) 50:216–22. doi: 10.1016/S0015-0282(16)60062-6

32. Bacci M, Capobianco A, Monno A, Cottone L, Di Puppo F, Camisa B, et al. Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. *Am J Pathol*. (2009) 175:547–56. doi: 10.2353/ajpath.2009.081011

33. Prather GR, MacLean JA 2nd, Shi M, Boadu DK, Paquet M, Hayashi K. Niclosamide as a potential nonsteroidal therapy for endometriosis that preserves reproductive function in an experimental mouse model. *Biol Reprod*. (2016) 95:76. doi: 10.1095/biolreprod.116.140236

34. Forster R, Sarginson A, Velichkova A, Hogg C, Dorning A, Horne AW, et al. Macrophage-derived insulin-like growth factor-1 is a key neurotrophic and nerve-sensitizing factor in pain associated with endometriosis. *FASEB J*. (2019) 33(10):11210–11222. doi: 10.1096/fj.201900797R

35. Wu J, Xie H, Yao S, Liang Y. Macrophage and nerve interaction in endometriosis. *J Neuroinflamm*. (2017) 14:53. doi: 10.1186/s12974-017-0828-3

36. Kiran S, Kumar V, Murphy EA, Enos RT, Singh UP. High fat diet-induced CD8 (+) T cells in adipose tissue mediate macrophages to sustain low-grade chronic inflammation. *Front Immunol*. (2021) 12:680944. doi: 10.3389/fimmu.2021.680944

37. Kiran S, Rakib A, Kodidela S, Kumar S, Singh UP. High-fat diet-induced dysregulation of immune cells correlates with macrophage phenotypes and chronic inflammation in adipose tissue. *Cells*. (2022) 11(8), 1327. doi: 10.3390/cells11081327

38. Dutta S, Sengupta P. Men and mice: Relating their ages. *Life Sci*. (2016) 152:244–8. doi: 10.1016/j.lfs.2015.10.025

39. Sanyaolu A, Okorie C, Qi X, Locke J, Rehman S. Childhood and adolescent obesity in the United States: A public health concern. *Glob Pediatr Health*. (2019) 6:2333794X19891305. doi: 10.1177/2333794X19891305

40. Speakman JR. Use of high-fat diets to study rodent obesity as a model of human obesity. *Int J Obes (Lond)*. (2019) 43:1491–2. doi: 10.1038/s41366-019-0363-7

41. Wilson CR, Tran MK, Salazar KL, Young ME, Taegtmeyer H, diet W. but not high fat diet, causes derangements of fatty acid metabolism and contractile dysfunction in the heart of Wistar rats. *Biochem J*. (2007) 406:457–67. doi: 10.1042/BJ20070392

42. Winzell MS, Ahren B. The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes*. (2004) 53 Suppl 3:S215–9. doi: 10.2337/diabetes.53.suppl_3.s215

43. van der Heijden RA, Sheedfar F, Morrison MC, Hommelberg PP, Kor D, Kloosterhuis NJ, et al. High-fat diet induced obesity primes inflammation in adipose tissue prior to liver in C57BL/6j mice. *Aging (Albany NY)*. (2015) 7:256–68. doi: 10.18632/aging.100738

44. Gallou-Kabani C, Vige A, Gross MS, Rabes JP, Boileau C, Larue-Achagiotis C, et al. C57BL/6J and A/J mice fed a high-fat diet delineate components of metabolic syndrome. *Obes (Silver Spring)*. (2007) 15:1996–2005. doi: 10.1038/oby.2007.238

45. Duan Y, Zeng L, Zheng C, Song B, Li F, Kong X, et al. Inflammatory links between high fat diets and diseases. *Front Immunol*. (2018) 9:2649. doi: 10.3389/fimmu.2018.02649

46. Cassado Ados A, D'Imperio Lima MR, Bortoluci KR. Revisiting mouse peritoneal macrophages: heterogeneity, development, and function. *Front Immunol*. (2015) 6:225. doi: 10.3389/fimmu.2015.00225

47. Montagna P, Capellino S, Villaggio B, Remorgida V, Ragni N, Cutolo M, et al. Peritoneal fluid macrophages in endometriosis: correlation between the expression of estrogen receptors and inflammation. *Fertility sterility*. (2008) 90:156–64. doi: 10.1016/j.fertnstert.2006.11.200

48. Ji RR, Xu ZZ, Gao YJ. Emerging targets in neuroinflammation-driven chronic pain. *Nat Rev Drug Discovery*. (2014) 13:533–48. doi: 10.1038/nrd4334

49. Ding S, Zhu T, Tian Y, Xu P, Chen Z, Huang X, et al. Role of brain-derived neurotrophic factor in endometriosis pain. *Reprod Sci*. (2017) 25(7):1045–1057. doi: 10.1177/1933719117732161

50. Kobayashi H, Yamada Y, Morioka S, Niiro E, Shigemitsu A, Ito F. Mechanism of pain generation for endometriosis-associated pelvic pain. *Arch Gynecol Obstet*. (2014) 289:13–21. doi: 10.1007/s00404-013-3049-8

51. Greaves E, Temp J, Esnal-Zufiurre A, Mechsner S, Horne AW, Saunders PT. Estradiol is a critical mediator of macrophage-nerve cross talk in peritoneal endometriosis. *Am J Pathol*. (2015) 185:2286–97. doi: 10.1016/j.ajpath.2015.04.012

52. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci U.S.A*. (2005) 102:11070–5. doi: 10.1073/pnas.0504978102

53. Chadchan SB, Cheng M, Parnell LA, Yin Y, Schriefer A, Mysorekar IU, et al. Antibiotic therapy with metronidazole reduces endometriosis disease progression in mice: a potential role for gut microbiota. *Hum Reprod*. (2019) 34:1106–16. doi: 10.1093/humrep/dez041

54. Hases L, Stepanauskaite L, Birgersson M, Brusselaers N, Schuppe-Koistinen I, Archer A, et al. High-fat diet and estrogen modulate the gut microbiota in a sex-dependent manner in mice. *Commun Biol*. (2023) 6:20. doi: 10.1038/s42003-022-04406-5

55. Lin H, An Y, Tang H, Wang Y. Alterations of bile acids and gut microbiota in obesity induced by high fat diet in rat model. *J Agric Food Chem*. (2019) 67:3624–32. doi: 10.1021/acs.jafc.9b00249

56. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertility sterility*. (1997) 67:817–21. doi: 10.1016/S0015-0282(97)81391-X

57. Vercellini P, Fedele L, Aimi G, Pietropaolo G, Consonni D, Crosignani PG. Association between endometriosis stage, lesion type, patient characteristics and severity of pelvic pain symptoms: a multivariate analysis of over 1000 patients. *Hum Reprod*. (2007) 22:266–71. doi: 10.1093/humrep/del339

58. Johnson NP, Hummelshoj L, Adamson GD, Keckstein J, Taylor HS, Abrao MS, et al. World Endometriosis Society consensus on the classification of endometriosis. *Hum Reprod*. (2017) 32:315–24. doi: 10.1093/humrep/dew293

59. Bishop CV, Takahashi DL, Luo F, Sidener H, Martin LD, Gao L, et al. The combined impact of testosterone and Western-style diet on endometriosis severity and progression in rhesus macaquesdagger. *Biol Reprod*. (2023) 108:72–80. doi: 10.1093/biolre/ioac183

60. Kim TH, Bae N, Kim T, Hsu AL, Hunter MI, Shin JH, et al. Leptin stimulates endometriosis development in mouse models. *Biomedicines*. (2022) 10(9), 2160. doi: 10.3390/biomedicines10092160

61. Heard ME, Melnyk SB, Simmen FA, Yang Y, Pabona JM, Simmen RC. High-fat diet promotion of endometriosis in an immunocompetent mouse model is associated with altered peripheral and ectopic lesion redox and inflammatory status. *Endocrinology*. (2016) 157:2870–82. doi: 10.1210/en.2016-1092

62. Heard-Lipsmeyer ME, Alhallak I, Simmen FA, Melnyk SB, Simmen RCM. Lesion genotype modifies high-fat diet effects on endometriosis development in mice. *Front Physiol*. (2021) 12:702674. doi: 10.3389/fphys.2021.702674

63. Dang AT, Marsland BJ. Microbes, metabolites, and the gut-lung axis. *Mucosal Immunol*. (2019) 12:843–50. doi: 10.1038/s41385-019-0160-6

64. Machairiotis N, Vasilakaki S, Thomakos N. Inflammatory mediators and pain in endometriosis: A systematic review. *Biomedicines*. (2021) 9(1), 54. doi: 10.3390/biomedicines9010054

65. Wan Y, Wang F, Yuan J, Li J, Jiang D, Zhang J, et al. Effects of dietary fat on gut microbiota and faecal metabolites, and their relationship with cardiometabolic risk factors: a 6-month randomised controlled-feeding trial. *Gut*. (2019) 68:1417–29. doi: 10.1136/gutjnl-2018-317609

66. Fan S, Chen S, Lin L. Research progress of gut microbiota and obesity caused by high-fat diet. *Front Cell Infect Microbiol*. (2023) 13:1139800. doi: 10.3389/fcimb.2023.1139800

67. Velazquez KT, Enos RT, Bader JE, Sougiannis AT, Carson MS, Chatzistamou I, et al. Prolonged high-fat-diet feeding promotes non-alcoholic fatty liver disease and alters gut microbiota in mice. *World J Hepatol*. (2019) 11:619–37. doi: 10.4254/wjh.v11.i8.619

68. Shin NR, Whon TW, Bae JW. Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol*. (2015) 33:496–503. doi: 10.1016/j.tibtech.2015.06.011

69. Zheng Z, Lyu W, Ren Y, Li X, Zhao S, Yang H, et al. Allobaculum involves in the modulation of intestinal ANGPTLT4 expression in mice treated by high-fat diet. *Front Nutr*. (2021) 8:690138. doi: 10.3389/fnut.2021.690138

70. Gill PA, Inniss S, Kumagai T, Rahman FZ, Smith AM. The role of diet and gut microbiota in regulating gastrointestinal and inflammatory disease. *Front Immunol*. (2022) 13:866059. doi: 10.3389/fimmu.2022.866059

71. Greaves E, Collins F, Esnal-Zufiaurre A, Giakoumelou S, Horne AW, Saunders PT. Estrogen receptor (ER) agonists differentially regulate neuroangiogenesis in peritoneal endometriosis via the repellent factor SLIT3. *Endocrinology*. (2014) 155:4015–26. doi: 10.1210/en.2014-1086

72. Greaves E, Cousins FL, Murray A, Esnal-Zufiaurre A, Fassbender A, Horne AW, et al. A novel mouse model of endometriosis mimics human phenotype and reveals insights into the inflammatory contribution of shed endometrium. *Am J Pathol*. (2014) 184:1930–9. doi: 10.1016/j.ajpath.2014.03.011

73. Greaves E, Horne AW, Jerina H, Mikolajczak M, Hilferty L, Mitchell R, et al. EP2 receptor antagonism reduces peripheral and central hyperalgesia in a preclinical mouse model of endometriosis. *Sci Rep*. (2017) 7:44169. doi: 10.1038/srep44169

74. Cousins FL, Murray A, Esnal A, Gibson DA, Critchley HO, Saunders PT. Evidence from a mouse model that epithelial cell migration and mesenchymal-epithelial transition contribute to rapid restoration of uterine tissue integrity during menstruation. *PloS One*. (2014) 9:e86378. doi: 10.1371/journal.pone.0086378

75. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. (2013) 41:D590–6. doi: 10.1093/nar/gks1219

76. Kuczynski J, Stombaugh J, Walters WA, Gonzalez A, Caporaso JG, Knight R. Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Curr Protoc Bioinf Chapter*. (2011) 10:10 7 1–10 7 20. doi: 10.1002/0471250953.bi1007s36

77. Lozupone C, Hamady M, Knight R. UniFrac–an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinf*. (2006) 7:371. doi: 10.1186/1471-2105-7-371

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 19 March 2024
DOI 10.3389/fendo.2024.1359182

OPEN ACCESS

EDITED BY
Iveta Yotova,
Medical University of Vienna, Austria

REVIEWED BY
Radosław Bernard Maksym,
Centre for Postgraduate Medical Education, Poland
Gabriele Centini,
University of Siena, Italy

*CORRESPONDENCE
Piotr Laudanski
piotr.laudanski@wum.edu.pl

†Memorable

RECEIVED 20 December 2023
ACCEPTED 04 March 2024
PUBLISHED 19 March 2024

CITATION
Zyguła A, Sankiewicz A, Sakowicz A, Dobrzyńska E, Dakowicz A, Mańka G, Kiecka M, Spaczynski R, Piekarski P, Banaszewska B, Jakimiuk A, Issat T, Rokita W, Młodawski J, Szubert M, Sieroszewski P, Raba G, Szczupak K, Kluza T, Kluza M, Pierzyński P, Wojtyla C, Lipa M, Warzecha D, Wielgos M, Cendrowski K, Gorodkiewicz E and Laudanski P (2024) Is the leptin/BMI ratio a reliable biomarker for endometriosis? *Front. Endocrinol.* 15:1359182. doi: 10.3389/fendo.2024.1359182

Is the leptin/BMI ratio a reliable biomarker for endometriosis?

Aleksandra Zyguła[1], Anna Sankiewicz[2], Agata Sakowicz[3], Ewa Dobrzyńska[4], Agnieszka Dakowicz[5], Grzegorz Mańka[6], Mariusz Kiecka[6], Robert Spaczynski[7,8], Piotr Piekarski[9], Beata Banaszewska[10], Artur Jakimiuk[11,12], Tadeusz Issat[13], Wojciech Rokita[14,15†], Jakub Młodawski[14,15], Maria Szubert[16,17], Piotr Sieroszewski[16,18], Grzegorz Raba[19,20], Kamil Szczupak[19,20], Tomasz Kluza[21], Marek Kluza[21], Piotr Pierzyński[1], Cezary Wojtyla[1,22], Michal Lipa[23], Damian Warzecha[1,24,25], Miroslaw Wielgos[23,26,27], Krzysztof Cendrowski[4], Ewa Gorodkiewicz[2] and Piotr Laudanski[1,4,22*]

[1]OVIklinika Infertility Center, Warsaw, Poland, [2]Bioanalysis Laboratory, Faculty of Chemistry, University of Bialystok, Bialystok, Poland, [3]Department of Medical Biotechnology, Medical University of Lodz, Lodz, Poland, [4]Chair and Department of Obstetrics, Gynecology and Gynecological Oncology, Medical University of Warsaw, Warsaw, Poland, [5]Department of Rehabilitation, Medical University of Bialystok, Bialystok, Poland, [6]Angelius Provita Hospital, Katowice, Poland, [7]Center for Gynecology, Obstetrics and Infertility Treatment Pastelova, Poznan, Poland, [8]Collegium Medicum, University of Zielona Gora, Zielona Gora, Poland, [9]Gynecological Obstetric Clinical Hospital of Poznan University of Medical Sciences, Minimally Invasive Gynecological Surgery, Poznan, Poland, [10]Chair and Department of Laboratory Diagnostics, Poznan University of Medical Sciences, Poznan, Poland, [11]Department of Reproductive Health, Institute of Mother and Child in Warsaw, Warsaw, Poland, [12]Department of Obstetrics and Gynecology, Central Clinical Hospital of the Ministry of Interior, Warsaw, Poland, [13]Department of Obstetrics and Gynecology, Institute of Mother and Child in Warsaw, Warsaw, Poland, [14]Collegium Medicum, Jan Kochanowski University in Kielce, Kielce, Poland, [15]Clinic of Obstetrics and Gynecology, Provincial Combined Hospital in Kielce, Kielce, Poland, [16]Department of Gynecology and Obstetrics, Medical University of Lodz, Lodz, Poland, [17]Department of Surgical Gynecology and Oncology, Medical University of Lodz, Lodz, Poland, [18]Department of Fetal Medicine and Gynecology, Medical University of Lodz, Lodz, Poland, [19]Clinic of Obstetric and Gynecology in Przemysl, Przemysl, Poland, [20]Medical College of Rzeszow University, Rzeszow, Poland, [21]Department of Gynecology, Gynecology Oncology and Obstetrics, Institute of Medical Sciences, Medical College of Rzeszow University, Rzeszow, Poland, [22]Women's Health Research Institute, Calisia University, Kalisz, Poland, [23]Departament of Obstetrics and Perinatology National Medical Institute of the Ministry of Interior and Administration, Warsaw, Poland, [24]City South Hospital, Warsaw, Warsaw, Poland, [25]Department of Biomedical Fundamentals of Development and Sexology, Faculty of Education, University of Warsaw, Warsaw, Poland, [26]Premium Medical Clinic, Warsaw, Poland, [27]Medical Faculty, Lazarski University, Warsaw, Poland

Background: The aim of this study was to analyze the concentration of leptin in peritoneal fluid and plasma and to assess their role as potential biomarkers in the diagnosis of endometriosis.

Materials & methods: Leptin adjusted for BMI (leptin/BMI ratio) was measured using surface plasmon resonance imaging (SPRI) biosensors. Patients with suspected endometriosis were included in the study. Plasma was collected from 70 cases, and peritoneal fluid from 67 cases. Based on the presence of endometriosis lesions detected during laparoscopy, patients were divided into a study group and a control group (patients without endometriosis).

Results: Leptin/BMI ratio in plasma did not differ between women with endometriosis and the control group (0.7159 ± 0.259 vs 0.6992 ± 0.273, p= 0,7988). No significant differences were observed in peritoneal leptin/BMI ratio

levels in patients with and without endometriosis (0.6206 ± 0.258 vs 0.6215 ± 0.264, p= 0,9896). Plasma and peritoneal leptin/BMI ratios were significantly lower in women with endometriosis - related primary infertility compared to women with endometriosis without primary infertility (0.640 ± 0.502 vs 0.878 ± 0.623, $p < 0.05$). The difference was observed in case of primary infertility, but not in terms of the secondary one. No significant differences were noted between leptin/BMI ratio in the proliferative phase and the secretory phase (0.716 ± 0.252 vs 0.697 ± 0.288, p= 0,7785).

