SEUNGBAEK LEE

Mining biomarkers for infertility-associated conditions

Studies on polycystic ovary syndrome and recurrent implantation failure through microbiome and AI-based approaches

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Lee, Seungbaek, Mining biomarkers for infertility-associated conditions. Studies on polycystic ovary syndrome and recurrent implantation failure through microbiome and AI-based approaches

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Abstract

The prevalence of female infertility has increased over the last decades. Reproductive disorders, such as polycystic ovary syndrome (PCOS) and recurrent implantation failure (RIF), are important contributors to female infertility. PCOS and RIF endometria have been shown to have altered immune profiles that may contribute to endometrial dysfunction. Furthermore, changes in the microbiome of the reproductive tract (RT) or the gut have also been observed in these conditions, potentially influencing host health as well as reproductive outcomes.

In this thesis, the RT microbiome, including the vagina and the endometrium, was investigated to characterize the landscape of microbial community across the menstrual cycle phases in women with PCOS and to identify microbial signatures associated with PCOS. Next, this thesis explored the gut microbiome among PCOS women who also experienced mood disorders (MDs), aiming to understand its association with both PCOS and MDs. Lastly, an artificial intelligence (AI) model was developed to perform histological assessments on the endometria of women with PCOS and women with RIF. The AI model examined endometrial gland proportions and inflammatory status evidenced as CD138+ plasma cell aggregation.

The studies conducted in this thesis revealed that the alpha diversity of the endometrial microbiome was significantly higher in women with PCOS during the early secretory phase compared to women without PCOS. Additionally, three bacterial taxa exhibited significantly different abundances in relation to PCOS. The gut microbiome varied based on MD status in women with PCOS, and specific bacteria correlated with common clinical traits of both PCOS and MDs. Last, the AI analysis showed variations in CD138+ cell percentages based on PCOS

phenotypes. On the other hand, endometrial receptivity did not affect either epithelial gland development or CD138+ cell aggregation in RIF patients.

These findings pave the way for future research on RT microbiome and adverse reproductive outcomes in PCOS and underline the possible roles of altered gut microbiome on MDs among women with PCOS. Additionally, the results indicate that AI could serve as a tool in clinical practice for screening inflammatory status and hold promise for large-scale sample analysis due to quick and accurate diagnostic potential.

Keywords: artificial intelligence, AI, female infertility, female reproductive tract, microbiome, mood disorder, polycystic ovary syndrome, PCOS, recurrent implantation failure, RIF

Lee, Seungbaek, Hedelmättömyyteen liittyvien biomarkkerien tunnistaminen. Tutkimuksia munasarjojen monirakulaoireyhtymästä ja toistuvasta alkion kiinnittymishäiriöstä mikrobiomin ja tekoälypohjaisten lähestymistapojen kautta

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Tiivistelmä

Naisesta johtuvan lapsettomuuden esiintyvyys on kasvanut viime vuosikymmeninä. Lisääntymishäiriöt, kuten munasarjojen monirakkulaoireyhtymä (polycystic ovary syndrome, PCOS) ja toistuva alkion kiinnittymishäiriö (recurrent implantation failure, RIF), ovat merkittäviä naisperäisessä lapsettomuuden taustatekijöitä. Aiempien tutkimusten perusteella on voitu todeta, että PCOS: ssä ja RIFssä kohdun limakalvon immuunisolujakaumat ovat muuntuneet, mikä saattaa edesauttaa kohdun limakalvon toimintahäiriöiden kehittymistä. Myös lisääntymiselinten ja suolen mikro-organismien ja bakteerikannan koostumuksella, mikrobiomilla, on todettu olevan merkitystä naisen terveyteen yleisesti mutta myös lisääntymisterveyteen.

Tässä väitöskirjassa tutkittiin emättimen ja endometriumin mikrobiomia naisilla, joilla on PCOS, tarkoituksena määrittää mikrobiyhteisön yleiskoostumus kuukautiskierron eri vaiheissa sekä etsiä oireyhtymään liittyviä tyypillisiä mikrobeja. Seuraavaksi väitöskirjassa tutkittiin suoliston mikrobiomia naisilla, joilla on sekä PCOS että mielenterveyshäiriö (mood disorder, MD), tavoitteena ymmärtää mikrobiomin yhteyttä näihin tiloihin. Viimeisissä töissä kehitettiin tekoälymalli (artificial intelligence, AI) suorittamaan histologisia arvioita PCOS: ää ja RIF: ia sairastavien naisten endometriumista. AI-mallin avulla tutkittiin endometriumin rauhasten määrää sekä tulehdustilaa, jonka merkkinä toimi CD138-positiivisten plasmasolujen kertyminen.

Tutkimukset paljastivat, että endometriumin mikrobiomin alfamonimuotoisuus oli varhaisessa sekretorisessa vaiheessa merkittävästi suurempi PCOS-naisilla, verrattuna naisiin, joilla ei ollut oireyhtymää. Lisäksi

kolmen bakteeritaksonin määrä oli merkittävästi erilainen PCOS:ään suhteutettuna. Havaitsimme myös, että naisilla, joilla on PCOS, suoliston mikrobiomin koostumus vaihteli sen mukaan, oliko naisella mielenterveyshäiriö. Tietyillä bakteereilla esiintyi korreltio sekä yleisten kliinisten piirteiden, että PCOS: n ja mielenterveyshäiriöiden kanssa. AI-analyysi puolestaan osoitti kuukautiskierron mukaisia muutoksia CD138+ solujen määrissä eri PCOS-fenotyypeissä. Toisaalta RIF-potilailla endometriumin reseptiivisyys ei vaikuttanut kohdun limakalvon epiteelirauhasrakenteiden kehitykseen tai CD138+ solujen kertymiseen.

Nämä löydökset avaavat mahdollisuuksia jatkotutkimuksille lisääntymiselinten mikrobiomin osalta ja korostavat muuntuneen suolistomikrobiomin mahdollista merkitystä mielenterveyshäiriöissä naisilla, joilla on PCOS. Lisäksi tulokset osoittavat, että AI voisi käytännön hoitotyössä toimia aputyökaluna tulehdustilan seulonnassa. AI vaikuttaa myös lupaavalta apuvälineeltä tutkia endometriumin häiriötiloja laajemmin erityisesti isoissa näytesarjoissa nopean ja osuvan diagnostiikan vuoksi.

Asiasanat: tekoäly, naisen lapsettomuus, naisen lisääntymiselimistö, mikrobiomi, mielialahäiriö, monirakkulainen munasarjaoireyhtymä, PCOS, toistuva implataatiohäiriö, RIF

Lee, Seungbaek, Viljatusega seotud biomarkerite tuvastamine: Polütsüstiliste munasarjade sündroomi ja korduvate implantatsiooni ebaõnnestumiste põhjuste mikrobioomi ja tehisintellektipõhised uuringud

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Abstrakt

Naiste viljatuse esinemissagedus on viimastel aastakümnetel suurenenud. Reproduktiivsed häired, nagu polütsüstiline munasarjade sündroom (PCOS) ja korduvad implanteerimise ebaõnnestumised (RIF), on olulised naise viljatuse põhjustajad. Varasemad uuringud on näidatud, et nii PCOS kui ka RIF patsientide endomeetriumi immuunprofiil on muutunud, mis võib aidata kaasa endomeetriumi talitluse häiretele ja viljatuse kujunemisele. Lisaks on nende seisundite puhul täheldatud ka reproduktiivse trakti ja/või soolestiku mikrobioomi muutuseid, mis võivad mõjutada patsiendi üldist tervist ja tema viljakust.

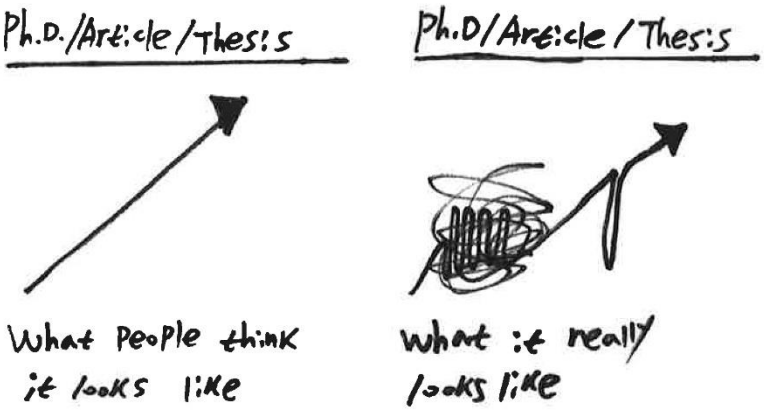
Käesolevas doktoritöös uuriti reproduktiivse trakti mikrobioomi, sealhulgas vaginaalseid ja endomeetriumi proove, et iseloomustada mikroobide kooslust menstruaaltsükli erinevates faasides PCOS-i diagnoosiga naistel ja tuvastada PCOS-iga seotud mikroobide profiil. Seejärel analüüsisime PCOS diagnoosiga naiste soolestiku mikrobioomi ja selle seost patsientidel esinevate meeleoluhäiretega. Viimase teemana arendati välja tehisintellektil (AI) baseeruv mudel, mis aitab anda histoloogilist hinnangut PCOS-i ja RIF diagnoosiga naiste endomeetriumi koe proovidele. AI-mudeliga uuriti menstruaaltsükli faasist sõltuvaid muutusi endomeetriumi näärmete proportsioonides ja põletikulises seisundis, mida iseloomustas CD138 positiivsete plasmarakkude esinemine.

Väitekirjas läbi viidud uuringud näitasid, et endomeetriumi mikrobioomi alfa mitmekesisus oli oluliselt suurem PCOS-iga naistel varases sekretsioonifaasis võrreldes naistega, kellel PCOS-i ei olnud. Lisaks tuvastati kolm bakteritaksonit, mille sagedused olid oluliselt erinevad PCOS-i diagnoosiga naistel. Lisaks varieerus soolestiku mikrobioom PCOS-ga naistel sõltuvalt nendel esinevast meeleoluhäirete diagnoosist ning spetsiifilised bakterid korreleerusid nii PCOS-i kui ka meeleoluhäirete ühiste kliiniliste tunnustega. Viimaks näitas AI analüüs

PCOS naistel muutusi CD138+ rakkude proportsioonis. Samas ei mõjutanud endomeetriumi retseptiivsus RIF patsientidel endomeetriumi koe epiteeli näärmete arengut ega CD138+ rakkude esinemist.

Tulemused sillutavad teed tulevastele uuringutele reproduktiivse trakti mikrobioomi ja viljatuse omavaheliste seoste kohta PCOS ja RIF patsientidel ning rõhutavad soolestiku mikrobioomi muutuste võimalikku rolli PCOS diagnoosiga naistel esinevate meeleoluhäirete tekkes. Lisaks näitavad tulemused, et AI võiks olla kliinilises praktikas kasutuses põletikuliste seisundite tuvastamisel emaka koes ja antud meetod on paljulubav endomeetriumi funktsionaalsete häirete tõhusamateks teadusuuringuteks.

Märksõnad: tehisintellekt, naiste viljatus, naiste reproduktiivtrakt, mikrobioom, meeleoluhäired, polütsüstiliste munasarjade sündroom, PCOS, korduvad implantatsiooni ebaõnnestumised, RIF.



I couldn't have reached here without you, the one holding this book.

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Oulu, June 2024 *Seungbaek Lee*

List of abbreviations and symbols

AE-PCOS	androgen excess and PCOS
AI	artificial intelligence
ALDEx	ANOVA-like differential expression
AMH	anti-Müllerian hormone
ANCOM-BC	Analysis of Compositions of Microbiomes with Bias Correction
ANOVA	analysis of variance
ASRM	American Society for Reproductive Medicine
BDI-II	Beck Depression Inventory Second Edition
BMI	body mass index
CD	cycle day
CE	chronic endometritis
CNN	convolutional neural network
CRP	C-reactive protein
E2	estradiol
EF	endometrial fluid
ESE	early secretory phase
ESHRE	European society for human reproduction and embryology
FAI	free androgen index
FDR	false discovery rate
FN	false negatives
FP	false positives
FSH	follicle-stimulating hormone
GAD-7	Generalized Anxiety Disorder assessment
GE	glandular epithelium
GnRH	gonadotropin-releasing hormone
HA	hyperandrogenism
HSCL-25	Hopkins Symptom Checklist-25
ICC	intra-class correlation
IR	insulin resistance
IVF	*in vitro* fertilization
LH	luteinizing hormone
LSE	late secretory phase

LPS	lipopolysaccharide
MD	mood disorder
MSE	mid-secretory phase
NFBC	Northern Finland Birth Cohort
NIH	national institutes of health
OA	oligo- and/or anovulation
P4	progesterone
PA	PCOS anovulatory
PCA	principal component analysis
PCOM	polycystic ovarian morphology
PCOS	polycystic ovary syndrome
PE	proliferative phase
PERMANOVA	Permutational Analysis of Variance
PO	PCOS ovulatory
RIF	recurrent implantation failure
RT	reproductive tract
SCFA	short-chain fatty acid
SE	secretory phase
SHBG	sex hormone-binding globulin
TN	true negatives
TP	true positives
VS	vaginal swab

List of original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:

I **Lee, S.**, Aasmets, O., Arffman, R.K., Laru, j., Rossi, H-R, Salumets, A., Piltonen, T.T., and Org., E, 2024. The reproductive tract microbiome in women with polycystic ovary syndrome and across different menstrual cycle phases, Submitted to Human Reproduction, **Shared lead author**

II **Lee, S.**, Tejesvi, M.V., Hurskainen, E., Aasmets, O., Plaza-Díaz, J., Franks, S., Morin-Papunen, L., Tapanainen, J.S., Ruuska, T.S., Altmäe, S., Org., E, Salumets, A., Arffman, R.K., and Piltonen, T.T., 2024. Gut bacteriome and mood disorders in women with PCOS. Human Reproduction, 10.1093/humrep/deae073, **Shared lead author**

III **Lee, S.**, Arffman, R.K., Komsi, E.K., Lindgren, O., Kemppainen, J., Kask, K., Saare, M., Salumets, A. and Piltonen, T.T., 2024. Dynamic changes in AI-based analysis of endometrial cellular composition: Analysis of PCOS and RIF endometrium. Journal of Pathology Informatics, 15, p.100364, 10.1016/j.jpi.2024.100364, **Lead author**

IV **Lee, S.**, Arffman, R.K., Komsi, E.K., Lindgren, O., Kemppainen, J.A., Metsola, H., Rossi, H.R., Ahtikoski, A., Kask, K., Saare, M. and Salumets, A., 2024. AI-algorithm training and validation for identification of endometrial CD138+ cells in infertility-associated conditions; polycystic ovary syndrome (PCOS) and recurrent implantation failure (RIF). Journal of Pathology Informatics, p.100380, 10.1016/j.jpi.2024.100380, **Lead author**

Author's contribution to the publications

The author participated in designing the studies and selecting the study populations in all publications. Specifically, in **Paper I**, the author was responsible for the extraction of microbial DNA from endometrial fluid and vaginal swab samples. For **Papers I and II**, the author contributed to the sequencing data analysis, statistical analysis, and visualization of the results. Furthermore, in **Papers III and IV**, the author was involved in AI model training, AI-based image analysis as well as the statistical analysis and visualization of the results. The author drafted the first versions of all the original publications and subsequently improved them in collaboration with the co-authors.