Conclusion: The results of present study do not support the relevance of leptin concentration determination as a biomarker of the endometriosis. Due to the limited number of samples in the tested group, further studies are needed to confirm its role.

KEYWORDS

endometriosis, leptin, plasma, peritoneal fluid, infertility

Introduction

Endometriosis is a common chronic gynecological disease characterized by the presence of endometrium – like tissue outside the uterine cavity (1). Symptomatic endometriosis is estimated to affect 5 - 15% of women, with around half of women experiencing fertility problems (2–4). The varying extent of the disease (peritoneal endometriosis, ovarian endometrioma, deep infiltrating endometriosis) contributes to a wide array of clinical symptoms such as infertility, chronic pelvic pain, dysmenorrhea, dyspareunia, anxiety, and depression (5). The nonspecific nature of these symptoms makes early diagnosis of endometriosis challenging, often leading to diagnostic delays of 7-12 years (6–8). Consequently, extensive research efforts are underway to identify reliable and non-invasive biomarkers for this disease.

Despite numerous studies have been conducted, the potential pathogenesis of endometriosis remains elusive and requires further elucidation. Sampson's widely recognized theory suggests that retrograde menstruation is a contributing factor (9). However, while approximately 90% of menstruating women experience retrograde menstruation, only around 10% develop endometriosis from this backward flow of menstrual fluid (10). Other theories emphasize the involvement of genetic, environmental, and immunological factors in predisposing the development of the disease (3). Disturbed immunoregulatory mechanisms, accompanied by various inflammatory markers such as immune cells, cytokines, chemokines, metalloproteinases, cathepsin, and other immunologically-related substances, have been implicated in the development, invasion, and angiogenesis of ectopic lesions (11–18). However, none of these theories fully explain the complexity underlying the pathogenesis of endometriosis.

Relatively little is known about the molecular background of metabolic processes involved in the development of this pathology (19). More recently, molecule such as leptin, which is known to regulate long-term energy balance, has been evaluated as a potential biomarker for endometriosis. Leptin a product of *ob* gene, is a peptide hormone produced by white adipose tissue (WAT). It is also synthesized by other tissues, including the endometrium, placenta and ovary (20–24). Leptin concentration are positively correlated with WAT mass (21). In the plasma, this hormone exists in two forms, free or bound to leptin-binding proteins. Its actions are mediated via binding to leptin receptors (LepRb/ObR, member of class I cytokine receptor family), located in the hypothalamus and various peripheral tissues (21, 22, 25, 26). Leptin was initially described as a key regulator of food intake and modulation of energy expenditure. Subsequently it has been shown, that leptin exert pleiotropic actions by regulating immune homeostasis, promoting neoangiogenesis and reproduction, so it is thought that this molecule may also play a role in pathogenesis of endometriosis (3, 5, 21, 26, 27).

Studies revealed the direct positive correlation between leptin levels and obesity, measured by Body Mass Index (BMI) (28). For this reason, in order to eliminate factors that may influence leptin levels, such as body weight, leptin/BMI ratio was used for measurements.

Interestingly, while leptin, primarily produced by adipocytes, might suggest a higher occurrence of endometriosis in women with an increased BMI, there exists an inverse correlation between BMI and the prevalence of endometriosis (29, 30). Contrary to the assumption, obesity does not act as a protective factor against endometriosis; instead, an elevated BMI may be associated with more severe forms of the disease. This may be due to the role played by leptin, which not only regulates energy balance but also exhibits pro-inflammatory and angiogenic function (31).

The aim of this study was to compare leptin/BMI ratio in biological fluids (plasma and peritoneal fluid) between women

diagnosed with endometriosis and a control group, using SPRi biosensors. Furthermore, the study assessed the potential role of leptin as a biomarker of endometriosis.

Surface plasmon resonance imaging (SPRi) is at the forefront of optical sensing. This technique real-time, direct, and 'label-free' detection and monitoring of biomolecular events. It involves a plasmon resonance-imaging-supporting metal surface which combines light energy with an electromagnetic field on cells and surface-associated leptin. The main advantage of the SPRi is specificity of molecule determination as well as highly sensitive measurement of the refractive index. This characteristic makes it ideal for the quantification of biomolecules, such as different types of proteins (32–38). Applying the stationary SPRi version in a model investigation and in the determination of various biomarkers in real clinical samples has demonstrated that this technique is suitable for use without signal enhancement or analyte preconcentration (13). Recent reviews highlight SPRi detection as the most promising among surface plasmon-based techniques (32).

Materials and methods

Study population

This multicenter, cross-sectional study was conducted across eight Departments of Obstetrics and Gynecology in Poland between 2018 and 2019. Comprehensive recruitment details are provided in our latest publication (39).

All participants, both patients with endometriosis and controls, provide written informed consent, and the study was approved by the Ethics Committee of the Medical University of Warsaw (KB/223/2017).

The study group included individuals aged between 19 and 45 years, who were qualified for planned laparoscopic surgeries due to one or more non-malignant conditions, including infertility, chronic pelvic pain syndrome, ovarian cysts, or suspicion of endometriosis. Exclusion criteria encompassed irregular menstruations cycles (less than 25 days or more than 35), recent hormonal treatment within three months preceding laparoscopy, previous and/or current pelvic inflammatory disease, uterine fibroids, polycystic ovary syndrome, autoimmune comorbidities, malignancies, or any previous history of prior surgical treatment. All patients underwent gynecological examination and vaginal ultrasonography before being referred for surgery. Routine additional radiological examinations to assess extraperitoneal endometriosis were not performed as part of the standard procedure. Each patient was assessed based on the revised American Fertility Society (AFS) classification of endometriosis, complemented by histological examination of collected specimens. Furthermore, all patients completed a World Endometriosis Research Foundation (WERF) clinical questionnaire. As controls, we recruited patients without visible endometriosis during laparoscopy.

The cycle phase was determined based on the last menstrual period and the average length of the menstrual cycle. To ascertain the phases of the menstrual cycle in both women with and without endometriosis, histological dating of eutopic endometrial samples was performed concurrently with the collection of pathological lesions.

The details of the collection and structure of study groups have already been published (39). Briefly, trained gynecologists performed diagnostic laparoscopy on all patients, conducting a comprehensive examination of the uterus, fallopian tubes, ovaries, pouch of Douglas, and pelvic peritoneum. Peritoneal fluid (PF) was aspirated using a Veress needle under direct visualization immediately upon the laparoscope's insertion to prevent blood contamination. The procedure meticulously followed the standard operating procedures outlined by the Endometriosis Phenome and Biobanking Harmonization Project (40). At all centers, the collected PF underwent centrifugation at 1000g for 10 minutes at 4°C. The resulting supernatant was transferred to fresh 10 ml Sarstedt tubes and divided into 500 ml aliquots. Blood samples were obtained before laparoscopy, always prior to anesthesia, using EDTA 10 ml Sarstedt tubes. Consistent tube types were utilized for both blood and PF across all centers. The time elapsed between sample collection (both PF and plasma) and processing was kept under 45 minutes. Blood samples were centrifuged at 2500g for 10 minutes at 4°C, and then plasma samples were divided into 500 µl aliquots. PF and plasma were stored at -80°C until further use. All samples were transported on dry ice to the Department of Obstetrics and Gynecology in Warsaw, serving as the coordinating center for the study.

Patients diagnosed with endometriosis during laparoscopy were allocated to the corresponding endometriosis stage subgroups (I–IV). Leptin concentration assessment involved the collection of plasma and peritoneal fluid. The procedures adhered to the Endometriosis Phenome and Biobanking Harmonization Project standard operating procedures (40). After exclusion of the outlier results, the final analysis included 70 plasma samples (40 from patients with and 30 from those without) and 67 samples of the peritoneal fluid (38 from patients with and 29 from those without).

Method of measuring leptin concentration

In present study leptin concentrations in plasma and peritoneal fluid were measured by biosensor SPRI (Surface Plasmon Resonance Imaging) as described in detail by Sankiewicz et al. (41). The base of the biosensor was a gold plate covered with a separating mask of polymer. The first step in preparing the biosensor was to create a cysteamine layer on gold (cysteamine hydrochloride, Sigma-Aldrich, Stieinhem, Germany). The next step was the binding of the leptin capture element, that is rabbit anti-leptin antibody (concertation= 60 ng mL1. pH = 7.4), (Abcam, Cambridge, United Kingdom). A leptin-specific rabbit antibody was immobilized by the formation of a covalent bond between the amine group of cysteamine and the carboxy group of the heavy chain of the antibody. The antibody was activated with the N-ethyl-N'-(3-dimethylaminopropyl) carbodimide (EDC)/N-hydroxysuccimide (NHS) (Sigma-Aldrich, Steinheim, Germany) mixture in carbonate buffer, and then 3 µl of the antibody solution prepared in this way was applied to the active sites (cysteamine layer). The prepared plate was placed in an incubator at 37°C. After 1 hour, the active sites of the biosensor were washed with distilled water and HBS-ES solution. To eliminate the risk of non-specific adsorption, a BSA solution (C = 1 mg/ml) was used.

The biosensor prepared in this way, the biosensor was capable of capturing leptin from a solution. The standard solutions of leptin (Abcam, Cambridge, United Kingdom), samples plasma but also peritoneal fluid (5 times diluted with PBS buffer) with a volume of 3 μL were applied on the active sites of the biosensor. After 10 minutes, the surface of the active sites of the biosensor was rinsed with distilled water and HBS-ES solution to remove excess unbound protein.

SPRI measurements were carried out on an apparatus constructed in our laboratory. The SPRi apparatus is composed of a optical part (laser HE-Ne, system of polarizers, lenses, mirror and prism) and detection part (CCD camera connected to computer). A previously prepared biosensor was placed on the prism of the SPRi device and the appropriate angle of incidence of light was selected. The SPR signal was measured at the selected fixed SPR angle. Surface plasmon resonance in the image version examines the change in the intensity of the reflected monochromatic and p-polarized light after applying successive layers of the biosensor. The SPRi signal is proportional to the immobilized mass on the biosensor surface. Signal SPRI are recorded using a CCD camera in the form of images that are subjected to mathematical analysis using a program ImageJ software (version 1.53, National Institutes of Health, NIH). Images are taken twice, for the antibody layer and the analyte layer. Concentrations for the leptin were determined on the basis of calibration curve (in range 0.1–5.0 ng mL1) considering the appropriate dilutions.

Statistical analyses

Statistical analysis was performed using the statistical package Statistica 13 (TIBCO Software Inc.; 2017). In this study, we assessed variables that adhered to a normal distribution, including age, leptin levels in plasma and peritoneal fluid, and the leptin/BMI ratio in both fluids, confirmed by the Shapiro-Wilk test. Differences between two groups for these variables were analyzed using an independent t-test. Comparisons of leptin levels in plasma and peritoneal fluid within subgroups were conducted using a paired t-test. Results are expressed as mean ± SD.

For variables that did not conform to a normal distribution, specifically BMI, the Mann-Whitney U test was used to assess the significance of differences between groups. In these cases results are expressed as median ± IQR.

When examining differences among three or more groups, we employed an analysis of variance (ANOVA), followed by Duncan's *post hoc* test for detailed group comparisons. The effect size for these analyses was estimated using the partial eta squared (η^2part.) measure.

The contrast analysis was used to highlight patterns of differences between the control and endometriosis groups according to three status of infertility (no infertility, primary infertility and secondary infertility). Contrasts were introduced for each factor separately as follows: Group (1,-1) Infertility (1,-2,1). To explore the correlations between variables, we applied either the simple correlation coefficient (r_{xy}) or Spearman's rank correlation coefficient (r_S), depending on their distribution of values. For qualitative features, significance of relationships were assessed using Fisher's exact 2-tailed test, and the effect size for these analyses was estimated with Yule's coefficient (φ). A significance level $\alpha = 0.05$ was adopted in this study.
was used for estimate p-value

Results

The 70 patients i.e. 40 women with diagnosed endometriosis and 30 controls were enrolled to the study. The age of patients ranged from 19 to 45 years, with a mean body mass index (BMI)of 22.3 kg/2 in the study group and 21.58 kg/m^2 in the control group. No statistically significant difference according age and BMI between groups was found.

According to rAFS classification 27,5% of women were diagnosed as I stage, 27,5% as II stage, 27,5% as III stage, and 17,5% of women as IV stage of endometriosis.

The patient clinical characteristics is presented in Table 1.

The mean plasma leptin concentration (ng/mL) were comparable in two groups, 15.578 ± 5.162 ng/mL in women with endometriosis, and 15.530 ± 6.404 ng/mL in the control group.

The levels of leptin in peritoneal fluid (ng/ml) did not differ significantly between the groups, measuring 13.615 ± 5.322 in the study group and 13.576 ± 6.209 in the control group, respectively.

Leptin/BMI ratio in body fluids

Leptin/BMI ratio in plasma did not show statistical differences between women with endometriosis and the control group, nor between

TABLE 1 Baseline characteristics of participants and leptin levels.

Demographic and anthropometric variables[1]	Endometriosis	Controls	*p-value*
Age [yr] (M ± SD; n)	31.60 ± 5.33; 40	30.87 ± 7.06; 30	0.6459
Body-mass index [kg/m^2] (Me ± IQR)	22.23 ± 4.20; 40	21.58 ± 5.38; 28	0.7178
rAFS stage of disease %; N			
I	27.50;11	-	-
II	27.50;11	-	-
III	27.50;11	-	-
IV	17.50;7	-	-
Menstrual cycle phase (n; %)			
Proliferative	24; 60%	21; 70%	0.4552
Secretive	16; 40%	9; 30%	
Leptin levels			
Plasma (ng/mL) (M ± SD; n)	15.578 ± 5.162; 40	15.530 ± 6.404; 30	0.9721
Peritoneal fluid (ng/mL) (M ± SD; n)	13.615 ± 5.322; 38	13.576 ± 6.209; 29	0.9785

patients with primary infertility (Table 2, Figure 1). Additionally, the interaction between these factors was not statistically significant, and the effect size was small, accounting for only 5% of the variability.

Similarly, concerning the leptin/BMI ratio in peritoneal fluid (PF), no significant differences were observed between women with endometriosis and the control group (Table 3). However, a statistically significant interaction was noted. Notably, within the primary infertility subgroup, the level of leptin/BMI in PF was significantly lower in the endometriosis group compared to the control group (Table 3, Figure 2). Conversely, in the subgroup without infertility, the level of leptin/BMI in PF was marginally higher in the study group than in the control group (Table 3, Figure 2).

Leptin/BMI ratio in endometriosis – associated infertility

Among women with endometriosis, 55% (n=22) were diagnosed with infertility (primary or secondary). In the control group, this percentage was 23% (n=7). A statistically significant relationship was observed between infertility and the health condition status, with a p-value of 0.0136. Additionally, a moderate relationship with a Yule's coefficient (φ) of 0.31 was found (Table 4).

The contrast analysis revealed statistically significant differences in trends (quadratic polynomial) between the endometriosis group and the control group, both in the distribution of mean leptin/BMI ratio in plasma and in PF (Table 5, Figures 3, 4). In endometriosis cases without infertility and with secondary infertility, the means were higher compared to endometriosis cases with primary infertility. Conversely, in the control group, the highest level of Leptin/BMI ratio was observed in the group with primary infertility, followed by the other subgroups. These findings indicate varying patterns of average leptin levels depending on fertility status and the type of infertility, which could contribute to a better understanding within the broader context of existing research on the subject. Nevertheless, it is crucial to note that these results should be interpreted with caution due to the relatively small size of the groups.

TABLE 2 Leptin/BMI ratio in plasma.

Factors	Level	M	n	SD	SE	Min	Max	F	*p*-value	$\eta^2_{part.}$
Group	Endometriosis	0.716	40	0.259	0.041	0.275	1.376	0.59	0.4440	0.01
	Control	0.699	28	0.273	0.052	0.289	1.372			
Primary infertility	Yes	0.674	21	0.272	0.059	0.275	1.376	0.13	0.7244	<0.01
	No	0.724	47	0.260	0.038	0.289	1.372			
Interaction										
Endometriosis	Yes	0.640	18	0.278	0.066	0.275	1.376	3.56	0.0639	0.05
	No	0.778	22	0.231	0.049	0.343	1.254			
Control	Yes	0.878	3	0.103	0.059	0.767	0.969			
	No	0.678	25	0.280	0.056	0.289	1.372			

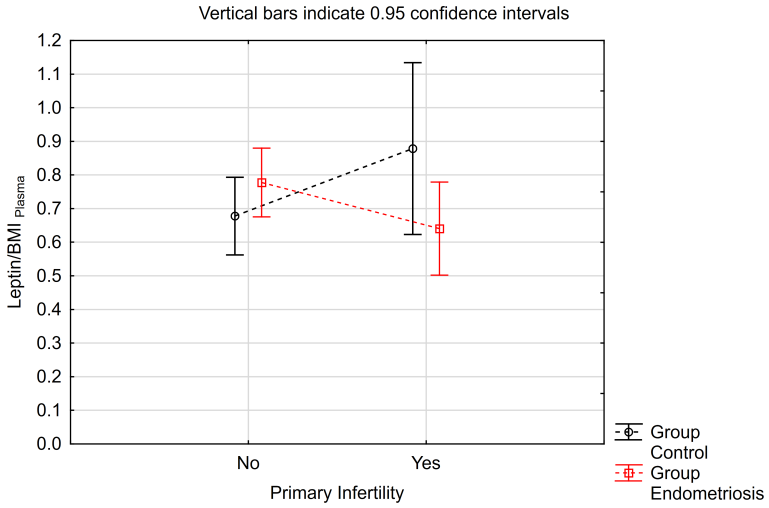


FIGURE 1
The means of leptin/BMI ratio in plasma depending of endometriosis and primary infertility.

TABLE 3 Leptin/BMI ratio in peritoneal fluid.

Factors	Level	M	n	SD	SE	Min	Max	F	*p-value*	$\eta^2_{part.}$
Group	Endometriosis	0.621	38	0.258	0.042	0.117	1.185	0.13	0.7173	<0.01
	Control	0.621	27	0.264	0.051	0.082	1.150			
Primary infertility	Yes	0.580	21	0.247	0.054	0.117	1.150	1.02	0.3169	0.02
	No	0.641	44	0.264	0.040	0.082	1.185			
Interaction										
Endometriosis	Yes#	0.541	18	0.225	0.053	0.117	0.921	4.36	0.0409	0.07
	No	0.693	20	0.271	0.061	0.155	1.185			
Control	Yes#	0.813	3	0.173	0.100	<0.001	1.150			
	No	0.597	24	1.511	0.308	<0.001	1.041			

M, mean; n, numbers; SD, standard deviation; SE, standard error; $\eta^2_{part.}$- eta_squared partial.
Means marked with # differed p<0.05 (Duncan's test).

Leptin correlation

A positive and statistically significant correlation of moderate strength was found between the concentration of leptin in peritoneal fluid and plasma within the study group (r_{xy}=0.39; p<0.05; Figures 5, 6). Conversely, no such association was observed in the control group (r_{xy}=0.05; p>0.05; Figures 5, 6).

Leptin/BMI ratio in reference to endometriosis stage, presence of endometrioma, menstrual cycle phase

There were no significant differences observed in the leptin/BMI ratio in plasma, peritoneal fluid leptin concentration, based on the endometriosis stage, presence of endometrioma, or menstrual cycle phase (Table 6).

The correlations between rAFS stage of disease & leptin/BMI ratio in plasma and rAFS stage of disease & leptin/BMI ratio in peritoneal fluid (PF) were not found (Figures 7, 8).

Discussion

In the present study, we found that plasma and peritoneal leptin concentrations did not differ between the studied groups. Furthermore, after adjusting for BMI in both the endometriosis and control groups, no significant difference was observed in leptin level between the groups, whether analyzing peritoneal or serum leptin concentrations.

These findings align with Wertel et al.'s study, supporting our thesis (42). However, Rathore et al. reported contrasting results, noting significantly higher leptin levels in peritoneal fluid among women with endometriosis compared to the control group, while serum leptin levels did not differ between the groups (43).

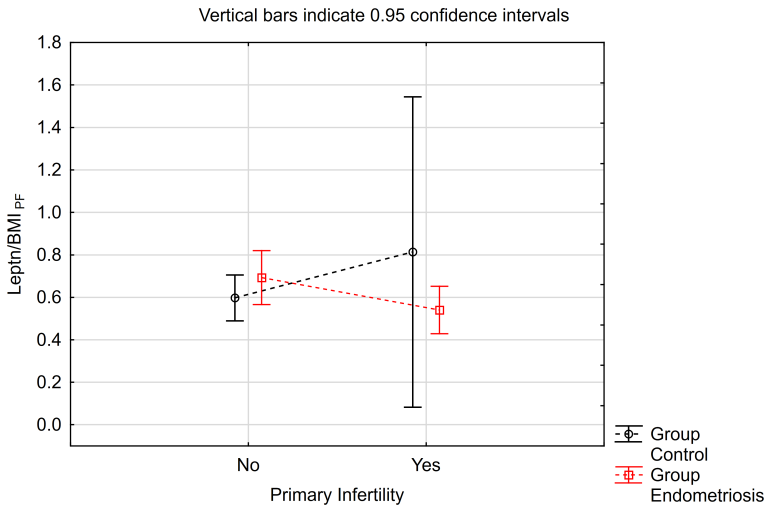


FIGURE 2
The means of leptin/BMI ratio in peritoneal fluid depending of endometriosis and primary infertility.

10.3389/fendo.2024.1359182

TABLE 4 Frequency distributions and percentages of fertility assessments in respective groups.

Group	Infertility		Σ	*p-value*	ϕ
	Yes	No			
Endometriosis, n(%)	22 (55.0%)	18 (45.0%)	40 (57.1%)	**0.0136**	0,32
Control, n(%)	7 (23.3%)	23 (76.7%)	30 (42.9%)		
Total, n (%)	29 (41.4%)	41 (58.6%)	70 (100%)		

Fisher's exact 2-tailed test was used for estimate p-value.
Bold values was to emphasize the statistical significance.

TABLE 5 Leptin/BMI ratio - contrast analysis (E vs C) x (trendy quadratic polynomial).

	Group	Infertility			F	*p-value*
		No M ± SE; n	Primary M ± SE; n	Secondary M ± SE; n		
plasma	Endometriosis	0.731 ± 0.051; 18	0.64 ± 0.066; 18	0.985 ± 0.091; 4	4.02	**0.0492**
	Control	0.667 ± 0.063; 22	0.878 ± 0.059; 3	0.753 ± 0.042; 3		
peritoneal fluid	Endometriosis	0.678 ± 0.073; 16	0.541 ± 0.053; 18	0.751 ± 0.084; 4	5.15	**0.0270**
	Control	0.613 ± 0.056; 21	0.813 ± 0.170; 3	0.490 ± 0.160; 3		

Bold values was to emphasize the statistical significance.

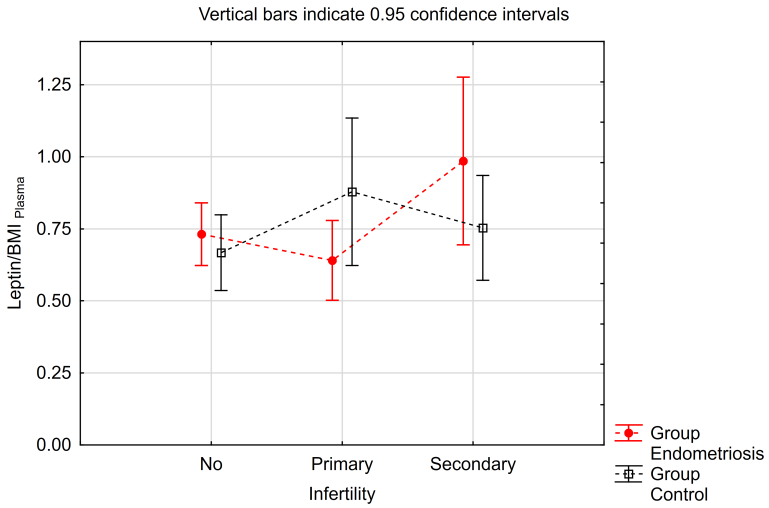


FIGURE 3
The means of leptin/BMI ratio in plasma depending of endometriosis and infertility.

Interestingly, in a previous clinical study positive correlation between PF and serum leptin concentrations was found among fertile patients with endometriosis (42). Moreover, another research study found no significant difference in peritoneal leptin levels between the endometriosis-associated infertility group and those with fallopian-associated infertility (44). These results are consistent with our study, which revealed that a subgroup of women with endometriosis related infertility had a significantly lower concentration of leptin adjusted for BMI in PE compared to women with endometriosis, but without primary infertility. However, it's important to note that the subgroups of patients with infertility in our study were small, so result may not lead to reliable premise. In order to overcome this limitation, further cooperation and research by a multi-center working group on endometriosis is planned. Hence, at present, we can only hypothesize that leptin might contribute to pelvic inflammation in endometriosis, potentially altering the local microenvironment of the peritoneal cavity, yet its direct impact on endometriosis-associated infertility is uncertain.

As previously mentioned, studies evaluating leptin levels in serum and peritoneal fluid among patients with endometrioma have shown conflicting results, reporting either elevated levels or no

10.3389/fendo.2024.1359182

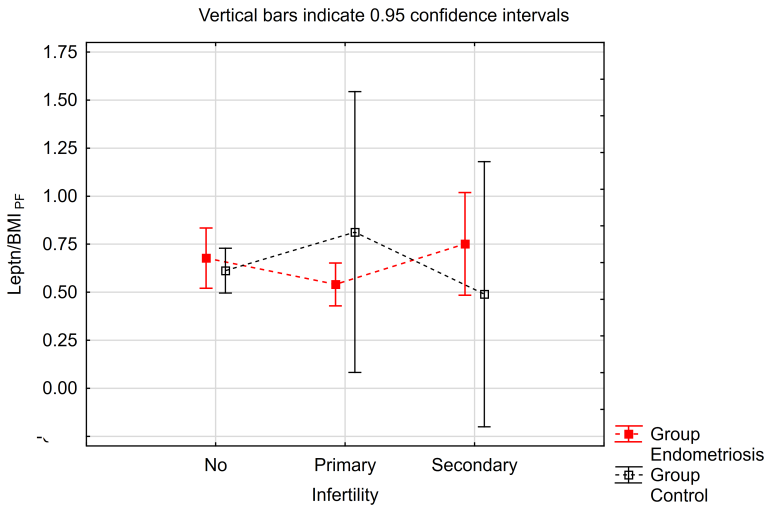


FIGURE 4
The means of leptin/BMI ratio in PE depending of endometriosis and infertility.

significant changes. In our study, we found no significant difference in both serum and PF leptin concentrations concerning the presence of endometrioma. Consistent, with our findings, Zendron et al. noted comparable leptin concentrations between patients with endometrioma and those in the control group (45).

However, recent research has revealed higher peritoneal fluid leptin concentrations among women displaying peritoneal lesions compared to those with endometrioma as the only finding (46). These differences observed among diverse phenotypes of the disease such as peritoneal versus ovarian endometriosis, suggest a potential role of leptin in the development of peritoneal endometriosis. This implies that different biochemical phenomena might be involved in the pathogenesis of the ovarian form of the disease.

Fluctuations in leptin concentration during the menstrual cycle remain a subject of controversy. Many studies have noted higher leptin levels in the midluteal phase compared to the follicular phase (47, 48). It is suggested that estradiol and progesterone may play an essential role in regulating leptin release during cycle. Ajala et al. demonstrated a significant increase in serum leptin levels on both day 14 (ovulatory phase) and day 21 (luteal phase) of the menstrual cycle (47). In comparison, another study noted higher leptin concentrations during midluteal phase (49), with observed positive correlations between leptin values and estradiol or progesterone levels (50). Conversely, our study findings indicated no significant changes in leptin/BMI ratio throughout the menstrual cycle. These result align with Stock et al.'s study, which similarly found no correlation between leptin values and estradiol or progesterone levels (51). Furthermore, Capobianco's research revealed no significant differences in leptin levels during the menstrual cycle (52). It needs to be underlined that the

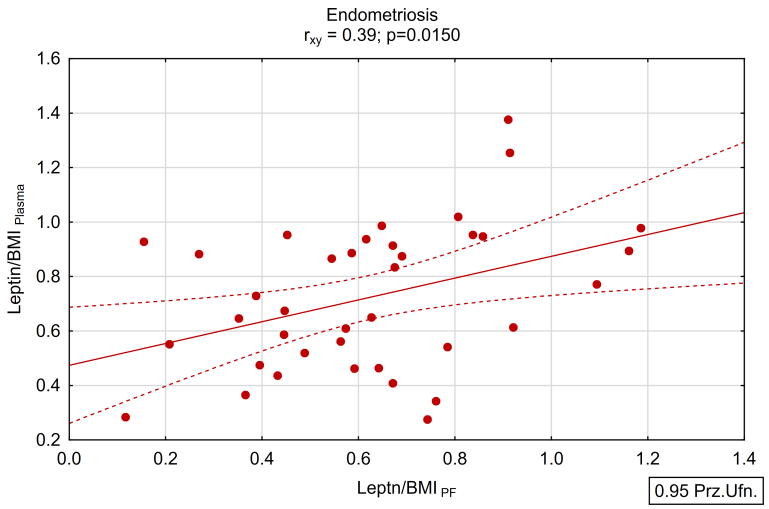


FIGURE 5
Correlations between plasma and peritoneal fluid Leptin/BMI ratio in endometriosis group (N=38).

10.3389/fendo.2024.1359182

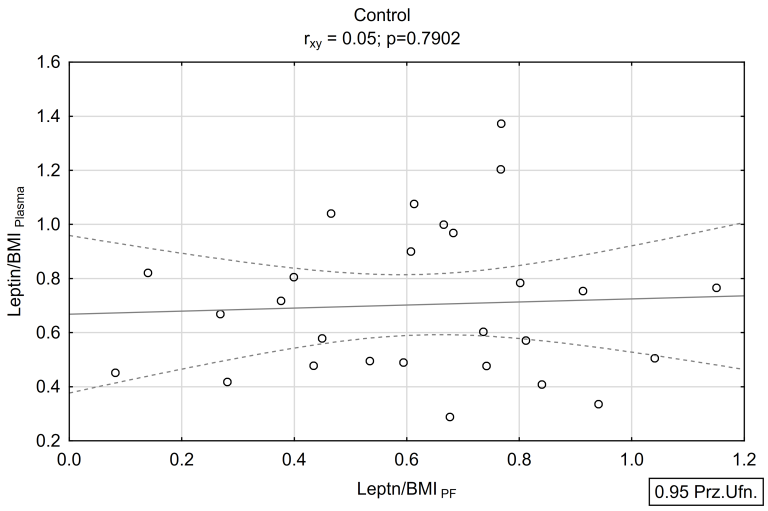


FIGURE 6
Correlations between plasma and peritoneal fluid Leptin/BMI ratio in control group (N=27).

aforementioned studies primarily focused on either healthy women or women with infertility problems, but not specifically on those with endometriosis. Considering numerous clinical reports highlighting aberrant progesterone signaling in patients with endometriosis, it is plausible that such regulation might be altered or missed in the context endometriosis (53).

Worth to note is that, a positive and statistically significant correlation of moderate strength was observed between the

TABLE 6 Leptin/BMI ratio according to the stage of endometriosis, endometrioma and cycle phase.

Factors		M	SD	n	Min	Max	F	*p*-value	$\eta^2_{part.}$
rAFS stage of disease									
Plasma (N=39)	1	0.64	0.33	11	0.28	1.38	0,99	0,4097	0,08
	2	0.73	0.21	11	0.44	1.02			
	3	0.69	0.19	11	0.41	0.98			
	4	0.85	0.30	7	0.28	1.25			
Peritoneal fluid (N=37)	1	0.56	0.22	11	0.21	0.91	0,62	0,6047	0,05
	2	0.71	0.25	11	0.39	1.16			
	3	0.59	0.32	9	0.15	1.19			
	4	0.61	0.27	7	0.12	0.91			
Endometrioma									
Plasma (N=68)	No	0.70	0.27	45	0.28	1.38	0.32	0.7507	<0.01
	Yes	0.72	0.25	23	0.28	1.25			
Peritoneal fluid (N=65)	No	0.60	0.26	44	0.08	1.16	0.81	0.4232	0.01
	Yes	0.66	0.26	21	0.12	1.19			
Cycle phase									
Plasma (N=68)	proliferative	0.72	0.25	44	0.28	1.37	-0.28	0.7785	<0.01
	secretive	0.70	0.29	24	0.28	1.38			
Peritoneal fluid (N=65)	proliferative	0.61	0.27	42	0.08	1.16	0.35	0.7306	<0.01
	secretive	0.64	0.24	23	0.27	1.19			

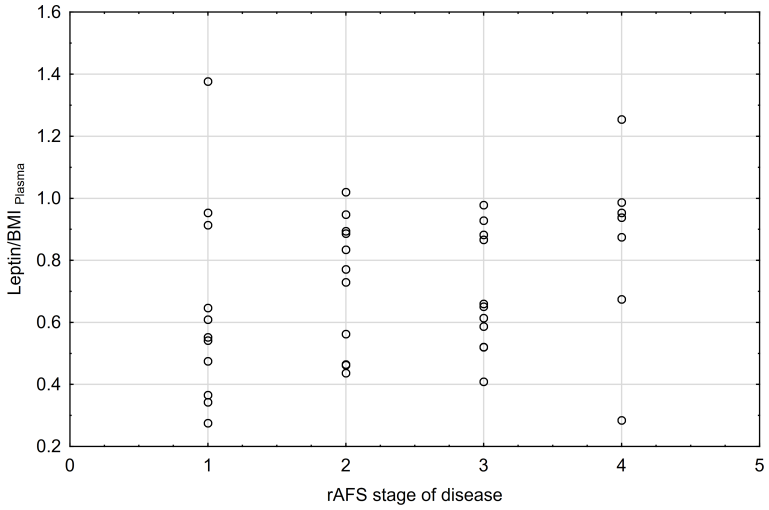


FIGURE 7
Correlations between rAFS stage of disease & Leptin/BMI ratio in plasma (rs=0.28; p=0.0871; N=39).

concentration of leptin in peritoneal fluid and plasma within the endometriosis group. These findings imply that plasma may serve as a viable alternative for assessing leptin concentration among women with endometriosis, presenting a less invasive method for evaluation.

However, we acknowledge limitation in this study. The relatively small sample size of both the study and the control groups poses a constraint. To address this, further studies based on a multicenter patient population in Poland are already planned, aiming to broaden and extend the analysis.

A strength of our study lies in the utilization of SPRI, a well-established and reliable technology for measurements. Additionally, through the assessment of both leptin concentrations in plasma and peritoneal fluid, we investigated their interrelationships and potential impact on the pathogenesis of endometriosis. Another advantage of our research was the meticulous sampling procedure, emphasizing the purity of the collected peritoneal fluid. Significantly, our study contributes to a series of publications examining the potential role of selected molecules as biomarkers for endometriosis.

In conclusion, the role of leptin as a reliable biomarker of endometriosis remains controversial. Our study aligns with the thesis, that peritoneal fluid and serum leptin level may not significantly differ between patients with endometriosis and the control group. However, our findings suggest an association between leptin concentration and endometriosis-associated infertility, albeit based on a limited number of samples. Hence, further research is imperative to elucidate the role of leptin in endometriosis.