Contents

1 Introduction

Infertility is described as the inability to achieve pregnancy within 12 months of unprotected intercourse or therapeutic donor insemination for women under 35 years old or within six months for women over 35 years old ("Infertility Workup for the Women's Health Specialist," 2019). Female infertility constitutes approximately 35% of infertility cases and tends to increase with age and body mass index (BMI) (B. Chu et al., 2023; Hart, 2016; Thurston et al., 2019). These factors impact the quality and quantity of oocytes, ovarian function, and embryo implantation (Best & Bhattacharya, 2015; Vander Borght & Wyns, 2018). Moreover, conditions, such as polycystic ovary syndrome (PCOS) and recurrent implantation failure (RIF) may contribute to female infertility (Cimadomo et al., 2023; Hart, 2016).

PCOS is a common endocrine and metabolic disorder affecting one in eight women, diagnosed by identifying characteristics, such as oligo/anovulation (OA), hyperandrogenism (HA), and polycystic ovarian morphology (PCOM) (Teede et al., 2023). PCOS can be categorized based on clinical manifestation. Phenotype A, the most severe case, displays all symptoms in the diagnostic criteria, while phenotype D, the mildest, presents with PCOM and OA but without HA (Jamil et al., 2016). In addtion to anovulation, impaired endometrial function in women with PCOS may contribute to adverse reproductive outcomes, such as increased miscarriage rate and pregnancy complications (Palomba et al., 2021). Additionally, chronic systemic and local inflammation in women with PCOS can further exacerbate metabolic and endocrine imbalances (Zhao et al., 2023). Moreover, attention has recently turned to the microbiome and its potential role in the development of PCOS-related disturbances, impacting inflammation, sex hormone regulation, and insulin resistance (IR) (Sola-Leyva et al., 2023; Tremellen & Pearce, 2012). The clinical features of PCOS, including irregular periods, hirsutism, metabolic dysfunction, and infertility problems, are believed to be linked to psychological distress in affected women (Karjula et al., 2017; Simpson et al., 2021).

RIF is a situation where multiple transfers of viable embryos fail to result in a positive pregnancy test (Cimadomo et al., 2023). Immunological abnormalities are considered to be closely linked to RIF (Q. Wang et al., 2022). For example, up to 67% of RIF patients have experienced chronic endometritis (CE), a condition

characterized by prolonged, persistent, and mild inflammation of the endometrium, as indicated by a higher occurrence of CD138+ plasma B cells in the stroma (Huang et al., 2020; Park et al., 2016). Additionally, there is evidence suggesting that inadequate endometrial development, characterized by delayed endometrial receptivity, may have a negative impact on implantation in RIF patients (Cimadomo et al., 2023; Meltsov et al., 2022). However, it is important to note that the underlying etiology and optimal treatment strategies for this condition are not yet fully understood.

The human microbiota refers to all microorganisms residing in and on the human body. The microbiota profoundly impacts the host, including nutrient metabolism, immune function, and the endocrine system (Hill & Round, 2021). Therefore, alterations in the microbiota are closely linked to various disorders and diseases. For example, shifts in gut microbiota can contribute to the development of inflammatory diseases, metabolic disorders, mood disorders (MDs), and even gynaecological and obstetrical issues (R. Liu et al., 2017; Simpson et al., 2021; Turjeman et al., 2021). Additionally, recent studies have suggested the associations between the reproductive tract (RT) microbiota and reproductive health, such as PCOS (Sola-Leyva et al., 2023).

The application of artificial intelligence (AI) in clinical research has increased significantly due to its predictive capabilities for disease diagnosis and therapeutic response (Kaul et al., 2020). In digital pathology, AI is gaining recognition for its potential to address the current limitations of microscopic assessments. It fortifies the reliability and reproducibility of analyses by reducing both intra- and inter-observer variation while also expeditiously inspecting whole-slide images (Ertosun & Rubin, 2015; Hallager et al., 2021).

The present thesis focused on the RT microbiome to characterize the landscape of the microbiome profiles in women with PCOS across the menstrual cycle phases and identify microbial signatures associated with PCOS. Shifting the focus from the RT to the gut, the thesis investigated the associations between MDs and PCOS within the context of the gut microbiome. Lastly, the thesis examined monthly epithelial gland development and inflammatory status in women with PCOS or RIF, employing an AI model.

2 Review of the literature

2.1 The menstrual cycle

The human uterus consists of two main layers: the smooth muscle layer, known as the myometrium, and the mucosal layer, known as the endometrium (**Fig. 1a**) (Aguilar & Mitchell, 2010). Throughout the menstrual cycle, the endometrial lining undergoes morphological changes (Mihm et al., 2011). The first day of menstruation is considered the first day of the menstrual cycle, and the cycle length is commonly around 28 days (Critchley et al., 2020; Maybin & Critchley, 2015; Mihm et al., 2011). Menstrual cycles can be considered irregular when intervals between periods fall below 21 days or exceed 35 days, the length of cycles varies more than seven days between cycles, or there are less than nine cycles per year.

2.1.1 The ovarian cycle

The ovarian cycle involves endocrine organs, including the hypothalamus, pituitary, and ovary (the HPO-axis), and consists of three phases: the follicular phase, ovulation, and the luteal phase (Richards, 2018) (**Fig. 1b**). During the follicular phase, follicle-stimulating hormone (FSH) induced by hypothalamic gonadotropin-releasing hormone (GnRH) stimulates follicle growth and prepares the eggs for ovulation (Holesh et al., 2024). As a result, matured follicles synthesize estradiol (E2) in ovarian granulosa cells, which then further stimulates the secretion of GnRH (Holesh et al., 2024). This leads to a boost in the release of luteinizing hormone (LH), and as a result, the mature follicle ruptures and releases an oocyte (Richards, 2018). A LH surge triggers the formation of the corpus luteum through luteinization, transforming the remaining ovarian granulosa and theca cells into lutein cells (Monis & Tetrokalashvili, 2024; Murphy, 2000). The fate of the corpus luteum hinges on the presence or absence of fertilization. If fertilization occurs, the corpus luteum persists and supports early pregnancy as a source of progesterone (P4). Conversely, in the absence of fertilization, the corpus luteum deteriorates, resulting in a sharp decline in P4 and E2 levels and consequent initiation of endometrial shedding (Holesh et al., 2024; Oliver & Pillarisetty, 2024; Toner, 2021).

2.1.2 The endometrial cycle

The endometrial cycle, also known as the uterine cycle, regulates the preparation and maintenance of the endometrial lining to facilitate embryo implantation. The structure and function of the human endometrium change dynamically during the menstrual cycle, influenced by fluctuating levels of steroid hormones (Giudice, 2006). The endometrial lining comprises two main parts: the functionalis and the basalis (**Fig. 1a**). During the proliferative phase (PE), the stratum functionalis regenerates from the underlying stratum basalis, which houses endometrial progenitor cells that differentiate into epithelial and stromal cells (Gargett, 2007). Functionalis thickens throughout the PE and also during the secretory phase (SE) (Yamaguchi et al., 2021).

The functionalis is composed of the epithelium and the stroma (**Fig. 1c**). The endometrial epithelium comprises the glandular epithelium (GE) and luminal epithelium (Filant & Spencer, 2014; Gargett, 2007). The GE forms an extensive network among the glands and facilitates glandular secretions (Filant & Spencer, 2014). The initial contact with the embryo occurs in the luminal epithelium, as it forms the border to the uterine cavity (Bergmann et al., 2021; Ye, 2020). The stromal compartment, which constitutes the largest proportion of the endometrium, contains the GE and luminal epithelium, along with various cell types, including stromal cells, endothelial cells, and immune cells (Critchley et al., 2020; Maybin & Critchley, 2015). Functionally, the stroma acts as a receptive niche and a source of nutrients for the implanting embryo while also supporting the growth and survival of epithelial cells (Yokomizo et al., 2021). In response to fluctuations in sex hormones during the menstrual cycle, the two major compartments undergo growth, maturation, and degeneration.

Menstruation is the first physiological process of the menstrual cycle, triggered by the withdrawal of E2 and P4 (Critchley et al., 2020). During the menstrual phase, the functionalis undergoes breakdown and shedding, after which the expelled endometrial linings and blood exit the uterus through the vagina (Critchley et al., 2020; Spencer et al., 2005).

Proliferative phase (PE)

The PE is the phase before ovulation, commonly the first 14 days of the menstrual cycle (Critchley et al., 2020). Under the influence of E2, the stroma becomes more compact, and the GE elongates and twists, driven by mitotic activity (**Fig. 1b,d**) (Critchley et al., 2020; Filant & Spencer, 2014). FSH also plays a role in thickening the endometrium and renewing the connective tissue with the development of glandular structures and helicine arteries, ensuring an adequate supply of blood flow and nutrients to the extended endometrial tissue (Acosta et al., 2000; Rock, 1937).

Secretory phase (SE)

Induced by the surge of LH, the ovary releases an egg on approximately cycle day (cd) 14, known as ovulation (Holesh et al., 2024). Subsequent to the LH surge, the SE is initiated by P4 action, characterized by the additional thickening of the endometrium, coiling of the glands, accumulation of glandular secretions, and further coiling and elongation of the helicine arteries (**Fig. 1b,d**) (Agostinis et al., 2019; Filant & Spencer, 2014). The SE can be divided into three distinct phases based on the specific days following the LH surge: the early SE (ESE; cd 15–21; LH+1–6), mid-SE (MSE; cd 22–25; LH+7–9), and the late SE (LSE; cd 26–28; LH+10–12) (Smith et al., 1989).

In the ESE, the GE cells exhibit an abundance of endoplasmic reticulum, accompanied by the accumulation of glycogen-rich vacuoles and a central displacement of nuclei (Gellersen & Brosens, 2014). At the end of the ESE, the vacuoles vanish from the cytoplasm and align with the peak of glandular secretory activity (Gellersen & Brosens, 2014; Mizutani et al., 2020). In the MSE, P4 produced by the corpus luteum inhibits cell mitosis and promotes the maturation and excretion of the GE (Hood et al., 2015). Furthermore, P4 facilitates mesenchymal-to-epithelial transition, leading to the transformation of stromal fibroblasts into decidualized and secretory fibroblasts (Bergmann et al., 2021). This decidualized stroma creates a beneficial environment for embryo implantation and development (Bergmann et al., 2021; H. Zhu et al., 2014). Consequently, there is a decrease in the stromal proportion within the endometrium (Filant & Spencer, 2014). In the LSE, if implantation does not occur, the reduction of P4 secretion

results in gland disruption, stromal breakdown, and tissue necrosis, all accompanied by a significant infiltration of leukocytes (Gellersen & Brosens, 2014).