Further studies are needed to delve into the underlying mechanisms through which leptin operates in endometriosis,

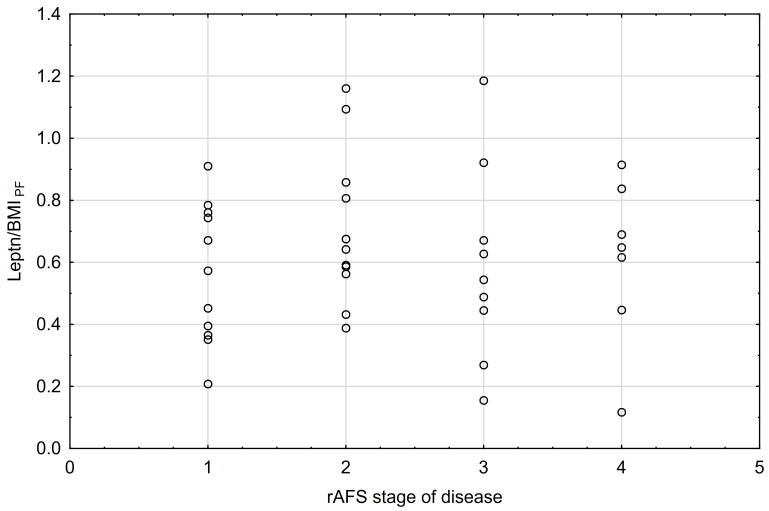


FIGURE 8
rAFS stage of disease & Leptin/BMI ratio in PF (rs=0.05; p=0.7570; N=37).

determining whether observed changes in leptin concentrations contribute to or are the result from the disease's pathogenesis. Additionally, more comprehensive data regarding leptin as a diagnostic biomarker are necessary for a clearer understanding of its potential utility in the diagnostic process of endometriosis.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Medical University of Warsaw (KB/223/2017). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AZ: Visualization, Validation, Formal analysis, Conceptualization, Writing – original draft. AnS: Writing – review & editing, Methodology. AgS: Writing – review & editing, Supervision. ED: Writing – review & editing. AD: Writing – review & editing, Resources. GM: Writing – review & editing, Resources. MKi: Writing – review & editing, Resources. RS: Writing – review & editing, Resources. PPk: Writing – review & editing, Resources. BB: Writing – review & editing, Resources. AJ: Writing – review & editing, Resources. TI: Writing – review & editing, Resources. WR: Writing – review & editing, Resources. JM: Writing – review & editing, Resources. MS: Writing – review & editing, Resources. PS: Resources, Writing – review & editing. GR: Writing – review & editing, Resources. KS: Writing – review & editing, Resources. TK: Writing – review & editing, Resources. MKl: Resources, Writing – review & editing. PPz: Writing – review & editing, Resources. CW: Writing – review & editing, Resources. ML: Writing – review & editing, Supervision, Methodology. DW: Writing – review & editing, Resources. MW: Writing – review & editing, Funding acquisition. KC: Writing – review & editing, Resources. EG: Writing – review & editing, Methodology. PL: Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was funded by a grant from Polish Ministry of Health (6/6/4/1/NPZ/2017/1210/1352) and MSCA-RISE-2020 project TRENDO (grant no. 101008193).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Becker CM, Bokor A, Heikinheimo O, Horne A, Jansen F, Kiesel L, et al. ESHRE guideline: endometriosis. *Hum Reprod Open*. (2022) 2022:hoac009. doi: 10.1093/hropen/hoac009

2. Lagana AS, Garzon S, Gotte M, Vigano P, Franchi M, Ghezzi F, et al. The pathogenesis of endometriosis: molecular and cell biology insights. *Int J Mol Sci*. (2019) 20. doi: 10.3390/ijms20225615

3. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *N Engl J Med*. (2020) 382:1244–56. doi: 10.1056/NEJMra1810764

4. Hansen KA, Eyster KM. Genetics and genomics of endometriosis. *Clin Obstet Gynecol*. (2010) 53:403–12. doi: 10.1097/GRF.0b013e3181db7ca1

5. Chapron C, Marcellin L, Borghese B, Santulli P. Rethinking mechanisms, diagnosis and management of endometriosis. *Nat Rev Endocrinol*. (2019) 15:666–82. doi: 10.1038/s41574-019-0245-z

6. Singh S, Soliman AM, Rahal Y, Robert C, Defoy I, Nisbet P, et al. Prevalence, symptomatic burden, and diagnosis of endometriosis in Canada: cross-sectional survey of 30 000 women. *J Obstet Gynaecol Can*. (2020) 42:829–38. doi: 10.1016/j.jogc.2019.10.038

7. Hudelist G, Fritzer N, Thomas A, Niehues C, Oppelt P, Haas D, et al. Diagnostic delay for endometriosis in Austria and Germany: causes and possible consequences. *Hum Reprod*. (2012) 27:3412–6. doi: 10.1093/humrep/des316

8. Wrobel M, Wielgos M, Laudanski P. Diagnostic delay of endometriosis in adults and adolescence-current stage of knowledge. *Adv Med Sci*. (2022) 67:148–53. doi: 10.1016/j.advms.2022.02.003

9. Sampson JA. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. *Am J Pathol*. (1927) 3:93–110 43.

10. Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. Retrograde menstruation in healthy women and in patients with endometriosis. *Obstet Gynecol*. (1984) 64:151–4.

11. Laudanski P, Szamatowicz J, Ramel P. Matrix metalloproteinase-13 and membrane type-1 matrix metalloproteinase in peritoneal fluid of women with endometriosis. *Gynecol Endocrinol*. (2005) 21:106–10. doi: 10.1080/09513590500154043

12. Laudanski P, Charkiewicz R, Kuzmicki M, Szamatowicz J, Swiatecka J, Mroczko B, et al. Profiling of selected angiogenesis-related genes in proliferative eutopic endometrium of women with endometriosis. *Eur J Obstet Gynecol Reprod Biol*. (2014) 172:85–92. doi: 10.1016/j.ejogrb.2013.10.007

13. Laudanski P, Gorodkiewicz E, Ramotowska B, Charkiewicz R, Kuzmicki M, Szamatowicz J. Determination of cathepsins B, D and G concentration in eutopic

proliferative endometrium of women with endometriosis by the surface plasmon resonance imaging (SPRI) technique. *Eur J Obstet Gynecol Reprod Biol*. (2013) 169:80–3. doi: 10.1016/j.ejogrb.2013.01.024

14. Szamatowicz J, Laudanski P, Tomaszewska I, Szamatowicz M. Chemokine growth-regulated-alpha: a possible role in the pathogenesis of endometriosis. *Gynecol Endocrinol*. (2002) 16:137–41. doi: 10.1080/713603033

15. Bartnik P, Kacperczyk-Bartnik J, Golawski K, Sierdzinski J, Manka G, Kiecka M, et al. Plasma and peritoneal fluid ZEB levels in patients with endometriosis and infertility. *Biomedicines*. (2022) 10. doi: 10.3390/biomedicines10102460

16. Kacperczyk-Bartnik J, Bartnik P, Golawski K, Sierdzinski J, Manka G, Kiecka M, et al. Plasma and peritoneal poly (ADP-ribose) polymerase levels in patients with endometriosis. *Biomedicines*. (2022) 10. doi: 10.3390/biomedicines10102451

17. Skarzynska E, Wrobel M, Zborowska H, Kolek MF, Manka G, Kiecka M, et al. The influence of lactoferrin in plasma and peritoneal fluid on iron metabolism in women with endometriosis. *Int J Mol Sci*. (2023) 24. doi: 10.3390/ijms24021619

18. Lisowska-Myjak B, Skarzynska E, Wrobel M, Manka G, Kiecka M, Lipa M, et al. Investigation of the changes in concentrations of vitamin D-binding protein and lactoferin in plasma and peritoneal fluid of patients with endometriosis. *Int J Mol Sci*. (2023) 24. doi: 10.3390/ijms24097828

19. Hwang JH, Wang T, Lee KS, Joo JK, Lee HG. Vitamin D binding protein plays an important role in the progression of endometriosis. *Int J Mol Med*. (2013) 32:1394–400. doi: 10.3892/ijmm.2013.1506

20. Margetic S, Gazzola C, Pegg GG, Hill RA. Leptin: a review of its peripheral actions and interactions. *Int J Obes Relat Metab Disord*. (2002) 26:1407–33. doi: 10.1038/sj.ijo.0802142

21. Mantzoros CS, Magkos F, Brinkoetter M, Sienkiewicz E, Dardeno TA, Kim SY, et al. Leptin in human physiology and pathophysiology. *Am J Physiol Endocrinol Metab*. (2011) 301:E567–84. doi: 10.1152/ajpendo.00315.2011

22. Friedman JM. Leptin and the endocrine control of energy balance. *Nat Metab*. (2019) 1:754–64. doi: 10.1038/s42255-019-0095-y

23. Gonzalez RR, Caballero-Campo P, Jasper M, Mercader A, Devoto L, Pellicer A, et al. Leptin and leptin receptor are expressed in the human endometrium and endometrial leptin secretion is regulated by the human blastocyst. *J Clin Endocrinol Metab*. (2000) 85:4883–8. doi: 10.1210/jc.85.12.4883

24. Lima-Couy I, Cervero A, Bonilla-Musoles F, Pellicer A, Simon C. Endometrial leptin and leptin receptor expression in women with severe/moderate endometriosis. *Mol Hum Reprod*. (2004) 10:777–82. doi: 10.1093/molehr/gah115

25. Hoffmann A, Ebert T, Kloting N, Dokas J, Jeromin F, Jessnitzer B, et al. Leptin dose-dependently decreases atherosclerosis by attenuation of hypercholesterolemia and induction of adiponectin. *Biochim Biophys Acta*. (2016) 1862:113–20. doi: 10.1016/j.bbadis.2015.10.022

26. Fasshauer M, Bluher M. Adipokines in health and disease. *Trends Pharmacol Sci*. (2015) 36:461–70. doi: 10.1016/j.tips.2015.04.014

27. Lempesis IG, van Meijel RLJ, Manolopoulos KN, Goossens GH. Oxygenation of adipose tissue: A human perspective. *Acta Physiol (Oxf)*. (2020) 228:e13298. doi: 10.1111/apha.13298

28. Martins Mdo C, Lima Faleiro L, Fonseca A. [Relationship between leptin and body mass and metabolic syndrome in an adult population]. *Rev Port Cardiol*. (2012) 31:711–9. doi: 10.1016/j.repce.2012.09.008

29. Rowlands IJ, Hockey R, Abbott JA, Montgomery GW, Mishra GD. Body mass index and the diagnosis of endometriosis: Findings from a national data linkage cohort study. *Obes Res Clin Pract*. (2022) 16:235–41. doi: 10.1016/j.orcp.2022.04.002

30. Goncalves HF, Zendron C, Cavalcante FS, Aiceles V, Oliveira MA, Manaia JH, et al. Leptin, its receptor and aromatase expression in deep infiltrating endometriosis. *J Ovarian Res*. (2015) 8:53. doi: 10.1186/s13048-015-0180-0

31. Pantelis A, Machairiotis N, Lapatsanis DP. The formidable yet unresolved interplay between endometriosis and obesity. *ScientificWorldJournal*. (2021) 2021:6653677. doi: 10.1155/2021/6653677

32. Puiu M, Bala C. SPR and SPR imaging: recent trends in developing nanodevices for detection and real-time monitoring of biomolecular events. *Sensors (Basel)*. (2016) 16. doi: 10.3390/s16060870

33. Peterson AW, Halter M, Tona A, Bhadriraju K, Plant AL. Using surface plasmon resonance imaging to probe dynamic interactions between cells and extracellular matrix. *Cytometry A*. (2010) 77:895–903. doi: 10.1002/cyto.a.20938

34. Sankiewicz A, Romanowicz L, Laudanski P, Zelazowska-Rutkowska B, Puzan B, Cylwik B, et al. SPR imaging biosensor for determination of laminin-5 as a potential cancer marker in biological material. *Anal Bioanal Chem*. (2016) 408:5269–76. doi: 10.1007/s00216-016-9621-x

35. Grzywa R, Gorodkiewicz E, Burchacka E, Lesner A, Laudanski P, Lukaszewski Z, et al. Determination of cathepsin G in endometrial tissue using a surface plasmon resonance imaging biosensor with tailored phosphonic inhibitor. *Eur J Obstet Gynecol Reprod Biol*. (2014) 182:38–42. doi: 10.1016/j.ejogrb.2014.08.029

36. Sankiewicz A, Laudanski P, Romanowicz L, Hermanowicz A, Roszkowska-Jakimiec W, Debek W, et al. Development of surface plasmon resonance imaging biosensors for detection of ubiquitin carboxyl-terminal hydrolase L1. *Anal Biochem*. (2015) 469:4–11. doi: 10.1016/j.ab.2014.09.021

37. Wrobel M, Zuzanna Z, Oldak L, Kalicka A, Manka G, Kiecka M, et al. Evaluation of proteasome and immunoproteasome levels in plasma and peritoneal fluid in patients with endometriosis. *Int J Mol Sci*. (2023) 24. doi: 10.3390/ijms241814363

38. Gorodkiewics E, Sankiewicz A, Laudański P. Surface plasmon resonance imaging biosensors for aromatase based on a potent inhibitor and a specific antibody: Sensor development and application for biological material. *centeurjchem*. (2014) 12::557–67. doi: 10.2478/s11532-014-0512-8

39. Laudanski P, Rogalska G, Warzecha D, Lipa M, Manka G, Kiecka M, et al. Autoantibody screening of plasma and peritoneal fluid of patients with endometriosis. *Hum Reprod*. (2023) 38:629–43. doi: 10.1093/humrep/dead011

40. Rahmioglu N, Fassbender A, Vitonis AF, Tworoger SS, Hummelshoj L, D'Hooghe TM, et al. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project: III. Fluid biospecimen collection, processing, and storage in endometriosis research. *Fertil Steril*. (2014) 102:1233–43. doi: 10.1016/j.fertnstert.2014.07.1208

41. Sankiewicz A, Hermanowicz A, Grycz A, Lukaszewski Z, Gorodkiewicz E. An SPR imaging immunosensor for leptin determination in blood plasma. *Anal Methods*. (2021) 13:642–6. doi: 10.1039/D0AY02047H

42. Wertel I, Gogacz M, Polak G, Jakowicki J, Kotarski J. Leptin is not involved in the pathophysiology of endometriosis-related infertility. *Eur J Obstet Gynecol Reprod Biol*. (2005) 119:206–9. doi: 10.1016/j.ejogrb.2004.08.004

43. Rathore N, Kriplani A, Yadav RK, Jaiswal U, Netam R. Distinct peritoneal fluid ghrelin and leptin in infertile women with endometriosis and their correlation with interleukin-6 and vascular endothelial growth factor. *Gynecol Endocrinol*. (2014) 30:671–5. doi: 10.3109/09513590.2014.920318

44. Tao Y, Zhang Q, Huang W, Zhu H, Zhang D, Luo W. The peritoneal leptin, MCP-1 and TNF-alpha in the pathogenesis of endometriosis-associated infertility. *Am J Reprod Immunol*. (2011) 65:403–6. doi: 10.1111/aji.2011.65.issue-4

45. Zendron C, Goncalves HF, Cavalcante FS, Pereira TR, Evangelista A, Ramos CF, et al. Increased expression of the leptin receptor in human ovaries affected by endometrioma and detection of high levels of leptin in the ovarian endometriomal fluid. *J Ovarian Res*. (2014) 7:2. doi: 10.1186/1757-2215-7-2

46. De Placido G, Alviggi C, Carravetta C, Pisaturo ML, Sanna V, Wilding M, et al. The peritoneal fluid concentration of leptin is increased in women with peritoneal but not ovarian endometriosis. *Hum Reprod*. (2001) 16:1251–4. doi: 10.1093/humrep/16.6.1251

47. Ajala OM, Ogunro PS, Elusanmi GF, Ogunyemi OE, Bolarinde AA. Changes in serum leptin during phases of menstrual cycle of fertile women: relationship to age groups and fertility. *Int J Endocrinol Metab*. (2013) 11:27–33. doi: 10.5812/ijem

48. Asimakopoulos B, Milousis A, Gioka T, Kabouromiti G, Gianisslis G, Troussa A, et al. Serum pattern of circulating adipokines throughout the physiological menstrual cycle. *Endocr J*. (2009) 56:425–33. doi: 10.1507/endocrj.K08E-222

49. Hardie L, Trayhurn P, Abramovich D, Fowler P. Circulating leptin in women: a longitudinal study in the menstrual cycle and during pregnancy. *Clin Endocrinol (Oxf)*. (1997) 47:101–6. doi: 10.1046/j.1365-2265.1997.2441017.x

50. Ludwig M, Klein HH, Diedrich K, Ortmann O. Serum leptin concentrations throughout the menstrual cycle. *Arch Gynecol Obstet*. (2000) 263:99–101. doi: 10.1007/s004040050004

51. Stock SM, Sande EM, Bremme KA. Leptin levels vary significantly during the menstrual cycle, pregnancy, and in *vitro* fertilization treatment: possible relation to estradiol. *Fertil Steril*. (1999) 72:657–62. doi: 10.1016/S0015-0282(99)00321-0

52. Capobianco G, de Muro P, Cherchi GM, Formato M, Lepedda AJ, Cigliano A, et al. Plasma levels of C-reactive protein, leptin and glycosaminoglycans during spontaneous menstrual cycle: differences between ovulatory and anovulatory cycles. *Arch Gynecol Obstet*. (2010) 282:207–13. doi: 10.1007/s00404-010-1432-2

53. Bulun SE, Cheng YH, Yin P, Imir G, Utsunomiya H, Attar E, et al. Progesterone resistance in endometriosis: link to failure to metabolize estradiol. *Mol Cell Endocrinol*. (2006) 248:94–103. doi: 10.1016/j.mce.2005.11.041

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 28 May 2024
DOI 10.3389/fendo.2024.1371393

OPEN ACCESS

EDITED BY
Iveta Yotova,
Medical University of Vienna, Austria

REVIEWED BY
Zhijing Na,
China Medical University, China
Preeti Khetarpal,
Central University of Punjab, India

*CORRESPONDENCE
Jinwei Miao
jinweimiao@ccmu.edu.cn

RECEIVED 16 January 2024
ACCEPTED 29 April 2024
PUBLISHED 28 May 2024

CITATION
Liu P, Wang Y, Ji X, Kong W, Pan Z, Xu C, Geng Y and Miao J (2024) Association between triglyceride-glucose index and risk of endometriosis in US population: results from the national health and nutrition examination survey (1999–2006). *Front. Endocrinol.* 15:1371393. doi: 10.3389/fendo.2024.1371393

Association between triglyceride-glucose index and risk of endometriosis in US population: results from the national health and nutrition examination survey (1999–2006)

Penglin Liu, Yixiao Wang, Xuechao Ji, Wenzhi Kong, Zangyu Pan, Chunyu Xu, Yuning Geng and Jinwei Miao*

Department of Gynecologic Oncology, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing Maternal and Child Health Care Hospital, Beijing, China

Background and Aims: The association of the triglyceride-glucose (TyG) index, a promising novel biomarker for insulin resistance, with the risk of endometriosis has not been investigated to date. This nationwide study aimed to explore the association between the TyG index and the endometriosis risk.