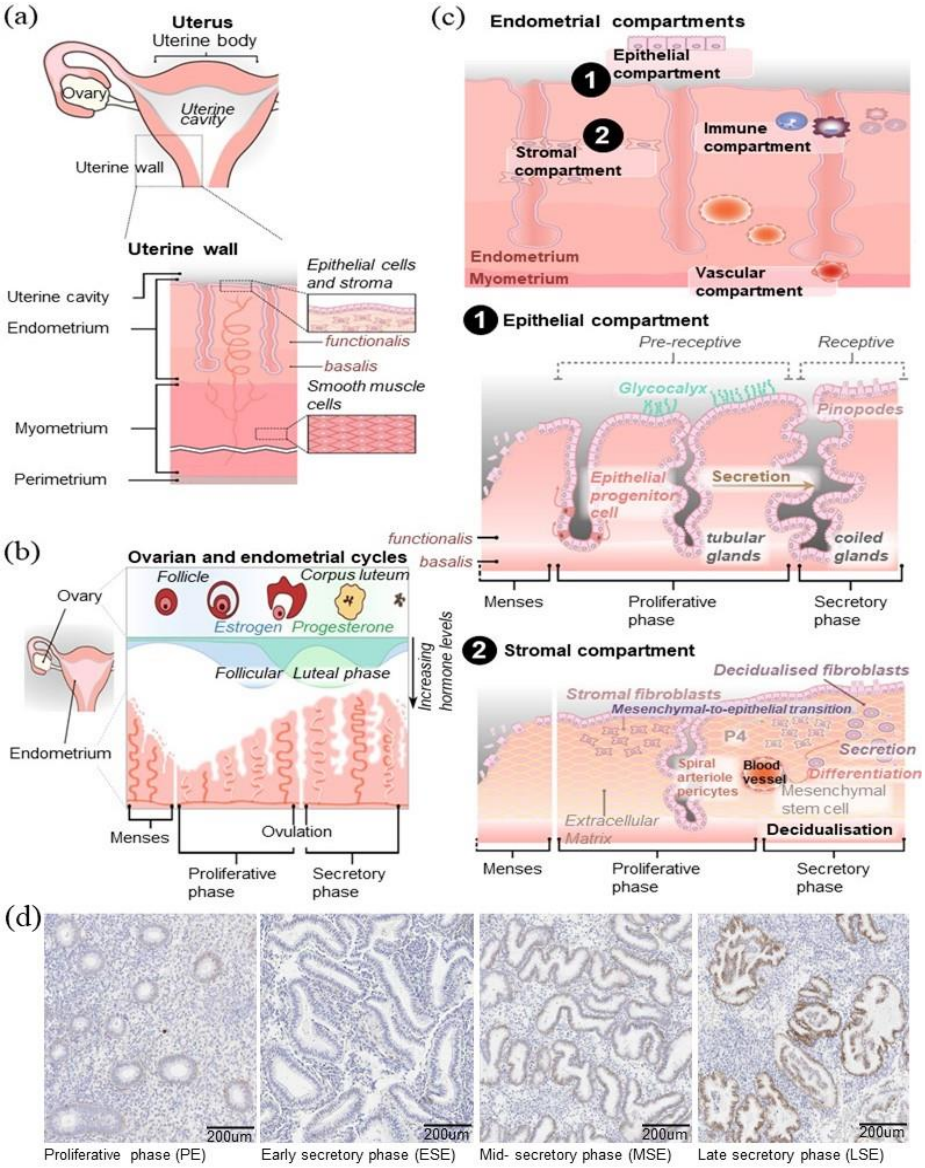

Fig. 1. The structure of the human (a) uterus. (b) The ovarian and endometrial cycles illustrating the development of follicles and the endometrium throughout the menstrual cycle in response to sex hormones. (c) The endometrial components: Structural and

functional changes in the (1) epithelial and (2) stromal compartment during the menstrual cycle. (d) Endometrial tissue images depicting the stages of the endometrial cycle. PE (proliferative phase), ESE (early secretory phase), MSE (mid-secretory phase), LSE (late secretory phase) (*Modified from* Bergmann et al., 2021)

2.2 Female infertility-associated conditions

Infertility refers to "a disease of the male or female reproductive system defined by the failure to achieve a pregnancy in one year" by the World Health Organization (WHO) (World Health Oragnization, 2006). According to a recent statement from the American Society for Reproductive Medicine (ASRM), infertility is defined as a disease characterized by any of the following criteria: (i) the inability to achieve a successful pregnancy based on a patient's medical, sexual, and reproductive history, age, physical findings, diagnostic testing, or any combination of those factors; (ii) the need for medical intervention, including but not limited to, the use of donor gametes or donor embryos to achieve a successful pregnancy, either as an individual or with a partner; (iii) in patients having regular, unprotected intercourse with no known cause of impaired reproductive ability for either partner, evaluation should be initiated after 12 months if the female partner is under 35 years old and after six months if the female partner is 35 years or older ("Definition of Infertility: A Committee Opinion," 2023). Female infertility, which accounts for approximately 35% of infertility, is influenced by various factors (Thurston et al., 2019). Even though ovarian aging and the decreasing number and quality of the oocytes are the main reasons for female infertility, reproductive disorders, such as PCOS and RIF, that link to impaired endometrial function also play a role (Vander Borght & Wyns, 2018).

2.2.1 Polycystic ovary syndrome (PCOS)

PCOS is a common endocrine disorder affecting one in eight women (Lim et al., 2012; Teede et al., 2023). The diagnosis of PCOS relies on three features: irregular menstrual cycles, the presence of polycystic ovaries, and HA (Teede et al., 2023).

Initially, in 1990, the National Institutes of Health (NIH) established diagnostic criteria that entail the presence of clinical and/or biochemical indications of HA and chronic anovulation (Zawadski & Dunaif, 1992). In 2003, the European

Society for Human Reproduction and Embryology (ESHRE) and the ASRM defined the widely adopted Rotterdam Criteria ("Revised 2003 Consensus on Diagnostic Criteria and Long-Term Health Risks Related to Polycystic Ovary Syndrome (PCOS)," 2004). The Rotterdam criteria have become the most commonly utilized PCOS diagnostic framework and are also endorsed by the recent update of the international PCOS guideline (Teede et al., 2023). The criteria require the presence of at least two out of three specific features: (1) OA, (2) clinical or biochemical signs of HA, and (3) PCOM on ultrasound ("Revised 2003 Consensus on Diagnostic Criteria and Long-Term Health Risks Related to Polycystic Ovary Syndrome," 2004). The Androgen Excess and PCOS (AE-PCOS) Society published the third recommendation in 2006, necessitating (1) the presence of hirsutism and/or HA, (2) ovarian dysfunction (OA and/or PCOM), and (3) the exclusion of other androgen access-related disorders (Azziz et al., 2006). All three definitions of PCOS mandate the exclusion of other endocrine disorders with overlapping symptoms, such as non-classic congenital hyperplasia, hyperprolactinemia, thyroid dysfunction, hypercortisolism, and androgen-secreting tumors (Teede et al., 2023).

There are four distinct phenotypes of PCOS based on clinical manifestation: Phenotype A: HA+OA+PCOM; Phenotype B: HA+OA; Phenotype C: HA+PCOM; and Phenotype D: OA+PCOM (Azziz et al., 2006; National Institutes of Health, 2016) (**Table 1**). Phenotypes A and B, or classic PCOS, feature more prominent irregular menstrual cycles and pose a higher risk for metabolic syndrome, with elevated levels of anti-Müllerian hormone (AMH) (Lizneva et al., 2016). These phenotypes are considered to indicate more severe symptoms of the condition compared to other phenotypes. Women classified as phenotype C, known as ovulatory PCOS, experience ovulation and display relatively milder symptoms compared to phenotypes A and B (Lizneva et al., 2016). Lastly, phenotype D, termed nonhyperandrogenic PCOS, shows the mildest symptoms, characterized by lower androgen levels and higher sex hormone-binding globulin (SHBG) levels than phenotypes A and B (Jamil et al., 2016; Lizneva et al., 2016).

Table 1. Phenotypes of PCOS

	Phenotypes			
Clinical manifestation	A	B	C	D
HA[1]	+	+	+	-
OA[2]	+	+	-	+
PCOM[3]	+	-	+	+

[1]hyperandrogenism, [2]oligoanovulation, [3]polycystic ovarian morphology

Pathophysiology of PCOS

The etiology of PCOS is multifactorial, resulting in diverse clinical characteristics, comorbidities, and reduced quality of life (Palomba et al., 2021; Stener-Victorin et al., 2024; Teede et al., 2023). Nevertheless, the exact underlying mechanisms of PCOS remain unknown.

Genetic factors—PCOS has a polygenic basis with a genetic predisposition, with >20 identified variants directly accounting for around 10% of its heritability (Bruni et al., 2022; J. Zhu et al., 2022). These genes are associated with inflammation, glucose metabolism, and insulin signaling (Tian, Li, et al., 2020). Maternal androgen exposure may increase susceptibility to PCOS in female offspring (Doherty et al., 2015; Risal et al., 2019; Roos et al., 2011). Lifestyle and environmental factors most likely contribute to epigenetic modulation and the development of PCOS (Hiam et al., 2019).

Hormonal imbalance and metabolic abnormalities—Hormonal imbalance is a key pathophysiological factor in the development of PCOS. Normally, GnRH stimulates the secretion of LH and FSH from the anterior pituitary (Marques et al., 2000; Wildt et al., 1981) (**Fig. 2a**). LH induces androgen secretion from the ovarian theca cells, whereas FSH facilitates E2 secretion from the granulosa cells, leading to follicular development and ovulation. P4 from the corpus luteum, along with E2, inhibits the pulsatile release of GnRH, downregulating LH and FSH levels (Marques et al., 2000). However, women with PCOS exhibit resistance to both E2

and P4, resulting in elevated levels of GnRH and LH pulse activities but lower FSH levels, culminating in androgen excess and anovulation (Coutinho & Kauffman, 2019; Wildt et al., 1981) (**Fig. 2b**).

PCOS is also associated with metabolic abnormalities. IR is marked by diminished efficiency in transferring glucose from the circulation into cells following activation of the insulin signaling pathway (Yaribeygi et al., 2019). Consequently, compensatory hyperinsulinemia occurs as the pancreas boosts insulin production to counteract the decreased insulin action and glucose absorption (Moghetti, 2016). Additionally, IR promotes HA by increasing androgen production in the ovaries and adrenal glands and by regulating SHBG production in the liver (Zeng et al., 2020).

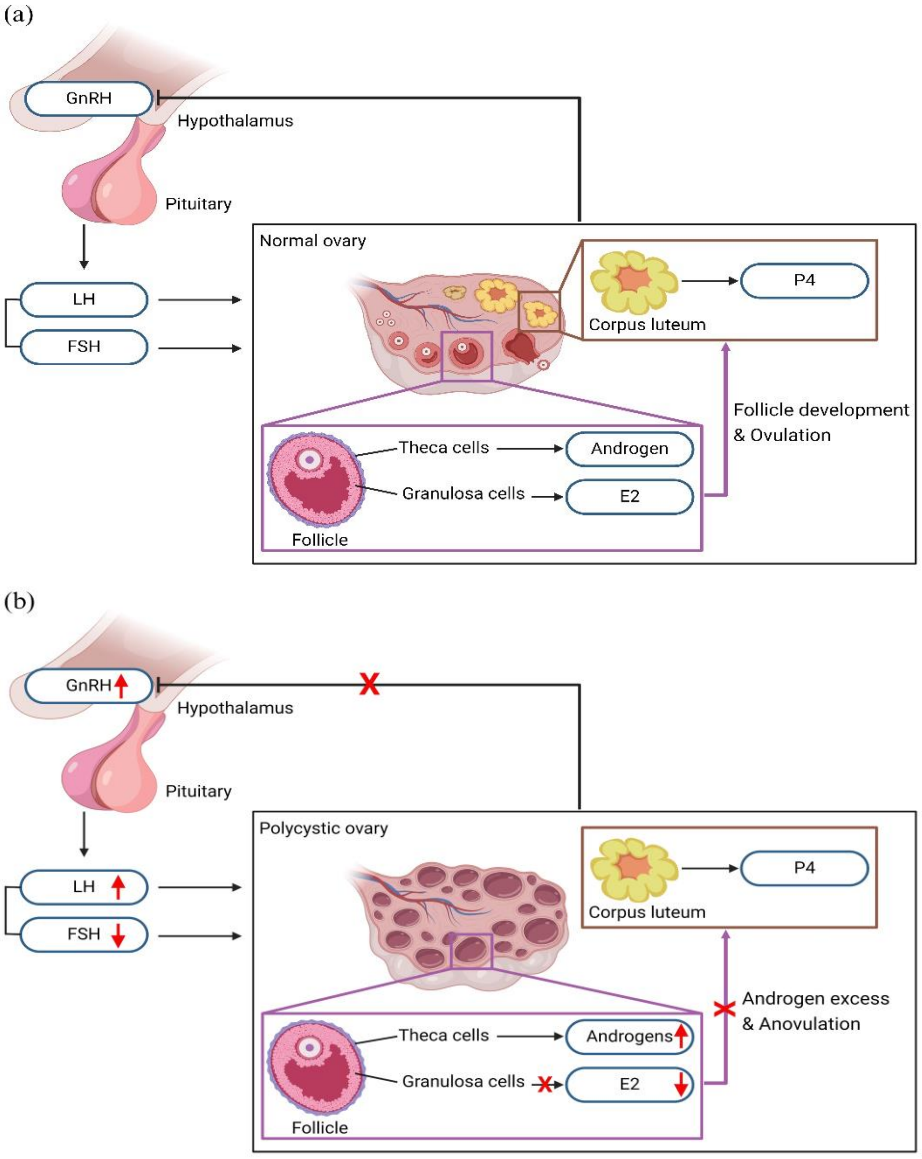

Fig. 2. Hypothalamus-pituitary-ovarian axis in (a) normal ovary and (b) polycystic ovary. GnRH (gonadotropin-releasing hormone), LH (luteinizing hormone), FSH (follicle-stimulating hormone), E2 (estradiol), P4 (progesterone)

Clinical features of PCOS

PCOS symptoms typically begin in adolescence and continue into postmenopause, manifesting in various ways (Stener-Victorin et al., 2024) (**Fig. 3**).