Methods: Data were obtained from the National Health and Nutrition Examination Survey (1999–2006). Female participants who provided complete data on the TyG index and endometriosis were enrolled in the analysis. Multivariate logistic regression analyses were utilized to assess the association of the TyG index with endometriosis, adjusted by multiple potential confounders. Meanwhile, in-depth subgroup analyses were conducted.

Results: A total of 1,590 eligible participants were included, among whom 135 (8.5%) women were diagnosed with endometriosis. The fully adjusted multivariate logistic model showed TyG index was significantly associated with the endometriosis risk (odds ratio [OR]$_{Q4\ versus\ Q1}$ 2.04, 95% confidence interval [CI]: 1.15–3.62; *P* for trend=0.013). In subgroup analyses, the significantly positive association between TyG index and the risk of endometriosis was also found in parous women (OR$_{Q4\ versus\ Q1}$ 2.18, 95% CI: 1.20–3.96), women without diabetes (OR $_{Q4\ versus\ Q1}$ 2.12, 95% CI: 1.19–3.79), women who smoke currently (OR $_{Q4\ versus\ Q1}$ 3.93, 95% CI: 1.33–11.58), women who drink currently (OR $_{Q4\ versus\ Q1}$ 2.54, 95% CI: 1.27–5.07), and in women who use oral contraceptives (OR $_{Q4\ versus\ Q1}$ 1.91, 95% CI: 1.04–3.51). Additionally, significantly increasing trends in the odds of endometriosis across the quartiles of the TyG index were observed in the above-mentioned subgroups (all *P* for trend<0.05).

Conclusions: This population-based study found that a higher TyG index, representing an increased level of insulin resistance, was associated with a higher risk of endometriosis among the US population. Our findings suggested TyG index might be a promising tool for the risk assessment of endometriosis. Prospective studies are warranted to further verify these findings.

KEYWORDS

endometriosis, triglyceride-glucose index, insulin resistance, cross-sectional study, National Health and Nutrition Examination Survey

1 Introduction

Endometriosis, as a chronic, systemic gynecologic disease, is characterized by the implantation of endometrial-like tissue outside the uterine cavity, commonly affecting the pelvic cavity and ovaries (1). It is estimated that endometriosis affects approximately 5–10% of women in their reproductive years and exerts significant psychological and physical effects on the quality of life (1–3). However, current treatments for endometriosis including surgical removal of lesions and drug therapy both have limited efficacy (4). The high occurrence and recurrence rates lead to tremendous healthcare expenses for long-term management, highlighting the urgent need to develop robust novel biomarkers for the risk assessment, diagnosis and monitoring of disease progression.

The exact pathological mechanism of endometriosis remains not fully comprehended despite the implication of various factors such as inflammation, hormones, metabolism and immunology (4). Recently, emerging evidence suggests that metabolic disturbances play a vital role in the development and progression of endometriosis (5, 6). In particular, dysregulation of glucose and lipid metabolism has been observed in women with endometriosis (7–9).

The triglyceride-glucose (TyG) index, derived from the fasting triglyceride and glucose levels, has been considered as a novel and reliable marker for assessing insulin resistance and shows comparable effectiveness to the commonly used homeostatic model assessment (HOMA) insulin resistance index (10). Due to its advantages of economic benefits and easy availability, the TyG index has gained attention as a comprehensive surrogate measure of insulin resistance.

Multiple epidemiological studies have demonstrated the association of TyG with a range of diseases, such as type 2 diabetes (11), cardiovascular disease (12), metabolic dysfunction-associated fatty liver disease (13) and gynecologic cancers (14, 15). Given the potential involvement of metabolic disturbances in endometriosis pathogenesis, it is plausible to explore the association of insulin resistance with the risk of endometriosis. However, to our knowledge, the association between the TyG index and endometriosis has not been explored till date.

This population-based study aims to investigate the association between the TyG index and the risk of endometriosis using the nationally representative American population from the National Health and Nutrition Examination Survey (NHANES) (16), to provide comprehensive epidemiological evidence for the clinical role of the TyG index in the risk assessment and prevention of endometriosis.

2 Materials and methods

2.1 Data source and population

The NHANES program is designed to assess the health and nutritional status of the American population through a series of interviews, physical examinations and laboratory tests. It is conducted by the Centers for Disease Control and Prevention (16) in the United States (US). In this current study, we collected publicly available data on a total of 21,210 female participants from NHANES (1999–2006). We excluded 15,653 participants with missing data on the diagnosis of endometriosis, and 3,049 participants with missing laboratory data on fasting blood triglyceride and fasting glucose levels. Additionally, 918 participants who had incomplete data on the potential confounding variables (described below) were also excluded. Consequently, a total of 1,590 female participants were included in the final analysis. The study flowchart is presented in Figure 1.

2.2 Ascertainment of endometriosis

The diagnosis of endometriosis was based on the "Questionnaire on Reproductive Health" at each survey circle in NHANES between 1999 and 2006. Participants who reported "yes" to the question "Told by the doctor having endometriosis?" were identified to have endometriosis.

2.3 TyG index and covariates

The TyG index was calculated using the formula Ln [fasting triglyceride (mg/dL) × fasting glucose (mg/dL)/2]. The data on

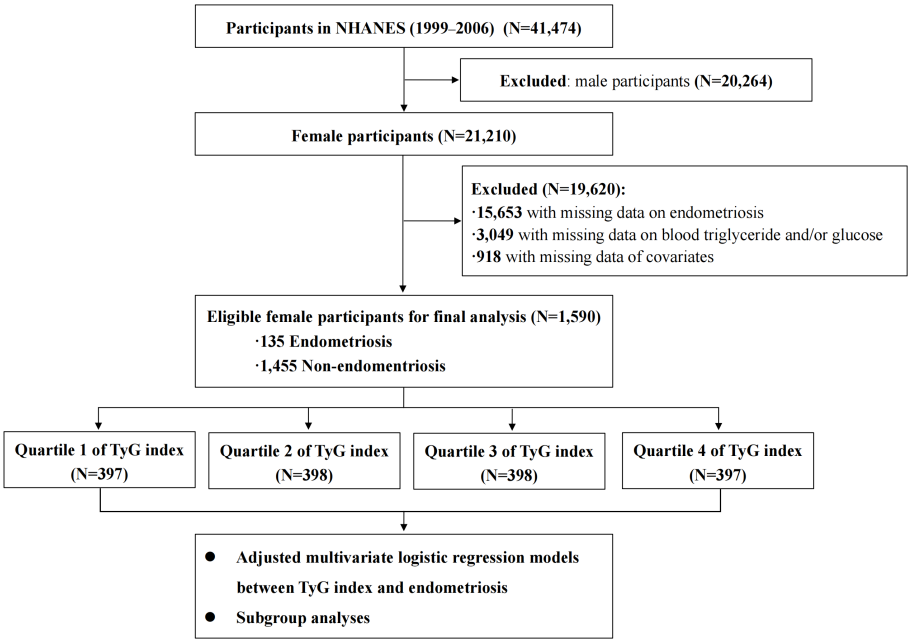


FIGURE 1
The flowchart of this study. NHANES, National Health and Nutrition Examination Survey; TyG index, triglyceride-glucose index.

fasting triglycerides and fasting glucose were obtained from the "Laboratory Data" in NHANES. In this study, the TyG Index ranged from 7.049 to 11.951, representing a continuous value.

Based on the previous studies (17, 18) and clinical experience, the following covariates were included in the analysis: age (years), ethnicity (non-Hispanic White, non-Hispanic Black, Mexican Americans, and other race), educational level (above high school, high school graduate, and less than high school), marital status (never married, married, and other), fertility status (≥ one birth or nulliparous), body mass index (BMI), diabetes (no or yes), drinking status (current drinker, former drinker, and non-drinker), smoking status (current smoker, former smoker, and non-smoker) and the use of oral contraceptives (no or yes).

BMI was determined by dividing the weight in kilograms by the square of height in meters (kg/m^2). Diabetes was assessed based on the following criteria: being told diabetes by a doctor, glycated hemoglobin A1c exceeding 6.5%, fasting glucose equal to or greater than 7.0mmol/L, random blood glucose equal to or greater than 11.1 mmol/L, blood glucose equal to or greater than11.1mmol/L during a 2-hour oral glucose tolerance test, and the use of diabetes medication or insulin.

2.4 Statistical analysis

All analyses were conducted in accordance with the NHANES analytic guidelines. Categorical variables were compared by the χ^2 test, and continuous variables were compared by the t-test or Wilcoxon rank sum nonparametric test based on the results of the normality test. The participants were categorized into four groups (Q1, Q2, Q3, Q4) according to the quartiles of the TyG index, using the Q1 group as the reference group. We utilized multivariate logistic regression models to calculate the odd ratios (OR) and 95% confidence intervals (CI) to assess the relationship between the TyG index and endometriosis. Initially, a crude model was applied, considering only the TyG index. Subsequently, three adjusted models were adopted. Model 1 was adjusted for age, ethnicity, education level and marital status. In Model 2, we further adjusted for BMI, diabetes, and fertility status in addition to the covariates included in Model 1. Lastly, Model 3 accounted for drinking status, smoking status, and use of oral contraceptives in addition to the covariates included in Model 2. Additionally, we estimated linear trends across quartiles of the TyG index by treating the median value in each quartile as a continuous variable in regression models. Moreover, we examined the association of the TyG index with the risk of endometriosis on a continuous scale using the restricted cubic spline (RCS) curve based on the logistic regression model. Subgroup analyses were performed stratified by fertility status, diabetes, drinking status, smoking status and the usage of oral contraceptives.

All statistical analyses were conducted using R software (version 4.2.2). All P values were two-sided with $P<0.05$ considered as statistically significant.

3 Results

3.1 Characteristics of the participants

The baseline characteristics between endometriosis and non-endometriosis groups are presented in Table 1. Among the 1,590 eligible participants, 135 (8.5%) women were diagnosed with

10.3389/fendo.2024.1371393

TABLE 1 Baseline characteristics between endometriosis and non-endometriosis groups.

Characteristics	Total (n=1,590)	Endometriosis (n=135)	Non-Endometriosis (n=1,455)	*P* value
Age (years), mean (SD)	39.20 (9.26)	41.96 (7.85)	38.95 (9.34)	<0.001
Ethnicity, n (%)				<0.001
Mexican American	367 (23.1)	9 (6.7)	358 (24.6)	
Non-Hispanic Black	382 (24.0)	25 (18.5)	357 (24.5)	
Non-Hispanic White	702 (44.2)	94 (69.6)	608 (41.8)	
Other Races	139 (8.7)	7 (5.2)	132 (9.1)	
Education level, n (%)				0.001
Less than high school	425 (26.7)	19 (14.1)	406 (27.9)	
High school graduate	374 (23.5)	42 (31.1)	332 (22.8)	
Above high school	791 (49.7)	74 (54.8)	717 (49.3)	
Marital status, n (%)				0.254
Never married	185 (11.6)	10 (7.4)	175 (12.0)	
Married	955 (60.1)	87 (64.4)	868 (59.7)	
Other	450 (28.3)	38 (28.1)	412 (28.3)	
Fertility status, n (%)				0.192
Nulliparous	95 (6.0)	12 (8.9)	83 (5.7)	
≥one birth	1495 (94.0)	123 (91.1)	1372 (94.3)	
BMI, mean (SD)	29.18 (7.36)	28.89 (6.66)	29.20 (7.43)	0.635
Diabetes, n (%)				0.793
No	1469 (92.4)	126 (93.3)	1343 (92.3)	
Yes	121 (7.6)	9 (6.7)	112 (7.7)	
Smoking status, n (%)				<0.001
Never	926 (58.2)	57 (42.2)	869 (59.7)	
Former	260 (16.4)	30 (22.2)	230 (15.8)	
Now	404 (25.4)	48 (35.6)	356 (24.5)	
Drinking status, n (%)				0.127
Never	255 (16.0)	15 (11.1)	240 (16.5)	
Former	278 (17.5)	30 (22.2)	248 (17.0)	
Now	1057 (66.5)	90 (66.7)	967 (66.5)	
Oral contraceptive, n (%)				0.054
No	319 (20.1)	18 (13.3)	301 (20.7)	
Yes	1271 (79.9)	117 (86.7)	1154 (79.3)	
Fasting glucose (mg/dl), median [IQR]	93.0 [87.0, 100.0]	92.3 [86.9, 100.4]	93.0 [87.1, 99.8]	0.935
Fasting triglyceride (mg/dl), median [IQR]	100.0 [70.0, 146.0]	119.0 [77.0, 179.0]	99.0 [69.0, 143.0]	0.001
TyG index, mean (SD)	8.51 (0.62)	8.67 (0.64)	8.49 (0.62)	0.002
TyG index Quartile, n (%)				0.029
Q1	397 (25.0)	24 (17.8)	373 (25.6)	
Q2	398 (25.0)	31 (23.0)	367 (25.2)	

(Continued)

TABLE 1 Continued

Characteristics	Total (n=1,590)	Endometriosis (n=135)	Non-Endometriosis (n=1,455)	*P* value
Q3	398 (25.0)	33 (24.4)	365 (25.1)	
Q4	397 (25.0)	47 (34.8)	350 (24.1)	

IQR, interquartile range; SD, standard deviation; TyG index, triglyceride-glucose index; BMI, body mass index.

endometriosis. The mean age of participants in the entire study was 39.20, ranging from 20 to 54 years. Participants diagnosed with endometriosis were found to be older, had a higher level of education, and were more likely to be a smokers. Furthermore, there were significant differences in ethnicity between the two groups.

In the overall study population, the mean TyG index was 8.51, with a significantly higher mean TyG index in cases with endometriosis compared to those without endometriosis (8.67 versus 8.49, *P*=0.002). Furthermore, a higher percentage of the highest quartile (Q4) TyG index (34.8% versus 24.1%) and a lower percentage of the lower quartile (Q1) TyG index (17.8% versus 25.6%) were found in the endometriosis group than the non-endometriosis group. Meanwhile, the baseline characteristics of the participants based on quartile categories of the TyG index are shown in Supplementary Table S1.

3.2 Association of the TyG index with endometriosis

The associations of TyG index with endometriosis in the overall cohort, are presented in Table 2. In the unadjusted model, the OR for participants with endometriosis in the highest quartile of the TyG index was 2.09 (95% CI: 1.25–3.49) compared to those in the lowest quartile (*P* for trend = 0.004). Similarly, in adjusted multivariate models 1, 2 and 3, the positive association of the TyG index with the risk of endometriosis persisted. After fully adjusting for the potential confounders, participants in the highest quartile of TyG had a 104% higher risk of endometriosis compared to those in the lowest quartile (OR $_{\text{Q4 versus Q1}}$ 2.04, 95% CI: 1.15–3.62). Additionally, significant increasing trends in the adjusted odds of endometriosis across the quartiles of the TyG index were observed in all models (Model 1: *P* for trend=0.010, Model 2: *P* for trend=0.004, Model 3: *P* for trend=0.013).

When assessing the association between the TyG index and the risk of endometriosis on a continuous scale, the fully adjusted logistic regression model also conveyed that the TyG index was positively related to the endometriosis (OR 1.62, 95% CI: 1.18–2.22) (Table 2). Moreover, we also observed a dose-response correlation between the TyG index and the endometriosis risk by the RCS analysis (*P*=0.013) (Figure 2).

3.3 Subgroup analysis

To explore the association of the TyG index with the risk of endometriosis in different populations, we performed in-depth subgroup analyses stratified by fertility status, diabetes, smoking status, drinking status, and usage of oral contraceptives (Figure 3; Supplementary Table S2).

The fully-adjusted logistic regression models also revealed a significant positive relationship between quartiles of TyG index and the risk of endometriosis in parous participants (OR $_{\text{Q4 versus Q1}}$ 2.18, 95% CI: 1.20–3.96; *P*=0.011), in participants without diabetes (OR $_{\text{Q4 versus Q1}}$ 2.12, 95% CI: 1.19–3.79; *P*=0.011), in participants who smoke currently (OR $_{\text{Q4 versus Q1}}$ 3.93, 95% CI: 1.33–11.58; *P*=0.013), in

TABLE 2 Associations between the TyG index and endometriosis in the total cohort.

TyG index	Cases with endometriosis/N	OR (95% CI)			
		Crude [a]	Model 1 [b]	Model 2 [c]	Model 3 [d]
Continuous Values (7.049–11.951)	135/1,590	1.52 (1.17–1.97)*	1.53 (1.15–2.03)*	1.73 (1.26–2.36)*	1.62 (1.18–2.22)*
Quartile Categories					
Q1 (7.049–8.061)	24/397	Reference	Reference	Reference	Reference
Q2 (8.063–8.452)	31/398	1.31 (0.76–2.28)	1.29 (0.74–2.25)	1.35 (0.76–2.37)	1.26 (0.71–2.23)
Q3 (8.453–8.865)	33/398	1.41 (0.81–2.42)	1.35 (0.77–2.34)	1.42 (0.81–2.51)	1.31 (0.74–2.32)
Q4 (8.866–11.951)	47/397	2.09 (1.25–3.49)*	1.99 (1.16–3.40)*	2.27 (1.29–4.00)*	2.04 (1.15–3.62)*
P for trend		0.004	0.010	0.004	0.013

*Statistically significant association.
[a]Crude model was adjusted for nothing.
[b]Model 1 was adjusted for age, ethnicity, education level and marital status.
[c]Model 2 included the covariates of Model 1 with additional adjustment for diabetes, body mass index and fertility status.
[d]Model 3 included the covariates of Model 2 with additional adjustment for drinking status, smoking status and use of oral contraceptives.
TyG index, triglyceride-glucose index; OR, odds ratio; CI, confidence interval.

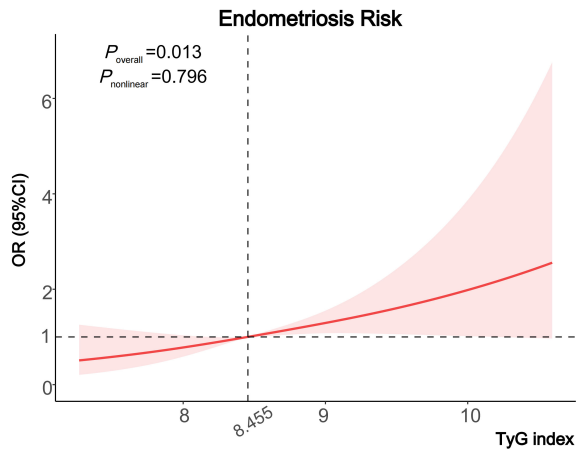


FIGURE 2
Cubic regression spline of the endometriosis risk by TyG index among the entire population. Cubic regression spline was adjusted for age, ethnicity, education level, marital status, fertility status, body mass index, diabetes, drinking status, smoking status and the use of oral contraceptives. ORs are indicated by solid lines and 95% CIs are presented by shaded areas. TyG index, triglyceride-glucose index; OR, odds ratio; CI, confidence interval.

participants who drink currently (OR $_{Q4\ versus\ Q1}$ 2.54, 95% CI: 1.27–5.07; *P*=0.008), and in participants who use oral contraceptives (OR $_{Q4\ versus\ Q1}$ 1.91, 95% CI: 1.04–3.51; *P*=0.038) (Figure 3). Significant increasing trends in the odds of endometriosis across the quartiles of the TyG index were observed in the above-mentioned subgroups (all *P* for trend <0.05) (Supplementary Table S2).

4 Discussion

To the best of our knowledge, this nationally representative study represents the first investigation into the correlation between the TyG index and the risk of endometriosis. We found a significantly positive association of the TyG index with the risk of endometriosis in the US adult population, highlighting the clinical role of the TyG index on the prevention and management strategies for endometriosis.

As a multifactorial and systemic disease, endometriosis is caused by many factors and the exact pathogenesis has not been clearly explained (5). Recently, metabolic abnormalities, including dyslipidemia and glucose metabolism dysfunction, were reported to be an increasingly significant etiology of endometriosis, with the development of metabolomics technology (6–9).

TyG index, measured based on blood fasting triglycerides and glucose, has been recommended as an effective surrogate marker for insulin resistance, and could integrate the effect of both triglycerides as well as glucose on endometriosis (19). Numerous studies have demonstrated the potential of the TyG index in predicting the risk of various metabolism-related disorders (11–15). However, the relationship between the TyG index and endometriosis has not been explored yet.

In our study, we observed a 104% higher risk of endometriosis in participants within the highest quartile of the TyG index, compared to those in the lowest quartile, after adjusting for various covariates. These findings provided novel insights into the association between insulin resistance and the development of endometriosis, suggesting that the TyG index could potentially serve as a valuable clinical tool for predicting and assessing the risk of endometriosis.

The potential underlying mechanisms linking the TyG index to endometriosis are still not well understood and there might be several possible explanations as follows. First, insulin resistance could lead to hyperinsulinemia and elevated levels of insulin-like growth factor-1 (IGF-1), which may potentially promote the growth and proliferation of endometrial tissue outside the uterus (20, 21). Additionally, insulin resistance is associated with chronic systematic inflammation, which has been implicated in the development and progression of endometriosis (1, 22). Moreover, hyperinsulinemia might promote the production of androgens, leading to hormonal imbalances between estrogen and progesterone, which may further facilitate the establishment and growth of ectopic endometrial lesions (23, 24).

In the stratified analysis of our study, we observed variations in the association between the TyG index and the risk of endometriosis across different subgroups. When stratified by fertility status, a significant relationship of TyG index with endometriosis was observed in participants who had given birth but not in nulliparous women. It is well-established that pregnancy could induce significant metabolic and hormonal changes, which might modulate the impact of insulin resistance on the progression

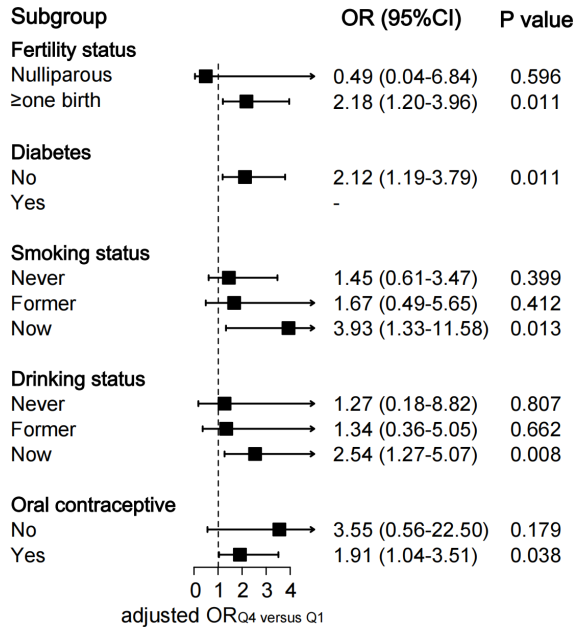


FIGURE 3
Subgroup analysis of association between quartiles of TyG index and endometriosis. The ORs were assessed by multivariate logistic regression models adjusted for age, ethnicity, education level, marital status, fertility status, body mass index, diabetes, drinking status, smoking status and the use of oral contraceptives. All Models were not adjusted for the stratified covariates on which the subgroup analyses were conducted. TyG index, triglyceride-glucose index; OR, odds ratio; CI, confidence interval.

of endometriosis (25). Meanwhile, parity has been regarded as an important protective factor for endometriosis (26). It is possible that the effect of insulin resistance on endometriosis might be attenuated among nulliparous women in our study.

Notably, in the subgroup analysis stratified by the smoking and drinking status, we found a considerable 3.93 and 2.54 times risk of endometriosis in women within the highest quartiles of the TyG index than those within the lowest quartiles, among women who smoke or drink currently, respectively. This observed phenomenon could potentially be ascribed to the hypothesis that smoking and alcohol consumption could exacerbate the adverse impact of insulin resistance on endometrial tissue, ultimately resulting in an increased susceptibility to the development of endometriosis (27, 28). Our findings conveyed that monitoring the TyG index could potentially be a useful tool in identifying individuals at a higher risk for endometriosis among the American female population who engage in smoking and alcohol consumption.

Regarding the stratified analysis by the use of oral contraceptives, a previous meta-analysis reported use of oral contraceptives could reduce the risk of endometriosis, which demonstrated that the use of oral contraceptives might serve as a protective factor against the development of endometriosis (29). In the present study, we also found a significantly positive correlation between the TyG index and the risk of endometriosis among those who used oral contraceptives. Nevertheless, no significant relationship was found in the women without the use of oral contraceptives. Prospective researches are necessary to further investigate the exact relationship between the TyG index and endometriosis in these specific women groups.

4.1 Strengths and limitations

The strengths of this study include its population-based dataset from NHANES, allowing the generalizability of our findings in the US population. Meanwhile, to explore the association of the TyG index with endometriosis preciously and thoroughly, in-depth adjustments for potential confounders and subgroups were performed, which could lead to more robust results. Moreover, to our knowledge, this study was the first to provide insights into the association of insulin resistance with the risk of endometriosis, highlighting the significant value of the TyG index in the risk assessment of endometriosis. We recommended that the TyG index should be calculated and listed routinely in laboratory biochemical tests, which could help gynecologists effectively identify individuals at a higher risk and facilitate early intervention and treatment. Additionally, targeting metabolic dysfunction, such as insulin resistance, through lifestyle modifications and pharmacological interventions, may present a novel therapeutic approach to managing endometriosis.

However, several limitations should be considered. First, the cross-sectional nature of the study limits the establishment of a causal relationship between the TyG index and endometriosis. Second, we relied on self-reported diagnosis of endometriosis in the NHANES, which might introduce recall bias. Prospective longitudinal studies are warranted to further confirm these findings.

5 Conclusion

To conclude, this nationally representative study found that elevated insulin resistance, as reflected by a higher TyG index, was associated with a higher risk of endometriosis in American adults. Our findings suggested TyG index may serve as an alternative tool to predict the risk of endometriosis, and potentially guide future prevention strategies. Further studies are needed to confirm these findings and elucidate the underlying mechanisms.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Health and Nutrition Examination Survey (https://www.cdc.gov/nchs/nhanes).

Ethics statement

The studies involving humans were approved by National Center for Health Statistics Ethics Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

PL: Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. YW: Investigation, Writing – review & editing. XJ: Investigation, Writing – review & editing. WK: Formal analysis, Software, Writing – review & editing. ZP: Visualization, Writing – review & editing. CX: Validation, Writing – review & editing. YG: Validation, Writing – review & editing. JM: Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by National Natural Science Foundation of China (82271677); Beijing Hospitals Authority's Ascent Plan (DFL20221201); Beijing Hospitals Authority Clinical Medicine Development of Special Funding Support (ZYLX202120); Laboratory for Clinical Medicine, Capital Medical University.

Acknowledgments

We appreciate all participants, physicians, medical technicians, interviewers, and the expert committees for contributing, organizing, and publishing the NHANES data.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1371393/full#supplementary-material

SUPPLEMENTARY TABLE 1
Baseline characteristics according to quartile categories of TyG index.

SUPPLEMENTARY TABLE 2
Subgroup analysis of the association between quartiles of TyG index and endometriosis.

References

1. Taylor HS, Kotlyar AM, Flores VA. Endometriosis is a chronic systemic disease: clinical challenges and novel innovations. *Lancet*. (2021) 397:839–52. doi: 10.1016/S0140–6736(21)00389–5

2. Giudice LC, Horne AW, Missmer SA. Time for global health policy and research leaders to prioritize endometriosis. *Nat Commun*. (2023) 14:8028. doi: 10.1038/s41467–023-43913–9

3. Simoens S, Dunselman G, Dirksen C, Hummelshoj L, Bokor A, Brandes I, et al. The burden of endometriosis: costs and quality of life of women with endometriosis and treated in referral centres. *Hum Reprod*. (2012) 27:1292–9. doi: 10.1093/humrep/des073

4. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *N Engl J Med*. (2020) 382:1244–56. doi: 10.1056/NEJMra1810764

5. Horne AW, Missmer SA. Pathophysiology, diagnosis, and management of endometriosis. *BMJ*. (2022) 379:e070750. doi: 10.1136/bmj-2022–070750

6. Lu J, Ling X, Liu L, Jiang A, Ren C, Lu C, et al. Emerging hallmarks of endometriosis metabolism: A promising target for the treatment of endometriosis. *Biochim Biophys Acta Mol Cell Res*. (2023) 1870:119381. doi: 10.1016/j.bbamcr.2022.119381

7. Li B, Zhang Y, Zhang L, Zhang L. Association between endometriosis and metabolic syndrome: a cross-sectional study based on the National Health and Nutrition Examination Survey data. *Gynecol Endocrinol*. (2023) 39:2254844. doi: 10.1080/09513590.2023.2254844

8. Lu C, Qiao P, Fu R, Wang Y, Lu J, Ling X, et al. Phosphorylation of PFKFB4 by PIM2 promotes anaerobic glycolysis and cell proliferation in endometriosis. *Cell Death Dis*. (2022) 13:790. doi: 10.1038/s41419–022-05241–6

9. Melo AS, Rosa-e-Silva JC, Rosa-e-Silva A, Poli-Neto OB, Ferriani RA, Vieira CS. Unfavorable lipid profile in women with endometriosis. *Fertil Steril*. (2010) 93:2433–6. doi: 10.1016/j.fertnstert.2009.08.043

10. Guerrero-Romero F, Simental-Mendía LE, González-Ortiz M, Martínez-Abundis E, Ramos-Zavala MG, Hernández-González SO, et al. The product of triglycerides and glucose, a simple measure of insulin sensitivity. Comparison with the euglycemic-hyperinsulinemic clamp. *J Clin Endocrinol Metab*. (2010) 95:3347–51. doi: 10.1210/jc.2010–0288

11. Park B, Lee HS, Lee Y-J. Triglyceride glucose (TyG) index as a predictor of incident type 2 diabetes among nonobese adults: a 12-year longitudinal study of the Korean Genome and Epidemiology Study cohort. *Transl Res*. (2021) 228:42–51. doi: 10.1016/j.trsl.2020.08.003

12. Jung M-H, Yi S-W, An SJ, Yi J-J, Ihm S-H, Han S, et al. Associations between the triglyceride-glucose index and cardiovascular disease in over 150,000 cancer survivors: a population-based cohort study. *Cardiovasc Diabetol*. (2022) 21:52. doi: 10.1186/s12933-022-01490-z

13. Wang J, Yan S, Cui Y, Chen F, Piao M, Cui W. The diagnostic and prognostic value of the triglyceride-glucose index in metabolic dysfunction-associated fatty liver disease (MAFLD): A systematic review and meta-analysis. *Nutrients*. (2022) 14:4969. doi: 10.3390/nu14234969

14. Shi H, Zhou L, Yang S, Zhou H. The relationship between Triglyceride and glycose (TyG) index and the risk of gynaecologic and breast cancers. *Clin Nutr ESPEN*. (2022) 51:345–52. doi: 10.1016/j.clnesp.2022.08.004

15. Shi H, Guo F, Zheng K, Li R, Zhou H. Triglyceride-glucose index (TyG index) and endometrial carcinoma risk: A retrospective cohort study. *Int J Gynaecol Obstet*. (2024) 164:298–304. doi: 10.1002/ijgo.15038

16. Centers for Disease Control and Prevention. About the National Health and Nutrition Examination Survey . Available online at: http://www.cdc.gov/nchs/nhanes/about_nhanes.htm.

17. Vercellini P, Viganò P, Somigliana E, Fedele L. Endometriosis: pathogenesis and treatment. *Nat Rev Endocrinol*. (2014) 10:261–75. doi: 10.1038/nrendo.2013.255

18. Shafrir AL, Farland LV, Shah DK, Harris HR, Kvaskoff M, Zondervan K, et al. Risk for and consequences of endometriosis: A critical epidemiologic review. *Best Pract Res Clin Obstet Gynaecol*. (2018) 51:1–15. doi: 10.1016/j.bpobgyn.2018.06.001

19. Brito A, Hermsdorff HHM, Filgueiras MDS, Suhett LG, Vieira-Ribeiro SA, Franceschini S, et al. Predictive capacity of triglyceride-glucose (TyG) index for insulin resistance and cardiometabolic risk in children and adolescents: a systematic review. *Crit Rev Food Sci Nutr*. (2021) 61:2783–92. doi: 10.1080/10408398.2020.1788501

20. Zhou Y, Zeng C, Li X, Wu P-L, Yin L, Yu X-L, et al. IGF-I stimulates ERβ and aromatase expression via IGF1R/PI3K/AKT-mediated transcriptional activation in endometriosis. *J Mol Med (Berl)*. (2016) 94:887–97. doi: 10.1007/s00109–016-1396–1

21. Kim JG, Suh CS, Kim SH, Choi YM, Moon SY, Lee JY. Insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs), and IGFBP-3 protease activity in the peritoneal fluid of patients with and without endometriosis. *Fertil Steril*. (2000) 73:996–1000. doi: 10.1016/s0015–0282(00)00493–3

22. Rohm TV, Meier DT, Olefsky JM, Donath MY. Inflammation in obesity, diabetes, and related disorders. *Immunity*. (2022) 55:31–55. doi: 10.1016/j.immuni.2021.12.013

23. Lin Y-K, Li Y-Y, Li Y, Li D-J, Wang X-L, Wang L, et al. SCM-198 prevents endometriosis by reversing low autophagy of endometrial stromal cell via balancing ERα and PR signals. *Front Endocrinol*. (2022) 13:858176. doi: 10.3389/fendo.2022.858176

24. Maturana MA, Spritzer PM. Association between hyperinsulinemia and endogenous androgen levels in peri- and postmenopausal women. *Metabolism*. (2002) 51:238–43. doi: 10.1053/meta.2002.29997

25. De Paoli M, Zakharia A, Werstuck GH. The role of estrogen in insulin resistance: A review of clinical and preclinical data. *Am J Pathol*. (2021) 191:1490–8. doi: 10.1016/j.ajpath.2021.05.011

26. Peterson CM, Johnstone EB, Hammoud AO, Stanford JB, Varner MW, Kennedy A, et al. Risk factors associated with endometriosis: importance of study population for characterizing disease in the ENDO Study. *Am J Obstet Gynecol*. (2013) 208:451.e1–11. doi: 10.1016/j.ajog.2013.02.040

27. Jeong SH, Joo HJ, Kwon J, Park E-C. Association between smoking behavior and insulin resistance using triglyceride-glucose index among South Korean adults. *J Clin Endocrinol Metab*. (2021) 106:e4531–41. doi: 10.1210/clinem/dgab399

28. Tatsumi Y, Morimoto A, Asayama K, Sonoda N, Miyamatsu N, Ohno Y, et al. Association between alcohol consumption and incidence of impaired insulin secretion and insulin resistance in Japanese: The Saku study. *Diabetes Res Clin Pract*. (2018) 135:11–7. doi: 10.1016/j.diabres.2017.10.021

29. Vercellini P, Eskenazi B, Consonni D, Somigliana E, Parazzini F, Abbiati A, et al. Oral contraceptives and risk of endometriosis: a systematic review and meta-analysis. *Hum Reprod Update*. (2011) 17:159–70. doi: 10.1093/humupd/dmq042

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 02 August 2024
DOI 10.3389/fendo.2024.1328403

OPEN ACCESS

EDITED BY
Achmad Kemal Harzif,
University of Indonesia, Indonesia

REVIEWED BY
Maria Stamou,
Massachusetts General Hospital and Harvard Medical School, United States
Zhibo Tan,
Heyou International Hospital, China

*CORRESPONDENCE
Yiqing Zhao
Yiqing_Zhao99@hotmail.com

†These authors have contributed equally to this work

RECEIVED 26 October 2023
ACCEPTED 09 July 2024
PUBLISHED 02 August 2024

CITATION
Shen L, Li J, Zhang H and Zhao Y (2024) Causal effects of endometriosis stages and locations on menstruation, ovulation, reproductive function, and delivery modes: a two-sample Mendelian randomization study. *Front. Endocrinol.* 15:1328403. doi: 10.3389/fendo.2024.1328403

Causal effects of endometriosis stages and locations on menstruation, ovulation, reproductive function, and delivery modes: a two-sample Mendelian randomization study

Lin Shen[1,2,3†], Jie Li[1†], Hanwang Zhang[1] and Yiqing Zhao[1*]

[1]Department of Reproductive Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2]National Research Institute for Family Planning, Beijing, China, [3]Graduate School, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

Background: Endometriosis is a chronic inflammatory disease of women during their reproductive years. The relationship between the severity and location of endometriosis and menstruation, ovulation, reproductive function, and mode of delivery remains unclear.