Menstrual irregularities, reproductive complications, and endometrial dysfunctions—Anovulation presents as either oligomenorrhea (periods exceeding 35 days or less than 8 periods annually) or amenorrhea and is the main cause of infertility in women with PCOS (Legro et al., 2013; Teede et al., 2023).

PCOS endometrium displays shifts in the expression patterns of sex hormone receptors, and these changes result in abnormal endometrial responses to sex steroid hormones, i.e., enhanced E2 and androgen action and compromised P4 action (Palomba et al., 2021). Overall, endometrial dysfunctions in women with PCOS can precipitate gynecological complications, such as endometrial cancer, as well as obstetrical challenges, including miscarriage and preeclampsia (Palomba et al., 2021).

Metabolic disorders—IR is prevalent in 50 to 90% of women with PCOS and plays a significant role in the development of metabolic disorders, including obesity, type 2 diabetes mellitus, and dyslipidemia (Barber et al., 2015; Diamanti-Kandarakis & Dunaif, 2012). Hormonal imbalances in PCOS can worsen metabolic disturbance, as evidenced by increased abdominal visceral adiposity and disrupted carbohydrate and lipid metabolism associated with HA (Cortón et al., 2007; Xu et al., 1991).

Hyperandrogenism (HA)—HA can occur through two primary mechanisms: gonadotropin-dependent functional ovarian androgen excess and adrenal HA (Escobar-Morreale et al., 2005). Diagnosis of HA typically involves assessing serum testosterone levels via mass spectrometry or radioimmunoassay techniques (Teede et al., 2023). Clinical presentation of HA includes hirsutism, characterized by the development of male-pattern hair growth in women (Yildiz, 2006). The severity of hirsutism can be evaluated through visual inspection of various skin areas, including the upper lip, chin, chest, upper and lower back, upper and lower abdomen, arms, and thighs (Ferriman & Gallwey, 1961; Teede et al., 2023).

Mood disorders (MDs)—Women with PCOS have an increased risk for MDs, such as depression and anxiety, significantly impacting their quality of life (Cooney et al., 2017; Karjula et al., 2017, 2021; Kolhe et al., 2022). Previous studies demonstrated inhibitory neurotransmitters of GnRH and LH (e.g., serotonin,

dopamine, gamma-aminobutyric acid) were found to be decreased, while stimulatory neurotransmitters (e.g., glutamate) were increased in both PCOS and PCOS-like animal models (McCartney & Campbell, 2020; Moore & Campbell, 2017). Inflammation, IR, and MDs form a vicious cycle. Elevated cytokine levels facilitate serotonin uptake, while reduced serotonin concentrations in the central nervous system can worsen IR by interfering with the inhibition of insulin secretion (Daut & Fonken, 2019; Samuvel et al., 2005; C.-B. Zhu et al., 2005). Additionally, cytokines activate the hypothalamic-pituitary-adrenal axis, leading to increased cortisol secretion (Hollinrake et al., 2007; Pomytkin et al., 2015).

Immunological dysregulation—Inflammation is the hallmark clinical characteristic of PCOS, irrespective of obesity (Escobar-Morreale et al., 2011). Women with PCOS present elevated circulatory markers of inflammation, such as C-reactive protein (CRP), pro-inflammatory cytokines and chemokines (e.g., TNF-α, macrophage inflammatory protein-1α), and mononuclear cells (e.g., lymphocytes, monocytes, neutrophils), alongside increased oxidative stress and endothelial dysfunction (Aboeldalyl et al., 2021; Ganie et al., 2019; Xiong et al., 2011).

The above-mentioned factors are also closely linked to metabolic and endocrine disturbances. For example, IR and hyperglycemia—a metabolic consequence of IR—are associated with oxidative stress, mononuclear cell counts, and endothelial dysfunction, further exacerbating inflammation in women with PCOS (Duleba & Dokras, 2012; González, 2012). Meanwhile, TNF-α promotes adiposity lipolysis, and IL-6 reduces the expression of glucose transporter-4 and insulin receptor substrate-1 (L. Chen et al., 2015).

The elevated estrogen levels in women with PCOS can stimulate the immune system, increasing susceptibility to autoimmune disorders and leading to higher production of inflammatory cytokines (Mobeen et al., 2016; Romitti et al., 2018). Additionally, mononuclear cell-derived macrophages in the polycystic ovary can be involved in ovarian androgen production in theca cells (González, 2012).

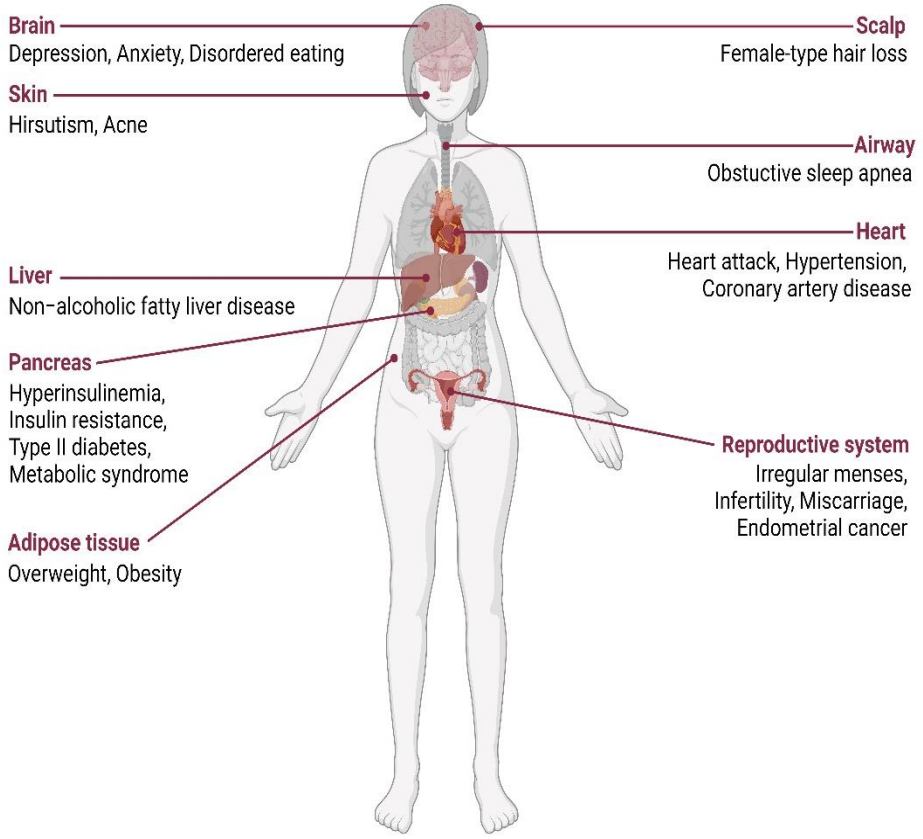

Fig. 3. Possible clinical features of PCOS.

2.2.2 Recurrent implantation failure (RIF)

RIF stands out as a significant factor impacting the success rate of *in vitro* fertilization (IVF) (Bashiri et al., 2018). Recently, the ESHRE Working Group on RIF defined RIF as the situation where multiple transfers of viable embryos fail to result in a positive pregnancy test (Cimadomo et al., 2023). However, the etiology of RIF is multifactorial, and the exact underlying mechanism remains unclear.

Risk factors of RIF

Genetic variations—Genetic variations observed in RIF patients, including chromosomal abnormalities and altered expression of genes involved in cell cycle and cytoskeleton formation, are believed to impact implantation success in these individuals (De Sutter et al., 2012; Koot et al., 2016; Raziel et al., 2002). Additionally, a delayed window of implantation has been observed in approximately 10% of women undergoing IVF and in at least 25% of RIF patients (Meltsov et al., 2022).

Maternal age—Maternal age significantly increases the risk of RIF. As women get older, the likelihood of genetic abnormalities in oocytes increases. Additionally, age affects both the quality and quantity of oocytes, ultimately impacting blastocyst formation (Coughlan et al., 2014; Zeyneloglu & Onalan, 2014).

Obesity and overweight—High BMI is also a relevant risk factor for RIF (Orvieto et al., 2009). In IVF patients with obesity, the number of oocytes retrieved is fewer than in non-obese women, even after undergoing more gonadotrophin stimulation cycles. This suggests a potential impact of obesity on oocyte quality and follicular development as well as endometrial receptivity (Bellver et al., 2021; Fedorcsák et al., 2000; Orvieto et al., 2009).

CE—The endometrial immune system plays a crucial role in ensuring the acceptance of implanted allogeneic embryos during embryo implantation. However, RIF patients exhibit heightened levels of immune cells in both peripheral blood and the uterus, suggesting a link between an altered immunological profile and this condition (Sacks et al., 2012; Santillán et al., 2015). For example, up to 67% of RIF patients have experienced CE, a persistent inflammation of the endometrium triggered by pathogen infections, such as Group B *Streptococcus*, *Escherichia coli*,

Enterococcus faecalis, *Mycoplasma*, or *Chlamydia* (Cicinelli et al., 2015; Huang et al., 2020).

The diagnosis of CE involves histological examination and bacterial culture (Y. Li et al., 2021). Immunohistochemistry (IHC) staining for syndecan-1 (CD 138) has been used for decades as a diagnostic method for CE (Bayer-Garner et al., 2004). CD138, a heparan sulfate proteoglycan on the plasma cell surface, functions as a receptor for growth factors and immune mediators, attracting peripheral plasma cells into the endometrium (Kitaya et al., 2018; Y. Xu et al., 2020). Notably, the abundance of CD138+ plasma cells shows a positive correlation with the severity of endometrial inflammation observed in patients experiencing reproductive failure, including RIF and recurrent miscarriage (Bayer-Garner & Korourian, 2001; Bouet et al., 2016; Y. Chen et al., 2016; Park et al., 2016). Identifying isolated CD138+ plasma cell aggregates, even without other histological features of CE, such as stromal edema, endometrial hyperemia, and the presence of micropolyps, could potentially detect cases with milder endometrial inflammation and associated endometrial dysfunction (Park et al., 2016). However, there is no consensus on CE diagnostic criteria and cut-off values. Previous studies have noted variations in the occurrence of CD138+ plasma cells in the endometrium across menstrual cycle phases, with higher occurrence observed during the PE compared to the SE (Y. Li et al., 2021; D. Song et al., 2018). This lack of agreement may stem from varying approaches to CD138+ cell quantification, which hinders consistent and reproducible results (Y. Li et al., 2021). Currently, counting CD138+ cells in selected high-power fields (HPF) is the predominant way. However, the size of these HPFs is often not provided and may vary significantly among studies (Y. Li et al., 2021). Additionally, manual microscopic assessments conducted in only a few randomly chosen areas limit the comprehensive evaluation of the entire slide (Park et al., 2016). Moreover, microscopic evaluation typically involves laborious and time-intensive processes, and these manual evaluations can introduce significant biases due to intra- and inter-observer variations (Park et al., 2016; Puente et al., 2020).

Anatomical abnormalities—Anatomical irregularities within the RT, such as myomas, hydrosalpinges, and Mullerian abnormalities, are recognized as factors associated with implantation failure (Bashiri et al., 2018). These irregularities hinder the embryo's attachment to the luminal surface and disrupt the provision of essential nutrients and energy to the embryo (Coughlan et al., 2014; Kodaman et

al., 2004). Insufficient endometrial thickness also contributes to implantation failure. For example, inadequate development of epithelial cell growth and angiogenesis results in a thin endometrium (<8 mm), which hampers implantation rates (Lebovitz & Orvieto, 2014; Miwa et al., 2009; Richter et al., 2007).

2.3 The human microbiome

The human microbiota includes bacteria, viruses, fungi, and archaea, constituting 10–100 trillion symbiotic microbial cells and possessing a gene set at least 150 times larger than human genes (Ley et al., 2006; Tierney et al., 2019; Turnbaugh et al., 2007). Considering that newborns receive seed microbiota from their mothers at birth, these tiny organisms are our oldest and lifelong companions (Enav et al., 2022; Gritz & Bhandari, 2015; Mutic et al., 2017). While microbiome denotes the collective genetic materials of microbes, the terms microbiota and microbiome are frequently used interchangeably.

Advanced sequencing technologies allow microbiome analysis even for unculturable taxa (Y. Kim et al., 2015). Next-generation sequencing, such as amplicon and shotgun sequencing, has revealed that the human microbiome varies based on geographic location, lifestyle, host genetics, and social interactions (Dill-McFarland et al., 2019; Hall et al., 2017; Y. Kim et al., 2015; Shin et al., 2016). In other words, each harbors a distinctive and unique microbiome (Wissel & Smith, 2019).

Furthermore, the microbiota plays a crucial role in maintaining human health by regulating nutrient metabolism, the immune systems, and endocrine functions (Cerf-Bensussan & Gaboriau-Routhiau, 2010; Devaraj et al., 2013; S. He et al., 2021). Research on the gut and vaginal microbiome has gained significance in female gynecological and reproductive health, such as PCOS, highlighting its potential connection to this condition (Sola-Leyva et al., 2023) (**Fig. 4**). However, the endometrial microbiome in women with PCOS has been significantly less explored compared to the vaginal microbiome.

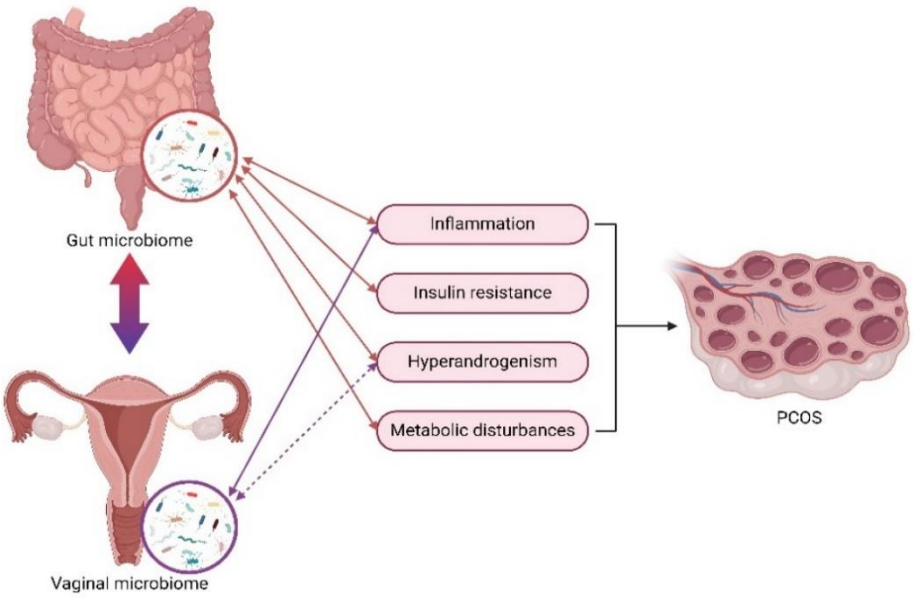

Fig. 4. Potential mechanisms of the pathophysiology of PCOS associated with the gut and vaginal microbiome. The gut microbiome can contribute to the development of PCOS by participating in systemic and chronic inflammation, IR, and HA, as well as metabolic disturbances. The vaginal microbiome can also play a role in inflammation. Other specific contributions have yet to be identified. IR (insulin resistance), HA (hyperandrogenism)

2.3.1 Gut microbiome

The gut microbiome has been extensively studied due to its significant impact on human health, leading to it being referred to as the "second genome" or the "forgotten organ" (O'Hara & Shanahan, 2006; B. Zhu et al., 2010).

The digestion of dietary carbohydrates involves a collaborative process between the gut microbiota and the host digestive system, yielding metabolites like short-chain fatty acids (SCFAs), bile acids, and trimethylamine (Devaraj et al., 2013). These metabolites significantly impact host physiology, involving inflammatory pathways, the synthesis and degradation of neurotransmitters, and

the metabolism of sex hormones (Devaraj et al., 2013; Peirce & Alviña, 2019; Simpson et al., 2021).

During the early stages of life, infants undergo a significant phase of immune development marked by the formation of lymphoid structures and lymphocyte differentiation. The gut microbiota trains the adaptive immune system through exposure to microbial antigens (Cerf-Bensussan & Gaboriau-Routhiau, 2010). In addition, the gut commensal microbiota contributes to immune defense by regulating mucus production, which serves as a physical barrier between bacteria and the intestinal epithelial cell surface, thereby preventing bacterial penetration (Hooper & Macpherson, 2010).

Sex hormones and gut microbial communities mutually influence each other (Colldén et al., 2019; Flores et al., 2012; Kornman & Loesche, 1982; Org et al., 2016). For instance, P4 promotes the growth of specific taxa, while E2 and androgens regulate the composition of the microbial community (Flores et al., 2012; Kornman & Loesche, 1982). Simultaneously, the gut microbiota regulates the levels of steroid hormones (Colldén et al., 2019; Hases et al., 2023).

In summary, alterations in the gut microbiota can influence the onset of inflammatory diseases, metabolic disorders, MDs, and even gynecological and obstetrical complications (Jian et al., 2022; R. Liu et al., 2017; Paone et al., 2022; Simpson et al., 2021; Turjeman et al., 2021).

2.3.2 Female RT microbiome

Numerous studies leveraging advanced sequencing technologies have yielded indisputable evidence of microbial proliferation in the female RT (Ng et al., 2018). The female RT consists of lower and upper compartments characterized by its unique environment. The vagina is predominantly inhabited by *Lactobacillus*, constituting over 90% of the microbial composition (C. Chen et al., 2017). Unlike the gut microbiome, having less diversity in the microbial community is more beneficial for vaginal health (N. M. Molina et al., 2021). *Lactobacillus* maintains an acidic environment with a pH ranging from 3.5 to 4.5, effectively preventing pathogen infections (Hawes et al., 1996; Skarin & Sylwan, 1986). E2 enhances vaginal secretions rich in mucins and glycoproteins, leading to vaginal epithelial thickening and glycogen accumulation (Nunn & Forney, 2016). Given that glycogen is a key nutrient for *Lactobacillus*, the proliferation of *Lactobacillus*

aligns with the fluctuations in E2 levels throughout the menstrual cycle (C. Chen et al., 2017; Nunn & Forney, 2016).

The endometrium maintains a pH range of 7.0-8.0 (Ng et al., 2018). Microbiota in the endometrium can originate through various routes, including hematogenous transmission from other organs (e.g., oral cavity, gastrointestinal tract), ascending migration from the vagina, the intervention of assisted reproductive technology and intrauterine device usage, and sexual activities (Baker et al., 2018; Garcia-Grau et al., 2019; N. Molina et al., 2020). The endometrial microbial community comprises a more diverse array of taxa beyond *Lactobacillus*, including *Flavobacterium, Pseudomonas*, *Acinetobacter*, *Vagococcus*, and *Sphingobium*, compared to the vaginal community (Baker et al., 2018; Garcia-Grau et al., 2019; N. Molina et al., 2020). Previous studies have demonstrated cyclic alterations in the endometrial microbial community across the menstrual cycle, with increased abundance of *Prevotella* spp. during the PE and *Sneathia* spp. during SE (Baker et al., 2018; C. Chen et al., 2017; Garcia-Grau et al., 2019; N. Molina et al., 2020; Pelzer et al., 2018).

Alterations in the RT microbial community have been linked to various gynecological and obstetrical conditions. For example, a decline in the abundance of *Lactobacillus* and an increase in *Gardnerella vaginalis* within the vaginal microbial community lead to bacterial vaginosis, often observed in women with a history of embryo implantation failure, preterm birth, and endometritis (Peuranpää et al., 2022; Salliss et al., 2021). In addition to CE, alterations in the endometrial microbiota can be associated with conditions, such as endometriosis, abnormal endometrial bleeding, and endometrial cancer or hyperplasia (Hernandes et al., 2020; Y. Liu et al., 2019; Pelzer et al., 2018; Sobstyl et al., 2022).

2.3.3 The impact of microbiome on PCOS

The microbiome has emerged as a potential factor in the pathophysiology of PCOS. Several studies have demonstrated altered microbiota in the blood, oral cavity, gut, and lower RT of women with PCOS (Sola-Leyva et al., 2023) (**Fig. 5**).

Alterations in the gut microbiota can trigger the development of PCOS via interactions with the immune, metabolic, and endocrine system (**Fig. 6**). Changes in the gut microbiota can compromise the gut mucosal barrier, allowing lipopolysaccharide (LPS) to enter the systemic circulation through a leaky gut

(Cani, Neyrinck, et al., 2007). This endotoxin upregulates inflammatory cytokines and mediators, hindering the insulin signaling pathway and contributing to IR (Cani, Amar, et al., 2007; Tremellen & Pearce, 2012).

Given the beneficial functions of SCFAs, such as producing anti-inflammatory mediators and improving gut barrier integrity, the decrease in the abundance of SCFA-producing bacteria and lower fecal concentrations of SCFAs observed in women with PCOS may contribute not only to chronic inflammation but also to IR (Arpaia et al., 2013; W. Chu et al., 2020; Cresci et al., 2017; Ratajczak et al., 2019; Torres et al., 2018; Zhang et al., 2019). Qi et al. (2019) demonstrated that an altered gut microbiota, characterized by an increased abundance of *Bacteroides vulgatus*, was correlated with decreased concentrations of bile acids, such as glycodeoxycholic acid and tauroursodeoxycholic acid, and reduced levels of IL-22, potentially improving both IR and ovarian function (Qi et al., 2019). Additionally, specific taxa, such as *Prevotella copri* and *Bacteroides vulgatus*, which are more abundant in women with PCOS compared to non-PCOS women, facilitate the biosynthesis of branched-chain amino acids, disrupting the insulin signaling pathway and thereby exacerbating IR (Gojda & Cahova, 2021; Pedersen et al., 2016; Qi et al., 2019).

Certain bacteria like *Bifidobacterium*, *Clostridium*, and *Lactobacillus* produce β-glucuronidases and β-glucuronides, which either deconjugate or conjugate E2 (Ervin et al., 2019; Yao et al., 2019). Additionally, HA can alter microbial diversity as well as the abundance of *Prevotella* and *Kandleria* (Kumar, 2013; Thursby & Juge, 2017; Torres et al., 2018). Changes in the microbiota modify gut microbiota-derived metabolites, which in turn influence gut peptides, such as glucagon-like peptide 1, ghrelin, and peptide YY (Sun et al., 2023; Zuo et al., 2022). Ghrelin regulates the synthesis and release of LH by delaying its pulse intensity (Cena et al., 2020; Hoover et al., 2021). Furthermore, gut microbiota-derived metabolites, acting as neurotransmitters, may disrupt gut-brain axis communication (Simpson et al., 2021). In women with PCOS, these altered neurotransmitters may contribute to the development of MDs, potentially synergizing with immune, metabolic, and endocrine abnormalities (Cooney et al., 2017; Peirce & Alviña, 2019; Y. Zhou et al., 2023).

However, the exact role of the gut microbiota in PCOS still remains unclear, with controversial findings among related studies (Lindheim et al., 2017; Lüll et al., 2021; Qi et al., 2019; Sola-Leyva et al., 2023; Torres et al., 2018).

Within studies focusing on the vaginal microbiome, women with PCOS exhibited a decrease in the abundance of *Lactobacillus* and an increase in *Gardnerella*, *Prevotella*, and *Atopobium* (Hong et al., 2020; Lu et al., 2021; Tu et al., 2020). Additionally, the vaginal microbial community diversity differs based on the presence or absence of PCOS (Sola-Leyva et al., 2023). Given the cyclic fluctuation of the richness of *Lactobacillus* aligning with E2 levels, irregular menstrual cycles and hormonal imbalance in women with PCOS may induce alterations in the vaginal microbiome (Gu et al., 2022). However, it is important to note that the previous studies did not adequately address potential confounding factors that may influence the microbiome, such as age, BMI, PCOS phenotypes, and menstrual cycle phases (Hong et al., 2020; Lu et al., 2021; Tu et al., 2020). Additionally, no previous studies have investigated the endometrial microbiome across the menstrual cycle in women with PCOS.

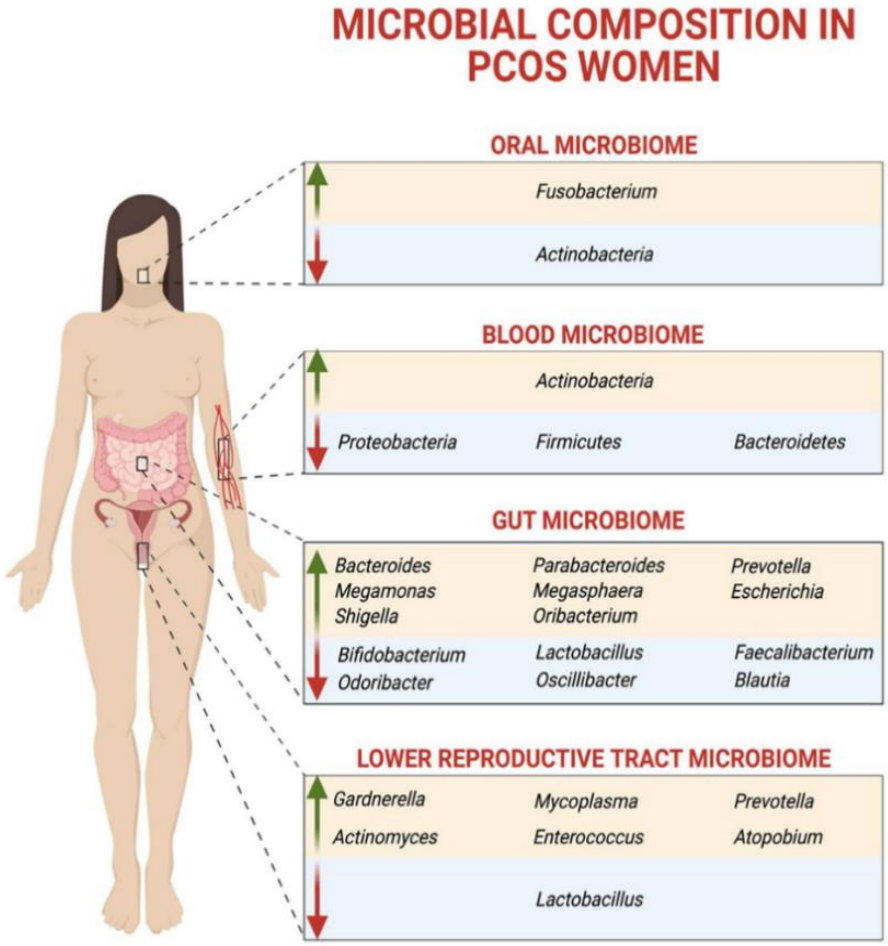

Fig. 5. Microbial compositional changes in women with PCOS compared to women without the syndrome. ***(Reprinted from*** Sola-Leyva et al., 2023 ***under the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND))*.**

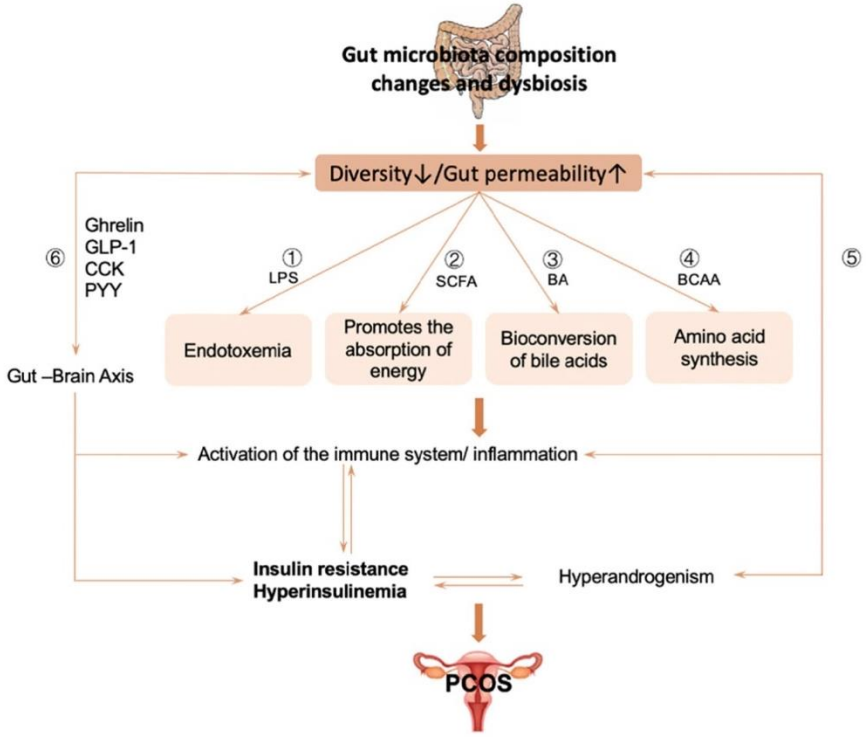

Fig. 6. Gut microbiota alterations and their implications in PCOS. IR can be exacerbated through elevated inflammatory status due to ① the entry of LPS into the circulation and ②-④ changes in gut microbiota-derived metabolites. Additionally, ⑤ HA and the gut microbial community influence each other, thereby contributing to IR. Last, ⑥ altered gut-brain peptides related to the gut microbiota affect neuro and systemic inflammation as well as hormone abnormalities. LPS (lipopolysaccharide), SCFA(short-chain fatty acid), BCAA(branch-chain amino acid), BA (bile acids) *(Reprinted from* F. He & Li, 2020 *under the Creative Commons CC BY license).*

2.4 AI application in histopathology

AI operates by extracting image representations and training a machine classifier for segmentation tasks (LeCun et al., 2015). A Convolutional Neural Network (CNN) consists of multiple layers that interact in a feedforward manner, allowing the network to learn and extract feature maps from images using filters positioned between the input and output layers (Bera et al., 2019). Hence, leveraging these inherent automated capabilities, AI can effectively tackle the limitations associated with current manual microscopic assessments. Most of all, AI allows for the analysis of a large number of whole slide images at high pixel resolution in a short time frame (Ertosun & Rubin, 2015). Intra- and inter-observer variations, even among observers with the same training, pose a significant issue in traditional histology because these discrepancies can result in diagnostic inconsistencies and potentially compromise the quality of patient care (Bera et al., 2019; Park et al., 2016). However, AI can decrease both intra- and inter-observer variations by eliminating the subjective nature of observation, thereby enhancing the reliability and reproducibility of analysis (Hallager et al., 2021).

In the gynecological and obstetrics research, histological evaluation stands as an important diagnostic tool encompassing conditions like CE, adenomyosis, and endometrial cancers (Bayer-Garner et al., 2004; Morice et al., 2016; Winkel, 2003). However, while AI has been primarily utilized in cancer research within uterine studies (Downing et al., 2020; Papke et al., 2021). Given the significance of endometrial CD138+ plasma cells in gynecological and obstetrical health, along with current challenges in measuring endometrial compartment areas and quantifying CD138+ cells, integrating AI could enhance analysis efficiency and reliability (Park et al., 2016).

3 Aims of the study

The present study aimed to identify biomarkers for infertility-related conditions, such as PCOS and RIF. First, microbial biomarkers associated with PCOS in the RT and the markers linked to both PCOS and MDs in the gut were investigated. Subsequently, endometrial histological characteristics were evaluated in women with PCOS and women with RIF using an AI model. To summarize, the specific aims were as follows:

1. To investigate the endometrial and vaginal microbiome profiles in women with PCOS compared to women without PCOS and to identify microbial signatures associated with PCOS.
2. To characterize the gut microbiome profile in women with PCOS who have experienced MDs and to explore its involvement in MDs among women with PCOS.
3. To assess endometrial epithelium-to-stroma proportions, respresenting epithelial gland development, using an AI model in women with PCOS and women with RIF.
4. To measure the accumulation of CD138+ cells in the stromal compartment, indicating endometrial inflammatory status, employing the AI model outlined in aim 3 in women with PCOS and women with RIF.

4 Materials and methods

This section provides a summary of the methodologies applied throughout the studies on which this doctoral thesis is based. For a comprehensive understanding of these methodologies, please refer to the descriptions below and the original publications in the 'List of original publications.'

4.1 Study subjects and study materials

All study subjects were non-smokers and had not used any hormonal medication for at least three months before sample collection. Controls were in good health, had regular menstrual cycles, and were not diagnosed with PCOS. PCOS diagnosis followed the Rotterdam criteria (Studies I, III, and IV) ("Revised 2003 Consensus on Diagnostic Criteria and Long-Term Health Risks Related to Polycystic Ovary Syndrome (PCOS)," 2004; Teede et al., 2023), while RIF samples were obtained from women who underwent three or more unsuccessful IVF attempts (Studies III, and IV) (Bashiri et al., 2018). In Study II, controls were defined as individuals who did not report OA or HA at age 31 and did not exhibit PCOM or PCOS at age 46. On the other hand, women who reported PCOS symptoms (i.e., OA and HA) at age 31 or had a PCOM/PCOS diagnosis at age 46 were categorized as PCOS subjects.

Age and BMI, recognized as confounding factors, were adjusted to alleviate inter-individual variability among women with and without PCOS or those with different receptivity status in RIF in all studies.

A total of 80 endometrial fluid (EF) and 83 vaginal swab (VS) samples for Study I were collected from healthy non-PCOS women and women with PCOS at the Oulu University Hospital (Oulu, Finland) from January 2017 to March 2020. Most study participants provided either EF or VS samples at a single time point, while others provided multiple samples from different cycle phases. Moreover, 39 women provided both sample types, EF and VS. By including samples from both the same women and different women, we aimed to increase sample size and improve data reliability.

The dataset utilized in Study II was obtained from the Northern Finland Birth Cohort 1966 (NFBC1966), which is a longitudinal birth cohort comprising individuals born in 1966 in the northernmost provinces of Finland (Oulu and Lapland) (described in Publication II). Cohort data collection commenced at the

24th gestational week for individuals born alive, with subsequent follow-ups conducted at ages 1, 14, 31, and 46. Postal questionnaires were distributed at ages 14, 31, and 46, while clinical examinations were carried out at ages 31 and 46. Additionally, MDs, including anxiety and depression, were evaluated using the following four criteria: Beck Depression Inventory Second Edition (BDI-II), Generalized Anxiety Disorder Assessment-7 (GAD-7), Hopkins Symptom Checklist-25 (HSCL-25), and a self-reported diagnosis with depression. Women who met at least two criteria were classified as MD subjects (Sinikumpu et al., 2023). A total of 205 control women and 102 women with PCOS were identified, all of whom provided stool samples for the gut microbiome analysis at age 46.

In Studies III and IV, a total of 164 endometrial tissue samples from non-PCOS and PCOS women were collected at the Oulu University Hospital and 29 samples from RIF patients at the Competence Centre on Health Technologies (CCHT, Tartu, Estonia). The RIF samples were obtained during a hormone replacement treatment cycle on day 5 after initiating P4 administration. All study subjects and materials used in this thesis are listed in **Table 2**.

All of the studies discussed in this thesis received approval from The Regional Ethics Committee of the Northern Ostrobothnia Hospital District, Finland (Studies I, III, IV: 65/2017; Study II: EETTMK 94/2011) and from the Research Ethics Committee of the University of Tartu, Estonia (Studies III, IV: 340T-12), and followed the principles of the Declaration of Helsinki.

Table 2. Study subjects and materials used in the thesis

Study	Study I	Study II	Studies III and IV
Study aim	RT[1] microbiome in women with PCOS	Gut microbiome in women with PCOS who have experienced MDs[2]	Epithelium and CD138 cell percentages in women with PCOS and women with RIF
Design	Prospective case-control study	Case-control study linking to a birth cohort data	Prospective case-control study
Study subjects	37 controls, 52 PCOS women	205 controls, 102 PCOS women	44 controls, 62 PCOS women, 29 RIF women
Age			
Controls	32.7±5.9	46	32.2±5.8
PCOS	34.0±5.4	46	33.0±5.3
RIF			38.4±5.7

BMI[3]			
Controls	27.0±5.0	27.9±5.4	26.5±4.9
PCOS	28.5±6.0	27.8±5.5	28.4±6.6
RIF			25.5±4.8
Study material	EF[4] and VS[5] samples using a syringe with a catheter and a swab, respectively	Fecal samples collected at age 46	Endometrial tissue biopsy samples using a suction curette (Pipelle®)

[1]reproductive tract, [2]mood disorders, [3]body mass index, [4]endometrial fluid, [5]vaginal swab

4.2 Biological sample collection and storage

Blood samples were collected during clinical visits to measure metabolic, hormonal, and inflammatory factors. The confirmation of menstrual cycle phases involved transvaginal ultrasonography and histological assessment of the endometrial tissues by experienced gynecologists and gyneco-pathologists.

One milliliter of sterile saline solution (0.9%) was injected into the uterine cavity using a syringe attached to a catheter (Pipelle®), and after ten seconds, the fluid was aspirated. The EF was transferred to a 1 ml sterile Eppendorf tube, and the tube was then immediately placed on ice and stored in a -80 °C deep freezer before microbial DNA extraction. A VS was inserted into the vagina, brushed on the vaginal wall, and then removed. The swab was promptly placed into a sterile tube and stored in a -80 °C deep freezer before DNA extraction. In this study, transvaginal ultrasonography was done first, followed by microbiome sample collection (VS), and the endometrial tissue biopsy was performed last.

In Study II, fecal samples were self-collected by the study participants at age 46 and transported in a cooler on the collection day or stored in a freezer at -20 °C for 1 or 2 days before shipment. Upon arrival at the hospital, the fecal samples were initially kept at -20 °C and later transferred to -70 °C for long-term storage before microbial DNA extraction (described in Publication II).

Endometrial biopsy specimens were obtained using an endometrial suction curette (Pipelle curette; CooperSurgical, Trumbull, CT). The tissue samples were fixed in 10% formaldehyde and stored before IHC staining (described in Publications III and IV).

4.3 Clinical characteristics of the study subjects

The clinical characteristics of all study subjects are documented in **Table 3** to **Table 5**.

Table 3. Clinical data of the study subjects used in Study I

	Controls (*n*=37)	Ovulatory women with PCOS (*n*=43)	Anovulatory women with PCOS (*n*=9)	*p*-value
Age (years)	31.0 [28.0;37.5] (37)	35.0 [30.0;38.0] (43)	32.0 [24.0;38.5] (9)	0.24
BMI[1] (kg/m2)	26.4 [23.2;31.7] (37)	27.6 [22.8;32.0] (43)	28.6 [23.8;37.7] (9)	0.43
Fasting glucose (mmol/L)	5.2 [4.8;5.5] (37)	5.3 [5.0;5.6] (42)	5.6 [5.3;6.4] (9)	**0.04**
Fasting insulin (mU/L)	7.1 [5.0;9.9] (37)	8.1 [5.5;10.8] (43)	9.9 [7.2;19.2] (9)	0.12
HOMA-IR[2]	1.6 [1.1;2.6] (37)	1.9 [1.2;2.6] (42)	2.8 [2.0;5.0] (9)	**0.02**
FSH[3] (IU/L)	7.1 [6.4;8.3] (31)	6.5 [5.6;7.6] (36)	6.6 [5.6;8.9] (9)	0.57
LH[4] (IU/L)	6.9 [5.9;10.2] (31)	7.9 [6.5;9.3] (36)	16.5 [11.6;25.2] (9)	**0.01**
AMH[5] (ng/ml)	2.5 [1.5;4.1] (37)	4.7 [2.5;5.8] (42)	9.1 [4.4;10.8] (9)	**<0.001**
SHBG[6] (nmol/L)	62.0 [41.1;89.6] (37)	49.4 [40.4;70.3] (42)	34.6 [20.0;57.0] (9)	**0.04**
Testosterone (nmol/L)	0.9 [0.8;1.3] (37)	1.2 [1.0;1.4] (43)	2.2 [1.8;2.7] (9)	**<0.001**
FAI[7]	1.9 [1.1;2.3] (37)	2.4 [1.5;3.1] (42)	6.4 [3.4;10.4] (9)	**<0.001**
P4[8] (nmol/L)	0.5 [0.3;0.8] (31)	0.5 [0.3;0.8] (37)	0.4 [0.2;0.7] (9)	0.13

Clinical characteristics are presented as median with interquartile range [Q1;Q3]. Blood samples were collected between cd[9] 2 and 8. The number of subjects is indicated in parentheses. A *p*-value was calculated by Kruskal–Wallis test, and bold values represent $p<0.05$. [1]body mass index, [2]Homeostatic Model Assessment for Insulin Resistance, [3]follicle-stimulating hormone, [4]luteinizing hormone, [5]anti-Müllerian hormone, [6]sex hormone-binding globulin, [7]free androgen index, [8]progesterone, [9]cycle day

Table 4. Clinical data of the study subjects used in Study II

	Controls (*n*=205)	Women with PCOS (*n*=102)	*p*-value
BMI[1] (kg/m^2)	26.2 [24.0;30.5] (205)	26.1 [24.1;30.4] (102)	0.93
Waist (cm)	88.5 [80.6;97.9] (204)	87.2 [80.8;95.3] (101)	0.61
Testosterone (nmol/L)	0.8 [0.6;1.1] (204)	0.9 [0.7;1.1] (102)	**0.05**
SHBG[2] (nmol/L)	52.9 [38.2;72.5] (203)	51.1 [34.2;70.9] (102)	0.71
FAI[3]	1.5 [1.1;2.2] (203)	1.7 [1.4;2.6] (102)	**0.01**
HOMA-IR[4]	1.8 [1.2;2.8] (198)	1.8 [1.3;3.2] (100)	0.43
Fasting glucose (mmol/L)	5.3 [5.1;5.6] (198)	5.4 [5.1;5.7] (100)	0.53
Fasting insulin (mU/l)	7.8 [5.3;11.3] (200)	7.7 [5.8;12.2] (100)	0.49
Hs-CRP[5] (mg/L)	0.8 [0.4;1.9] (192)	0.9 [0.4;1.8] (99)	0.87
Zonulin (ng/mL)	128.5 [116.4;137.2] (203)	131.0 [121.1;140.6] (102)	0.13
FABP2[6] (ng/mL)	1.3 [1.0;1.8] (203)	1.3 [1.0;1.8] (102)	0.59

Clinical characteristics are presented as median with interquartile range [Q1;Q3]. The number of subjects is indicated in parentheses. A *p*-value was determined by Mann–Whitney U-test, and bold values represent $p<0.05$. [1]body mass index, [2]sex hormone-binding globulin, [3]free androgen index, [4]Homeostatic Model Assessment for Insulin Resistance, [5]high sensitive C-reactive protein, [6]fatty acid-binding protein 2

Table 5. Clinical data of the study subjects used in Study III

PCOS diagnosis and ovulatory status	Controls (*n*=44)	Ovulatory women with PCOS (*n*=50)	Anovulatory women with PCOS (*n*=12)	*p*-value
Age (years)	34.0 [28.0;38.0] (44)	35.0 [30.0;38.0] (50)	31.0 [23.5;32.0] (12)	0.06
BMI[1] (kg/m2)	25.6 [22.1;31.2] (44)	26.9 [23.4;32.0] (50)	28.5 [23.3;36.8] (12)	0.25
FSH[2] (IU/L)	7.2 [6.6;8.5] (39)	6.3 [5.2;7.6] (38)	6.6 [5.8;7.6] (12)	**0.01**
LH[3] (IU/L)	6.8 [5.9;9.8] (39)	7.6 [6.4;9.2] (38)	15.7 [10.7;20.0] (12)	**<0.001**
AMH[4] (IU/L)	2.3 [1.5;3.8] (39)	5.0 [3.4;6.0] (38)	8.6 [4.7;12.3] (12)	**<0.001**
SHBG[5] (nmol/L)	62.0 [40.8;89.9] (39)	47.3 [36.8;63.9] (38)	38.6 [22.4;64.4] (12)	**0.05**
Testosterone (nmol/L)	0.9 [0.8;1.2] (39)	1.2 [1.0;1.4] (42)	2.3 [1.7;3.0] (12)	**<0.001**
FAI[6]	1.6 [1.2;2.2] (39)	2.4 [1.6;3.2] (40)	6.4 [3.3;11.2] (12)	**<0.001**
P4[7] (nmol/L)	0.5 [0.3;0.7] (39)	0.5 [0.3;0.8] (42)	0.4 [0.2;0.5] (12)	0.12
PCOS phenotype	Phenotype A (*n*=38)	Phenotype D (*n*=20)		*p*-value
Age (years)	34.0 [30.8;38.0] (38)	32.0 [28.3;36.8] (20)		0.18
BMI (kg/m2)	26.5 [23.2;32.6] (38)	28.3 [23.6;31.3] (20)		0.67
FSH (IU/L)	5.9 [5.0;7.3] (34)	6.8 [6.2;7.8] (16)		0.17
LH (IU/L)	8.2 [6.4;10.7] (34)	8.6 [7.4;10.6] (16)		0.33
AMH (IU/L)	5.2 [4.3;7.7] (34)	5.6 [3.4;8.7] (16)		0.79
SHBG (nmol/L)	41.2 [33.0;64.2] (34)	51.8 [42.9;65.8] (16)		0.12
Testosterone (nmol/L)	1.3 [1.0;1.7] (34)	1.3 [1.0;1.8] (18)		0.92
FAI	2.8 [1.6;4.2] (34)	2.3 [1.6;3.4] (16)		0.28
P4 (nmol/L)	0.5 [0.3;0.6] (34)	0.5 [0.3;0.9] (18)		0.44

Clinical characteristics are presented as median with interquartile range [Q1;Q3]. Blood samples were collected between cd[8] 2 and 8. The number of subjects is indicated in parentheses. A *p*-value was calculated Kruskal–Wallis test, and bold values represent $p<0.05$. [1]body mass index, [2]follicle-stimulating hormone, [3]luteinizing hormone, [4]anti-Müllerian hormone, [5]sex hormone-binding globulin, [6]free androgen index, [7]progesterone, [8]cycle day

4.4 Laboratory methods

A summary of all methodologies used in the four studies and discussed in this thesis is provided in **Table 6**. Detailed information about these methods can be found in original Publications I–IV.

In Study I, microbial DNA was extracted from the frozen EF and VS samples using the DNeasy PowerSoil PRO kit (Qiagen). The hypervariable V4-V5 regions of the 16S rRNA gene were amplified with primer 519F and primer 926R. Polymerase chain reactions were conducted in duplicates, and the PCR products were then purified and sequenced at the Biocenter Oulu Sequencing Center (Oulu, Finland) on an Ion Torrent PGM sequencer. During the sequencing step, two negative controls, consisting of sterile water, and one saline kit control were incorporated into each run.

In Study II, the microbial DNA from the frozen fecal samples was extracted using the QIAamp Stool Mini Kit (Qiagen) and sequenced using the V3–V4 regions of the 16S rRNA gene on an Illumina MiSeq sequencing instrument.

In Studies III and IV, endometrial receptivity in RIF samples was assessed using 57 receptivity-related gene expressions at CCHT (Tartu, Estonia). RNA extraction was conducted using the miRNeasy Mini kit (Qiagen), and based on the test results, the RIF samples were categorized into pre-receptive, receptive, and post-receptive endometrium. The endometrial tissues fixed in 10% formaldehyde were stained with 40x diluted mouse anti-human monoclonal antibody CD138 (MS-1793-S; Thermo Fisher Scientific), followed by H&E staining. All IHC slides were scanned using a Leica SCN 400 Slide Scanner (Leica, Biosystems, United States), and the scanned images were uploaded to a cloud-based image processing and analysis platform (Aiforia Technologies Oy, Helsinki, Finland). The images were used both for AI model training and subsequent AI analysis. The AI model comprised two CNN layers. CNN1 was trained to segment both epithelium and stroma, and the areas measured by CNN1 were utilized to analyze epithelial gland development in Study III. CNN2 was trained to detect CD138+ cells in the endometrial stroma, and the occurrence of CD138+ cells identified by CNN2 was analyzed in Study IV. Before the analysis, the AI training was validated by two external pathologists, and the AI performance was validated by three external pathologists.

Table 6. Methodologies used in the studies.

Methodologies	Original publication
Microbial DNA extraction	I, II
16S rRNA sequencing	I, II
Microbiome data analysis	I, II
Endometrial receptivity testing	III, IV
IHC[1]	III, IV
AI[2] algorithm training, validation, and analysis	III, IV

[1]immunohistochemistry, [2]artificial intelligence

4.5 Statistical analysis and data visualization

The results are presented as the mean±standard deviation or the median with the interquartile ranges, depending on data distribution and normality. For continuous variables, the independent samples *t*-test or Mann–Whitney U-test was used for paired comparisons, and Kruskal–Wallis test was used for multiple comparisons. A statistically significant difference was considered if $p<0.05$.

Linear mixed-effects models were used to adjust the microbiome profiles of samples from the same donors. Alpha diversity of a microbial community describes variations of microbes in a sample (Jost, 2007). It can be measured by species richness, defined as the number of unique taxa, and species evenness, which is the distribution balance of these taxa relative to each other. Beta diversity of a microbial community is defined as the variation of microbial communities between samples, mainly focusing on the differences in taxonomic abundance profiles (Jost, 2007). In Studies I and II, alpha diversity was evaluated by the observed number of unique genera, referred to as observed features, and the Shannon diversity index, which indicates both species richness and evenness, using the vegan package (Study I: v2.5.6; Study II: v2.6-4). Beta diversity was measured using the Euclidean distance on the unfiltered centered log-ratio transformed genus-level microbiome profile in Study I and using principal component analysis (PCA) with Bray–Curtis Dissimilarity at the genus level in Study II. Permutational Analysis of Variance (PERMANOVA) was utilized with 10,000 permutations for *p*-value calculations, using the adonis function in the vegan package (v.2.5.6) (Anderson, 2001). Differential abundance analysis was conducted using two methods: the ANOVA-Like Differential Expression tool for compositional data (ALDEx2, v1.30.0) and the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC,

v2.0.2) in Studies I and II (Fernandes et al., 2014; Lin & Peddada, 2020). The Benjamini–Hochberg method was utilized to correct for multiple testing.

In Study II, Pearson's Chi-square test was employed for the statistical analysis of categorical variables, and a *p*-value for multiple testing was adjusted with the Benjamini–Hochberg false discovery rate (FDR). A partial correlation coefficient was calculated using Spearman's correlation coefficient (ppcor, v.1.1) (S. Kim, 2015).

In Studies III and IV, a mixed-model analysis of variance (ANOVA) was used to estimate the effects of PCOS and cycle phases on (i) epithelium percentages and (ii) CD138+ cell percentages. On the other hand, Kruskal–Wallis test was employed to evaluate the impact of endometrial receptivity in RIF endometrium on (i) epithelium percentages and (ii) CD138+ cell percentages. The interobserver variability (i) between the pathologists and the AI model in the training validation and (ii) among the pathologists in the performance validation was evaluated using an intra-class correlation (ICC) estimate. The ICC value was calculated using a two-way mixed effects model with an absolute agreement model, in which reliability levels were categorized as follows: poor ($ICC<0.5$), moderate ($0.5\leq ICC<0.75$), good ($0.75\leq ICC<0.9$), and excellent ($ICC\geq 0.9$) (Koo & Li, 2016). Correlations with clinical characteristics were determined by a linear mixed model for the epithelium percentages and by Spearman correlations for the CD138 cell percentages.

Data analyses were performed using IBM SPSS Statistics v27 and v28 (IBM Corp., Armonk, NY, United States), GraphPad Prism (version 9.3.0), and RStudio (version 2022.12.0.353 with R version 4.2.2).

5 Results and discussion

5.1 RT microbiome profile in women with PCOS across the menstrual cycle (Study I)

As described earlier in sections 2.3.2 (Female RT microbiome) and 2.3.3 (The impact of microbiome on PCOS), alterations in the RT microbiome are associated with several gynecological conditions and obstetrical complications (N. Molina et al., 2020; Saraf et al., 2021; Sola-Leyva et al., 2023). However, previous studies did not fully account for confounding factors that may affect the microbiome, such as age, BMI, and menstrual cycle phases. Additionally, our understanding of the endometrial microbiome in women with PCOS remains significantly limited. Study I aimed to characterize the endometrial and vaginal microbiome in women with PCOS throughout the menstrual cycle and identify PCOS-related microbial signatures.

5.1.1 Overview of the endometrial and vaginal microbiome

The endometrial and vaginal microbiome were obtained from women with and without PCOS. A total of 545 genera in the EF microbiome and 114 genera in the VS microbiome were detected. *Lactobacillus* was the dominant genus in both EF and VS samples, followed by *Atopobium*, *Streptococcus*, *Prevotella*, *Shuttleworthia*, and *Sneathia* (**Fig. 7a**). There are variations in the microbial compositions of the endometrium and vagina among previous studies regarding the prevalence of genera, such as *Prevotella*, *Bacteroides*, *Atopobium*, and *Streptococcus*. These inconsistencies may be attributed to variations in sequencing techniques and the specific regions targeted within hypervariable sequences, potentially leading to amplification biases and reduced taxonomic precision (Abellan-Schneyder et al., 2021; N. Molina et al., 2020; Peric et al., 2019). Additionally, differences in bioinformatics data analysis pipelines across studies could contribute to discrepancies in the microbiome profile (N. M. Molina et al., 2021). In particular, the endometrial microbiome can exhibit markedly different profiles depending on the analysis pipelines employed, primarily due to its low biomass of microbes (Allali et al., 2017; Marizzoni et al., 2020; N. M. Molina et al., 2021; Peuranpää et al., 2022).

The EF microbial community showed higher observed features and Shannon index, indicating that it consisted of a greater variety of bacterial taxa and had more evenness in the distribution of these taxa compared to the VS microbial community ($p_{\text{Observed richness}}<0.001$; $p_{\text{Shannon}}<0.001$) (**Fig. 7b**). Additionally, the EF microbial community was significantly distinct from the VS community, indicating that the bacterial taxa present in the EF community differed from those in the VS community ($R^2=0.04$, PERMANOVA<0.001) (**Fig. 7c**). The differences in community diversity between the EF and VS samples may stem from the abundance of *Lactobacillus*. The relative abundance of *Lactobacillus* was greater in the VS community compared to the EF community (EF: median 87.7%; VS: median 99.1%; $p<0.001$) (**Fig. 8a**). Given that *Lactobacillus* hinders the growth of other bacteria in the vagina by maintaining an acidic environment and exerting antimicrobial activity, a higher abundance of *Lactobacillus* in the VS microbial community compared to the EF community is likely linked to lower alpha diversity and distinct community clustering (Aroutcheva et al., 2001; C. Chen et al., 2017; Eschenbach et al., 1989; Mansour et al., 2011). *Lactobacillus*-dominant communities observed in the EF microbial community, despite a less favorable environment with a higher pH compared to the vagina, may be attributable to bacterial ascension from the vagina to the endometrium through peristaltic contractions (Hansen et al., 2014; Winters et al., 2019). Furthermore, contact with the cervical canal was unavoidable during the insertion and removal of the catheter for EF collection. This could explain the dominance of *Lactobacillus* in EF samples, possibly due to contamination from vaginal microbiota, particularly *Lactobacillus* (Lüll & Org, 2023; N. M. Molina et al., 2021). Considering that the endometrial microbial communities obtained during hysteroscopy, laparoscopy, and/or during cesarean section did not exhibit a *Lactobacillus*-dominant composition, it is crucial to carefully consider the sampling method for the uterine microbiome (C. Chen et al., 2017; Leoni et al., 2019; Verstraelen et al., 2016; Winters et al., 2019).

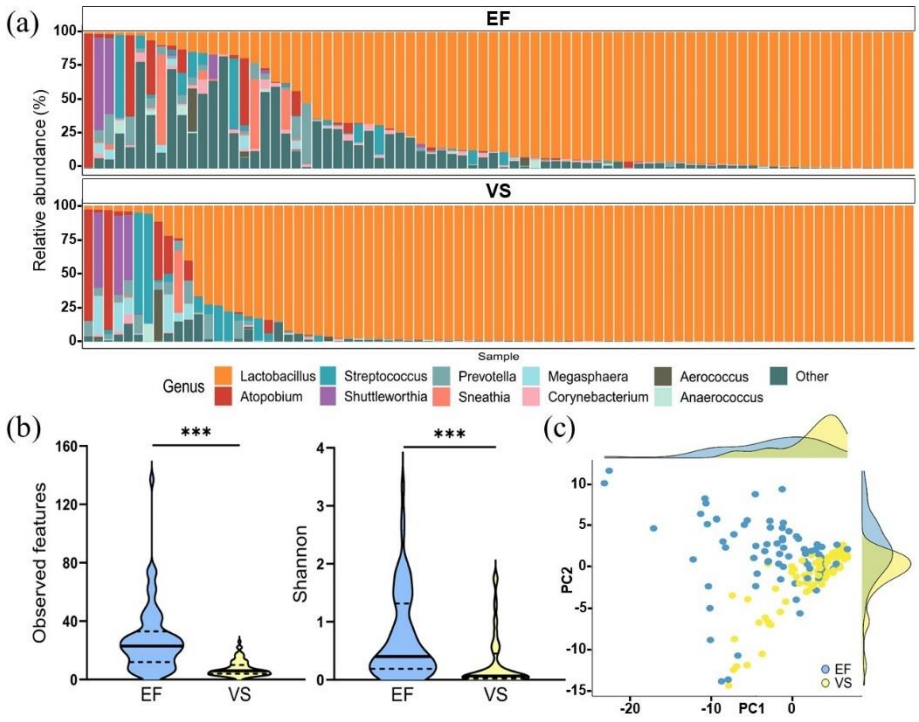

Fig. 7. Landscape of the endometrial and vaginal microbial communities. (a) Composition of the EF and VS microbial communities at the genus level. The top 10 most abundant genera are shown, while the remaining genera are grouped under "other." (b) Alpha diversity between the EF and VS communities was represented by the observed features and Shannon index. The violin plots are shown to display the distribution of the values. The median values are indicated by the middle line, while the interquartile ranges are represented by the dashed lines. Blue represents EF samples, and yellow represents VS samples. A *p*-value was determined using Mann–Whitney U-test for comparing EF and VS samples. (c) Beta diversity was calculated using PCA. A single sample is presented as a dot. Blue dot represents EF samples, and yellow dot represents VS samples. A *p*-value was defined by PERMANOVA analysis. EF (endometrial fluid), VS (vaginal swab), PCA (principal component analysis)

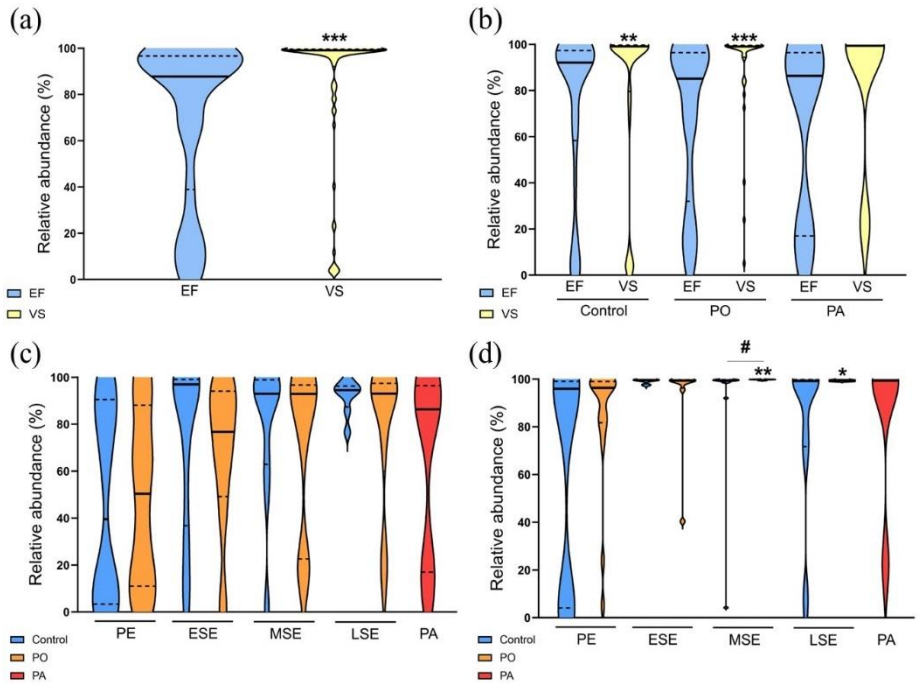

Fig. 8. The relative abundance of *Lactobacillus*. (a) The abundance of *Lactobacillus* in whole EF and whole VS samples. (b) The abundance of *Lactobacillus* in control, PO, and PA samples within each sample type. In (a) and (b), blue represents EF samples, and yellow represents VS samples. A *p*-value was calculated by Mann–Whitney U-test for comparing EF and VS samples. ***p*<0.01 and ****p*<0.001. The relative abundance of *Lactobacillus* at different cycle phases in control, PO, and PA samples in (c) the EF and (d) the VS communities. In (c) and (d), blue indicates control, orange PO, and red PA samples. The violin plots display the distribution of the values, with the median values indicated by the middle line and the interquartile ranges represented by dashed lines. **p*<0.05 and ***p*<0.01 were calculated by Kruskal–Wallis test when comparing the PE to the SE samples within each group. A *p*-value was calculated by Mann–Whitney U-test for comparing control and PO samples at the same cycle phase. #*p*<0.05. EF (endometrial fluid), VS (vaginal swab), PE (proliferative phase), ESE (early secretory phase), MSE (mid-secretory phase), LSE (late secretory phase), PA (PCOS anovulatory), PO (PCOS ovulatory)

5.1.2 Influence of PCOS and menstrual cycle phases on the RT microbiome

After demonstrating the distinct microbiome profiles based on their location within the RT, further analyses were conducted separately for each location. *Lactobacillus* was the most abundant taxon, regardless of the presence or absence of PCOS and ovulatory phenotypes in PCOS (**Fig. 9a**). Except for PCOS anovulatory (PA) samples, control and PCOS ovulatory (PO) samples showed higher *Lactobacillus* abundance in the VS samples compared to the EF samples (EF vs. VS: control, p=0.003; PO, p<0.001; PA, p=0.25) (**Fig. 8b**). This may be due to the smaller sample size of PA (n_{EF}=6; n_{VS}=5).

Similarly to the findings in section 5.1.1, alpha diversity indices were significantly higher in the EF samples compared to the VS samples; however, there were no differences observed between the sample types in the PA samples (EF vs. VS: control, $p_{\text{observed richness}}$<0.001, p_{Shannon}=0.013; PO, $p_{\text{observed richness}}$<0.001, p_{Shannon}<0.001; PA, $p_{\text{observed richness}}$=0.22, p_{Shannon}=0.53). PCOS diagnosis did not seem to affect community diversity (control vs. PO vs. PA: EF, $p_{\text{observed richness}}$=0.36, p_{Shannon}=0.69; VS, $p_{\text{observed richness}}$=0.85, p_{Shannon}=0.86; R^2=0.01, PERMANOVA=0.26) (**Fig. 9b,c,d**). Although previous studies have reported a decreased abundance of *Lactobacillus* and increased alpha diversity in the vaginal microbiome of women with PCOS compared to that of women without PCOS, these findings were not replicated in our study (Hong et al., 2020; Tu et al., 2020). These discrepancies could stem from the careful adjusting of confounding factors, such as age and BMI, between women with and without PCOS in this study. These factors can influence PCOS manifestations, including HA and IR, as well as microbiome profiles (Allen et al., 2022; Hsu, 2013; Medina-Bastidas et al., 2022; Moran et al., 2015).

Next, the microbial communities were investigated across the menstrual cycle. Cycle-related changes in the composition of the community were observed only in the PO VS samples, showing an increase in abundance of *Lactobacillus* from the PE to the SE (PE vs.: p_{MSE}=0.01; p_{LSE}=0.04) (**Fig. 8c,d**). Even, the PO samples showed a significantly higher abundance of *Lactobacillus* than the control samples at MSE (control vs. PO: p=0.04) (**Fig. 8d**).

Control EF samples showed significantly higher alpha diversity in the PE compared to the SE, with a clear decline from the PE to the ESE (**Fig. 10a,c**). On the other hand, PCOS EF samples exhibited comparable values regardless of the

cycle phases and ovulatory phenotypes. In particular, the alpha diversity in the EF samples was significantly higher in PCOS samples compared to control samples (control vs. PO: $p_{\text{observed richness}}$=0.03, p_{Shannon}=0.03) (**Fig. 10a,c**). This may be due to the lower *Lactobacillus* abundance in PCOS endometrium than in control endometrium during the ESE, although a statistical difference was not observed (**Fig. 8c**). Considering the elevated levels of LH and testosterone in the PCOS subjects, this finding aligns with previous probiotic intervention studies in women with PCOS (**Table 3**). Previous studies have shown that probiotics containing *Lactobacillus* and *Bifidobacterium* have beneficial effects on hormone imbalance in women with PCOS and a PCOS-like rat model, evidenced by negative correlations between the abundance of *Lactobacillus* and serum LH and testosterone levels (Jamilian et al., 2018; I. Kaur et al., 2022; Nasri et al., 2018; Ostadmohammadi et al., 2019). However, these studies focused on the gut microbiome, and conflicting findings exist regarding the role of *Lactobacillus* in the clinical features of PCOS (Łagowska & Kapczuk, 2022; X. Liu et al., 2024).

For the VS communities, alpha diversity remained consistent across cycle phases, except for significantly lower observed features in the MSE and LSE compared to the PE in PO VS samples (PE vs.: MSE, $p_{\text{observed richness}}$=0.01; LSE, $p_{\text{observed richness}}$=0.004) (**Fig. 10b,d**). Additionally, control EF community exhibited distinct clusters based on the cycle phases (R^2=0.14, PERMANOVA<0.001), unlike control VS and PCOS samples (**Fig. 11**). Again, it may be related to the increased abundance of *Lactobacillus* during the SE.

The cycle phase-related changes in the microbial communities, including composition and diversity, may be linked to sex hormone fluctuation during the menstrual cycle. E2 and P4 can enhance the immune function and promote nutrient supply for specific taxa, as discussed in 2.4.2 (Female RT microbiome) (H. Kaur et al., 2020; Nunn & Forney, 2016). Specifically, glycogen accumulated in the vaginal epithelium due to the action of E2 is released into the vaginal lumen through the action of P4, which lyses the epithelium (Shen et al., 2022). This process results in the proliferation of *Lactobacillus*, thereby leading to decreased species richness and evenness within the community (C. Chen et al., 2017; Krog et al., 2022; S. D. Song et al., 2020). Therefore, samples collected during the SE, characterized by elevated P4 levels following the peak of E2, may indicate a more stable and less diverse microbial community compared