Methods: We explored the association between the various phenotypes of endometriosis and menstruation, ovulation, reproductive function, and mode of delivery, using two-sample Mendelian randomization (MR) and summary data on endometriosis stages and locations from the FinnGen consortium and women's menstruation, ovulation, reproductive function, and mode of delivery from OpenGWAS and ReproGen. Inverse-variance weighting was used for the primary MR analysis. In addition, a series of sensitivity analyses, confounding analyses, co-localization analyses, and multivariate MR analyses were performed.

Results: MR analysis showed a negative effect of moderate to severe endometriosis on age at last live birth (OR = 0.973, 95% CI: 0.960–0.986) and normal delivery (OR = 0.999, 95% CI: 0.998–1.000; values for endpoint were excluded), ovarian endometriosis on age at last live birth (OR = 0.976, 95% CI: 0.965–0.988) and normal delivery (OR = 0.999, 95% CI: 0.998–1.000; values for endpoint were excluded), and fallopian tubal endometriosis on excessive irregular menstruation (OR = 0.966, 95% CI: 0.942–0.990). Bidirectional MR analysis showed that age at menarche had a negative causal effect on intestinal endometriosis (OR = 0.417, 95% CI: 0.216–0.804). All MR analyses were confirmed by sensitivity analyses, and only the genetic effects of moderate to severe endometriosis on normal delivery and age at last live birth were supported by co-localization evidence.

Conclusion: Our findings deepen the understanding of the relationship between various types of endometriosis and menstruation, ovulation, reproductive function, and mode of delivery and clarify the important role of moderate to severe endometriosis.

KEYWORDS

endometriosis, menstruation, ovulation, reproductive function, mode of delivery, Mendelian randomization

Introduction

Endometriosis is a chronic inflammatory disease that primarily affects women of reproductive age, with a prevalence of up to 10% (1, 2). It is characterized by the presence of endometrial tissue outside the body of the uterus and is associated with pelvic pain, dysmenorrhea, and infertility and its treatment is limited to hormonal therapy or surgical removal of disease (1, 3). Endometriosis is typically staged according to the revised American Society of Reproductive Medicine (ASRM) criteria, with milder lesions in stages 1–2 and more severe lesions in stages 3–4. In addition, ectopic endometrium can invade almost any part of the body, including the lungs and pleura, but most commonly the pelvic organs and parietal peritoneum (4).

The etiology of endometriosis remains unknown, but several risk factors have been reported. To date, it is known that earlier age at menarche and shorter menstrual cycles are associated with an increased risk of endometriosis, whereas higher parity is associated with a lower risk (5). However, these studies have lacked associations between various phenotypic features of endometriosis, including ASRM stages and locations, and menstruation, ovulation, reproductive function, and mode of delivery, consistently focusing on the broad category of endometriosis, with far less information on its subcategories. Moreover, most of these studies only included risk factors for endometriosis and lacked findings on the possible consequences of endometriosis.

In addition, studies on factors such as menstruation, ovulation, reproductive function, and mode of delivery in relation to endometriosis have been mostly limited to epidemiologic observations and cohort studies. Therefore, it is difficult to draw causal conclusions from these studies due to possible biases and potential confounders. In this manuscript, we investigated the risk factors and consequences of endometriosis based on GWAS using Mendelian randomization (MR). MR is an analytical method for assessing causal inference in epidemiologic studies in which endometriosis can be either an exposure or an outcome, and uses genetic variants that are strongly associated with exposure as instrumental variables (IVs) to assess the causal relationship between exposure and outcome.

Here, we reported a comprehensive GWAS-based MR analysis of the association between different stages and locations of endometriosis and menstruation, ovulation, reproductive function, and mode of delivery, elucidating the causal effects of endometriosis sub-phenotypes leading to various components of the complex pathophysiology and improving our understanding of the risk factors and consequences of endometriosis.

Methods

We selected genetic variants that were strongly associated with different ASRM stages and locations of endometriosis from the FinnGen consortium with a sample size of 210,870 women, including endometriosis ASRM stages 1–2 and ASRM stages 3–4, and endometriosis of ovarian, fallopian tube and pelvic peritoneum, rectovaginal septum and vagina, and intestine. At the same time, we collected the summary level data from OpenGWAS and ReproGen study for GWAS on menstruation (excessive irregular menstruation, and menstrual cycle length), ovulation (age at menarche, age at natural menopause, years ovulating), reproductive function (age at first live birth, age at last live birth, number of live births, spontaneous abortion), and mode of delivery (normal delivery, caesarean section) (6, 7). In this study, we used two-sample MR analysis to investigate the genetic basis between different stages and locations of endometriosis and menstruation, ovulation, reproductive function, and mode of delivery and assess the causal effects among them.

Data information

Phenotypes were described in detail as follows: (1) different ASRM stages and locations of endometriosis; (2) menstruation (excessive irregular menstruation and menstrual cycle length); (3) ovulation (age at menarche, age at natural menopause, and years ovulating); (4) reproductive function (age at first live birth, age at last live birth, number of live births, and spontaneous abortion); (5) mode of delivery (normal delivery and caesarean section). All genetic association estimates were derived from GWAS studies conducted in European populations, and the design and analysis protocols (such as adjustment for covariates) were available for query in publications (7–9). An overview of the data is shown in Figure 1, and detailed information can be found in Additional File 2: Table 1.

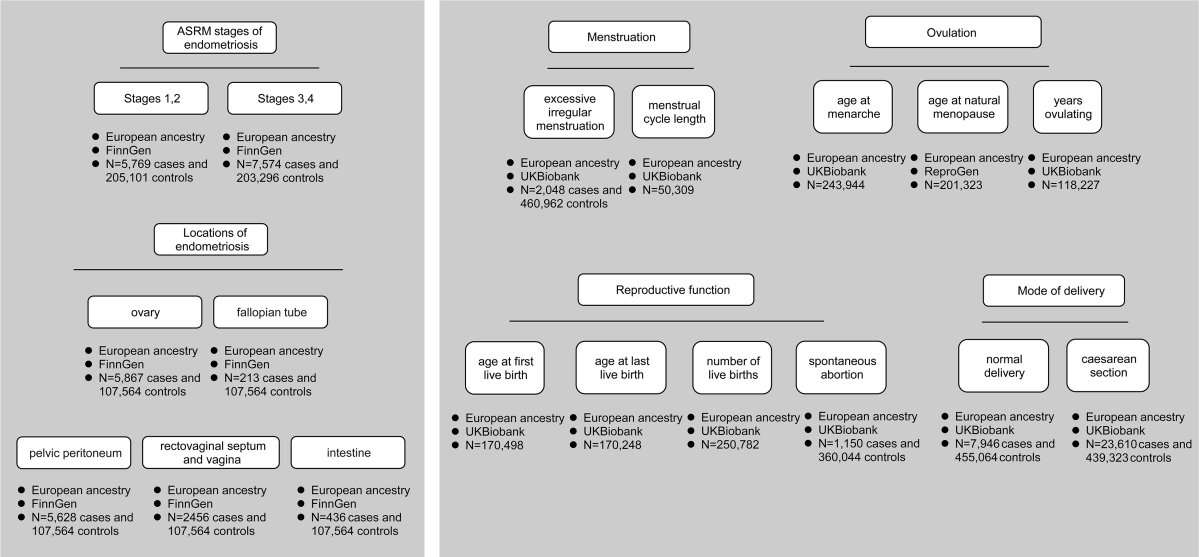


FIGURE 1
Data overview. It shows different ASRM stages and locations of endometriosis, menstruation, ovulation, reproductive function, and mode of delivery. Each phenotype labels the data source and the number of cases and controls.

Endometriosis

In FinnGen, N14 codes for endometriosis. The subitems of N14 were obtained, which were ASRM stages 1–2 and ASRM stages 3–4, as well as endometriosis of ovary, fallopian tube, pelvic peritoneum, rectovaginal septum and vagina, and intestine. Controls for a particular phenotype of endometriosis were those individuals who were not cases.

Menstruation

In OpenGWAS, ICD10: N92.1 was included in the analysis, and women with this code were diagnosed with excessive and frequent menstruation with irregular cycles (N = 2,048). It is referred to in this manuscript as excessive irregular menstruation. The length of menstrual cycle refers to the time since the last menstrual period (N = 50,309).

Ovulation

A total of 243,944 individuals of European ancestry who self-reported their age at menarche, ranging from 5 to 25 years, were included in OpenGWAS. Summary data on the age at natural menopause were reported by Ruth et al., who reported an age of 40 to 60 years, and were stored in the ReproGen database (N = 201,323) (8). Years ovulating was defined as the time between age at menarche and menopause, calculated by D'Urso et al. using UK Biobank data, as detailed in the publication (10).

Reproductive function

Age at first and last live birth was collected from women who reported having more than one child. The number of live births ranged from 0 to 22, with a median of 2. ICD10: O03 coded for spontaneous abortion.

Mode of delivery

GWAS summary data on normal deliveries and caesarean sections were available in OpenGWAS, which included 7,946 normal deliveries and 23,610 caesarean sections.

Selection of genetic instruments and data harmonization

We selected all single-nucleotide polymorphisms (SNPs) strongly associated with exposure as instrumental variables (IVs), with a genome-wide significance threshold of $p < 5 \times 10^{-8}$, which was $p < 5 \times 10^{-6}$ for endometriosis of the fallopian tube and intestine. Moreover, the strength of each IV was assessed, and when F >10, the SNP was included (10). All SNPs in linkage disequilibrium (LD) were trimmed with a window of $r^2 < 0{\cdot}001$ and size <10,000 kb, and at the same time, SNPs with minor allele frequency (MAF) >0.5% were retained. IVs that met the above quality control are shown in Additional File 1: Table 1. Then, SNP exposure and SNP outcome were harmonized with the use of the "harmonise_data" function (11). SNPs for incompatible alleles and being palindromic with intermediate allele frequencies were removed during harmonization, if present.

Mendelian randomization analysis

Univariate MR analyses were performed to estimate the causal effect of each type of endometriosis on (1) menstruation, (2) ovulation, (3) reproductive function, and (4) mode of delivery. Inverse variance weighted (IVW) MR was used as the primary analysis method unless only one SNP was available (in which case

the Wald ratio was used). IVW can provide unbiased estimates of outcomes in the absence of horizontal pleiotropy in IVs (12). In addition, the IVW method can account for the heterogeneity of causal estimates obtained from individual variants (13, 14).

Bidirectional Mendelian randomization analysis

To investigate the possibility of reverse causality between exposure and outcome, bidirectional MR analyses were performed (exposures and outcomes were reversed in the analyses). We used a $p < 5 \times 10^{-8}$ to select the IVs associated with exposure, and the p value threshold was appropriately relaxed to 5×10^{-6} for excessive irregular menstruation, menstrual cycle length, spontaneous abortion, normal delivery, and cesarean section. Other analysis details were the same as for the MR analysis. The available IVs are shown in Additional File 1: Table 2.

Sensitivity analyses

For each causal effect detected, we used a series of sensitivity analyses to assess the robustness of the MR analysis. First, Cochran's Q statistic, including both IVW and MR–Egger methods, was used to evaluate the heterogeneity of IVs in the causal effects. Second, MR–Egger regression was used to evaluate the presence of horizontal pleiotropy; if the intercept term equals zero, it indicates that horizontal pleiotropy does not exist. Third, the MR-pleiotropy residual sum and outlier (MR-PRESSO) analysis obtained horizontal pleiotropy by detecting abnormal IVs in causal effects. If abnormal IVs were present, outliers were removed, and MR analyses and sensitivity analyses were repeated. When <50% of the instruments showed horizontal pleiotropy, the MR-PRESSO test was most appropriate (15). Finally, we performed a leave-one-out sensitivity analysis by stepwise elimination of each IV to see if the results changed after each SNP was removed.

Confounding analysis

We used the "phenoscanner" package (version 1.0) for confounding analysis (16). Diseases/physical conditions significantly associated with IV were explored using $p < 5 \times 10^{-8}$ as the threshold.

Colocalization analysis

The coloc R package (version 5.2.2) was used to perform a Bayesian test for co-localization of the two traits to estimate the posterior probability of shared variation (17). We retrieved all SNPs within the upper and lower 50 kb of each IV for co-localization analysis to analyze the posterior probability of H4 (PP.H4), with PP.H4 >0.95 indicating co-localization of the two traits.

Multivariable Mendelian randomization

The exposures included in the multivariable MR (MVMR) analysis were ASRM stages 3–4 and ovarian endometriosis. Univariate MR analysis suggested a causal relationship between these exposures and age at last live birth and normal delivery. We used MVMR to account for potential horizontal pleiotropy (18). The inclusion of IVs for MVMR was the same as for MR analysis.

Statistical analysis

The results of MR analysis were presented as odds ratio (OR) and 95% confidence interval (CI). Due to the presence of multiple comparisons with causal effects, we corrected the p-values with the false discovery rate (FDR), with a threshold of an adjusted p-value < 0.1. This work was performed under R packages "TwoSampleMR" (version 0.5.7), "MR-PRESSO" (version 1.0), and "MVMR" (version 0.4) (11, 15).

Results

Causal effects of endometriosis stage and location on menstruation, ovulation, reproductive function, and mode of delivery

As shown in Figure 2, MR analysis showed a negative effect of ASRM stages 3–4 endometriosis on age at last live birth (OR = 0.973, 95% CI: 0.960–0.986) and normal delivery (OR = 0.999, 95% CI: 0.998–1.000, excluding the value of the endpoint 1.000). We also assessed the effect of different locations of endometriosis on various outcomes. IVW estimates showed a negative effect of ovarian endometriosis on age at last live birth (OR = 0.976, 95% CI: 0.965–0.988) and normal delivery (OR = 0.999, 95% CI: 0.998–1.000, values for endpoint 1.000 were excluded). Endometriosis in the fallopian tube was also observed to have a negative effect on excessive irregular menstruation (OR = 0.966, 95% CI: 0.942–0.990) (Figure 2). All MR estimates can be viewed in Additional File 1: Table 3.

Horizontal pleiotropy and heterogeneity analyses showed that the causal effects of ASRM stages 3–4 endometriosis and ovarian endometriosis on normal labor and tubal on excessive menstruation were robust (Additional File 2: Tables 2, 4, 6). MR-PRESSO analyses showed significant horizontal pleiotropy between IVs in the causal effect of ASRM stages 3–4 endometriosis and ovarian endometriosis on age at last live birth, identifying three abnormal IVs, respectively (Additional File 2: Tables 3, 5). After removing the outliers, the MR analysis was re-performed, and horizontal pleiotropy and heterogeneity between IVs were corrected (Additional File 2: Tables 3, 5). The leave-one-out test did not identify SNPs with abnormal effects (Additional File 2: Figure 1). This manuscript only listed the corrected MR Results (the same below).

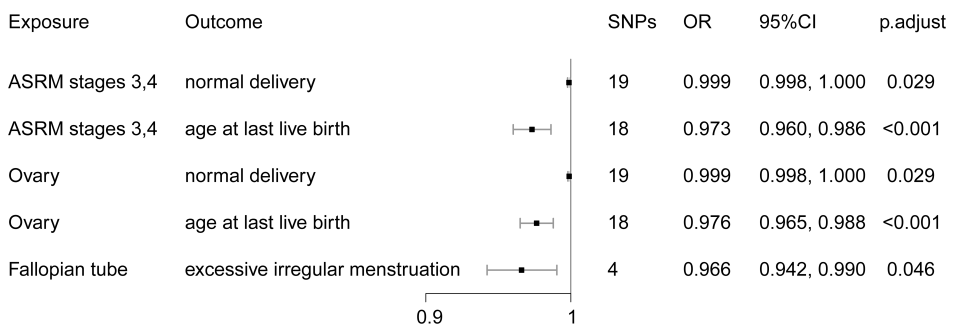


FIGURE 2
MR estimates for the association between endometriosis stages and locations and menstruation, ovulation, reproductive function, and mode of delivery.

Bidirectional Mendelian randomization analysis

Bidirectional MR analysis showed that age at menarche had a negative causal effect on intestinal endometriosis (OR = 0.417, 95% CI: 0.216–0.804), with an increase in age at menarche and a decrease in risk of the disease (Figure 3). No other causal effects were detected. Additional File 1: Table 4 demonstrated all MR estimates.

There was horizontal pleiotropy in the IVs for age at menarche; MR-PRESSO analysis excluded one SNP (Additional File 2: Table 7). After removal of outliers, horizontal pleiotropy of IVs was corrected, whereas heterogeneity among IVs remained (Additional File 2: Table 7). However, our results were generated based on the random-effects model (IVW), which remained robust in the presence of heterogeneity (13, 14). The leave-one-out test showed no outliers in the IVs (Additional File 2: Figure 1).

Confounding analysis

IVs in causal effects were summarized and analyzed with various confounders by Phenoscanner. We found that potential confounders of ASRM stages 3–4 and ovarian endometriosis mainly included trunk predicted mass, birth weight, coagulation dysfunction, height, heel bone mineral density, atopic dermatitis, potential confounders of fallopian tubal endometriosis mainly including mean plasma volume, mean hemoglobin, and erythrocyte distribution width, and potential confounders of age at menarche mainly including body mass index, birth weight, and triglycerides (Additional File 2: Figures 2, 3, 4, 5).

Colocalization analysis

By co-localization analysis (PP.H4 >0.95), we found evidence to support the existence of shared causal variants between ASRM stages 3 and 4 endometriosis with normal delivery and age at last live birth (Additional File 2: Table 8). There was no evidence of co-localization for the remaining four causal effects.

Multivariable Mendelian randomization

We further assessed the association between ASRM stages 3–4 and ovarian endometriosis with normal delivery and age at last live birth. Both ASRM stages 3–4 and ovarian endometriosis were not associated with normal delivery and age at last live birth in the MVMR framework (Additional File 2: Tables 9, 10).

Discussion

In this study, we performed a two-sample MR analysis using large genome-wide association datasets to evaluate putative causal relationships between seven types of endometriosis and menstruation, ovulation, reproductive function, and mode

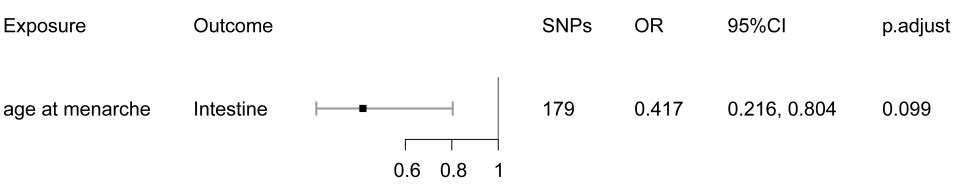


FIGURE 3
Bidirectional MR analysis.

of delivery. We found negative causal effects of ASRM stage 3–4 endometriosis on age at last live birth and on normal delivery, ovarian endometriosis on age at last live birth, and normal delivery, and fallopian tubal endometriosis on excessive irregular menstruation. In addition, bidirectional MR showed that age at menarche had a negative causal effect on intestinal endometriosis. All MR analyses were confirmed by sensitivity analyses, and further, the genetic effects of ASRM stage 3–4 endometriosis on normal delivery and age at last live birth were supported by co-localization evidence. Our findings highlight the genetic association between stages and locations of endometriosis and menstruation, ovulation, reproductive function, and mode of delivery.

Previous efforts, including cohort studies and observational studies, have focused on the effect of the severity of endometriosis on the ability of assisted reproductive technology (ART) to achieve fertility (19–22). We reported an association between endometriosis severity and normal delivery and age at last live birth, with moderate-to-severe endometriosis impairing normal delivery and advancing the age at last live birth. Moreover, our findings supported that menstruation, ovulation, reproductive function, and mode of delivery were not associated with mild endometriosis.

We also investigated the causal effects of endometriosis locations on menstruation, ovulation, reproductive function, and mode of delivery. In this study, our analyses found that ovarian endometriosis showed a greater impact on women, followed by fallopian tube, and endometriosis of pelvic peritoneum, rectovaginal septum and vagina, and intestine were unrelated exposures. In brief, ovarian endometriosis brought forward the age at last live birth and impaired normal delivery, whereas fallopian tubal endometriosis reduced the risk of excessive irregular menstruation.

The association between ovarian endometriosis and age at first live birth was noted by Tuominen et al. They found that the age at first live birth was greatest in women with ovarian endometriosis compared with those with peritoneal and deep endometriosis (23). This is inconsistent with our finding that we did not find such a correlation. First, we compared ovarian endometriosis with non-endometriosis and endometriosis elsewhere, whereas Tuominen et al. compared ovarian endometriosis with peritoneal and deep endometriosis. Second, our study included a more general population, whereas the study by Tuominen et al. was limited to the first live birth before surgical confirmation of endometriosis and excluded women with mild symptoms who were conservatively diagnosed and treated for endometriosis. In addition, Parazzini et al. also suggested that age at first live birth was not associated with endometriosis (24).

Menstrual cycle length has been widely reported to be associated with endometriosis (5). Short menstrual cycles increase the risk of endometriosis (25, 26). However, we did not find an association between menstrual cycle length and endometriosis. In fact, the effect of the menstrual cycle length on endometriosis is inconsistent across studies, and several studies suggested that the menstrual cycle length was not related to endometriosis (27, 28). Similarly, we did not find an association between endometriosis and the number of live births, although it was previously thought that the risk of endometriosis decreased with increasing number of births (24, 28, 29).

In addition to menstrual cycle length, age at menarche has also been reported. The earlier the age at menarche, the higher the risk of endometriosis (25, 30, 31). However, it has also been shown that there was no association between endometriosis and age at menarche (24, 28, 32). We found that the effect of age at menarche on endometriosis may be limited. The earlier the age at menarche, the higher the risk of intestinal endometriosis. This finding may explain the discrepancy between studies.

In addition, negative effects on age at last live birth and normal delivery were observed for both moderate-to-severe endometriosis and ovarian endometriosis. These two types of endometriosis can lead to abnormal delivery and an earlier age at last live birth, shortening reproductive life. This may suggest that clinical attention needs to be paid to the early detection and timely intervention of these two types of endometriosis to ameliorate their adverse effects on women's labor and reproductive life span.

When both exposures (moderate-to-severe endometriosis and ovarian endometriosis) were considered in the same multivariate model, neither was associated with outcomes, indicating collinearity of moderate-to-severe endometriosis and ovarian endometriosis. In fact, there was substantial overlap between IVs in moderate-to-severe endometriosis and ovarian endometriosis. We speculated that there might be a causal effect between moderate-to-severe endometriosis and ovarian endometriosis, i.e., ovarian endometriosis tends to be more severe. This is beyond the scope of this manuscript, and therefore we did not explore it further, but it could be explored in the future.

The main strength of this study is the utilization of summary-level data on non-overlapping exposures and outcomes in the two-sample MR framework for causal inference. This is a comprehensive analysis of the genetic association between the stages and locations of endometriosis and menstruation, ovulation, reproductive function, and mode of delivery. In addition, we conduct a series of sensitivity analyses, confounding analyses, and co-localization analyses to explore the bias introduced by violating the MR assumption. We also used MVMR to explore specific biases caused by horizontal pleiotropy via moderate-to-severe endometriosis and ovarian endometriosis.

Several limitations of this study should be considered when interpreting the results. First, our findings may have been affected by cohort selection bias, with the population in the sample representing a subset of the total population. In addition, because all genetic variants were derived from genomic studies of European populations, it is not known whether the findings of this study apply to other ethnic populations. Second, endometriosis may occur not only at the five locations described in this manuscript but also at other locations, such as lungs and pleura. We only performed the analyses of the five common locations of endometriosis, and the reason for not analyzing other locations was the lack of corresponding data. Next, due to the lack of IVs, SNPs used for several exposures in the analysis did not meet the traditional GWAS significance threshold ($p < 5 \times 10^{-8}$), which was relaxed to $<5 \times 10^{-6}$. This did not affect the reliability of the study's conclusions, and our main conclusions are based on strict thresholds and a series of subsequent analyses.

In conclusion, this two-sample MR study provided a comprehensive analysis of the genetic associations between stages and locations of endometriosis and menstruation, ovulation, reproductive function, and mode of delivery. Our study elucidates that moderate-to-severe endometriosis and ovarian endometriosis can lead to abnormal deliveries and a shortened reproductive life span. Both types of endometriosis should be detected promptly and intervened early in clinical practice.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Only summary level data were used in this study. Ethical approval is available in the publications (6–8).

Author contributions

LS: Data curation, Investigation, Validation, Writing – original draft. JL: Methodology, Visualization, Writing – review & editing. HZ: Conceptualization, Writing – review & editing. YZ: Conceptualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by the Basic Research Project for Young and Middle-Aged Physicians of the Beijing Health Promotion Association (BJHPA-2022-SHZHYX ZHQNYJ-JICH-002) and Scientific Research Starting Foundation for Returned Overseas Scholars (2020HGRY004) of Tongji Hospital.

Acknowledgments

We want to acknowledge the participants and investigators of the FinnGen, OpenGWAS, ReproGen, and UK Biobank study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1328403/full#supplementary-material

References

1. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *N Engl J Med*. (2020) 382:1244–56. doi: 10.1056/NEJMra1810764

2. Rowlands IJ, Abbott JA, Montgomery GW, Hockey R, Rogers P, Mishra GD. Prevalence and incidence of endometriosis in Australian women: A data linkage cohort study. *BJOG*. (2021) 128:657–65. doi: 10.1111/1471-0528.16447

3. Marquardt RM, Tran DN, Lessey BA, Rahman MS, Jeong J-W. Epigenetic dysregulation in endometriosis: implications for pathophysiology and therapeutics. *Endocr Rev*. (2023) 44:1074–95. doi: 10.1210/endrev/bnad020

4. Arafah M, Rashid S, Akhtar M. Endometriosis: A comprehensive review. *Adv Anat Pathol*. (2021) 28:30–43. doi: 10.1097/PAP.0000000000000288

5. Shafrir AL, Farland LV, Shah DK, Harris HR, Kvaskoff M, Zondervan K, et al. Risk for and consequences of endometriosis: A critical epidemiologic review. *Best Pract Res Clin Obstet Gynaecol*. (2018) 51:1–15. doi: 10.1016/j.bpobgyn.2018.06.001

6. Lyon MS, Andrews SJ, Elsworth B, Gaunt TR, Hemani G, Marcora E. The variant call format provides efficient and robust storage of gwas summary statistics. *Genome Biol*. (2021) 22:32. doi: 10.1186/s13059-020-02248-0

7. Kurki MI, Karjalainen J, Palta P, Sipilä TP, Kristiansson K, Donner KM, et al. Finngen provides genetic insights from a well-phenotyped isolated population. *Nature*. (2023) 613:508–18. doi: 10.1038/s41586-022-05473-8

8. Ruth KS, Day FR, Hussain J, Martínez-Marchal A, Aiken CE, Azad A, et al. Genetic insights into biological mechanisms governing human ovarian ageing. *Nature*. (2021) 596:393–7. doi: 10.1038/s41586-021-03779-7

9. Available online at: http://www.nealelab.is/uk-biobank/.

10. D'Urso S, Arumugam P, Weider T, Hwang L-D, Bond TA, Kemp JP, et al. Mendelian randomization analysis of factors related to ovulation and reproductive function and endometrial cancer risk. *BMC Med*. (2022) 20:419. doi: 10.1186/s12916-022-02585-w

11. Hemani G, Zheng J, Elsworth B, Wade KH, Haberland V, Baird D, et al. The mr-base platform supports systematic causal inference across the human phenome. *Elife*. (2018) 7:e34408. doi: 10.7554/eLife.34408

12. Burgess S, Dudbridge F, Thompson SG. Combining information on multiple instrumental variables in mendelian randomization: comparison of allele score and summarized data methods. *Stat Med*. (2016) 35:1880–906. doi: 10.1002/sim.6835

13. Burgess S, Davey Smith G, Davies NM, Dudbridge F, Gill D, Glymour MM, et al. Guidelines for performing mendelian randomization investigations. *Wellcome Open Res*. (2019) 4:186. doi: 10.12688/wellcomeopenres.15555.2

14. Burgess S, Butterworth A, Thompson SG. Mendelian randomization analysis with multiple genetic variants using summarized data. *Genet Epidemiol*. (2013) 37:658–65. doi: 10.1002/gepi.21758

15. Verbanck M, Chen C-Y, Neale B, Do R. Detection of widespread horizontal pleiotropy in causal relationships inferred from mendelian randomization between complex traits and diseases. *Nat Genet*. (2018) 50:693–8. doi: 10.1038/s41588-018-0099-7

16. Kamat MA, Blackshaw JA, Young R, Surendran P, Burgess S, Danesh J, et al. Phenoscanner V2: an expanded tool for searching human genotype-phenotype associations. *Bioinformatics*. (2019) 35:4851–3. doi: 10.1093/bioinformatics/btz469

17. Giambartolomei C, Vukcevic D, SChadt EE, Franke L, Hingorani AD, Wallace C, et al. Bayesian test for colocalisation between pairs of genetic association studies using summary statistics. *PloS Genet*. (2014) 10:e1004383. doi: 10.1371/journal.pgen.1004383

18. Sanderson E, Spiller W, Bowden J. Testing and correcting for weak and pleiotropic instruments in two-sample multivariable mendelian randomization. *Stat Med*. (2021) 40:5434–52. doi: 10.1002/sim.9133

19. Horton J, Sterrenburg M, Lane S, Maheshwari A, Li TC, Cheong Y. Reproductive, obstetric, and perinatal outcomes of women with adenomyosis and endometriosis: A systematic review and meta-analysis. *Hum Reprod Update*. (2019) 25:592–632. doi: 10.1093/humupd/dmz012

20. Soriano D, Adler I, Bouaziz J, Zolti M, Eisenberg VH, Goldenberg M, et al. Fertility outcome of laparoscopic treatment in patients with severe endometriosis and repeated *in vitro* fertilization failures. *Fertil Steril*. (2016) 106:1264–9. doi: 10.1016/j.fertnstert.2016.06.003

21. Zimmermann A, Faust C, Miquel L, Berbis J, Perrin J, Courbiere B. Impact of moderate-to-severe endometriosis on ivf cumulative live birth rate: A retrospective matched cohort study. *Reprod BioMed Online*. (2023) 47:103186. doi: 10.1016/j.rbmo.2023.02.012

22. Huang X-d, Sun Z-Y, Yu Q. Impact of endometriosis on embryo quality and endometrial receptivity in women undergoing assisted reproductive technology. *Reprod Biol*. (2023) 23:100733. doi: 10.1016/j.repbio.2023.100733

23. Tuominen A, Saavalainen L, Niinimäki M, Gissler M, But A, Härkki P, et al. First live birth before surgical verification of endometriosis-a nationwide register study of 18 324 women. *Hum Reprod*. (2023) 38:1520–8. doi: 10.1093/humrep/dead120

24. Parazzini F, Ferraroni M, Fedele L, Bocciolone L, Rubessa S, Riccardi A. Pelvic endometriosis: reproductive and menstrual risk factors at different stages in lombardy, northern Italy. *J Epidemiol Community Health*. (1995) 49:61–4. doi: 10.1136/jech.49.1.61

25. Missmer SA, Hankinson SE, Spiegelman D, Barbieri RL, Malspeis S, Willett WC, et al. Reproductive history and endometriosis among premenopausal women. *Obstet Gynecol*. (2004) 104:965–74. doi: 10.1097/01.AOG.0000142714.54857.f8

26. Matalliotakis IM, Cakmak H, Fragouli YG, Goumenou AG, Mahutte NG, Arici A. Epidemiological characteristics in women with and without endometriosis in the yale series. *Arch Gynecol Obstet*. (2008) 277:389–93. doi: 10.1007/s00404-007-0479-1

27. Blum S, Fasching PA, Hildebrandt T, Lermann J, Heindl F, Born T, et al. Comprehensive characterization of endometriosis patients and disease patterns in a large clinical cohort. *Arch Gynecol Obstet*. (2022) 305:977–84. doi: 10.1007/s00404-021-06200-w

28. Peterson CM, Johnstone EB, Hammoud AO, Stanford JB, Varner MW, Kennedy A, et al. Risk factors associated with endometriosis: importance of study population for characterizing disease in the endo study. *Am J Obstet Gynecol*. (2013) 208:451.e1–.11. doi: 10.1016/j.ajog.2013.02.040

29. Sangi-Haghpeykar H, Poindexter AN. Epidemiology of endometriosis among parous women. *Obstet Gynecol*. (1995) 85:983–92. doi: 10.1016/0029-7844(95)00074-2

30. Nnoaham KE, Webster P, Kumbang J, Kennedy SH, Zondervan KT. Is Early Age at Menarche a Risk Factor for Endometriosis? A Systematic Review and Meta-Analysis Of case-Control Studies. *Fertil Steril*. (2012) 98:702–12. doi: 10.1016/j.fertnstert.2012.05.035

31. Kim HJ, Lee HS, Kazmi SZ, Hann HJ, Kang T, Cha J, et al. Familial Risk for Endometriosis and Its Interaction with Smoking, Age at Menarche and Body Mass Index: A Population-Based Cohort Study among Siblings. *BJOG*. (2021) 128:1938–48. doi: 10.1111/1471-0528.16769

32. Ashrafi M, Sadatmahalleh SJ, Akhoond MR, Talebi M. Evaluation of risk factors associated with endometriosis in infertile women. *Int J Fertil Steril*. (2016) 10:11–21. doi: 10.22074/ijfs.2016.4763

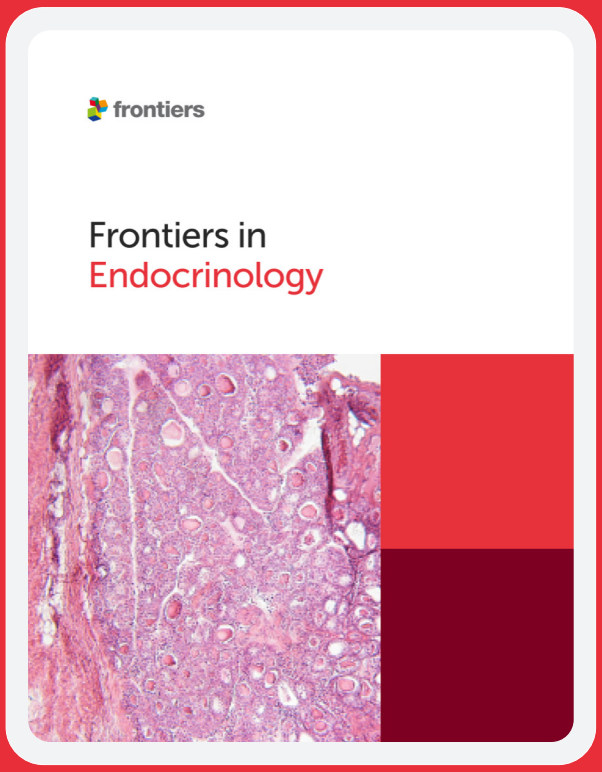
Frontiers in Endocrinology

Explores the endocrine system to find new therapies for key health issues

The second most-cited endocrinology and metabolism journal, which advances our understanding of the endocrine system. It uncovers new therapies for prevalent health issues such as obesity, diabetes, reproduction, and aging.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact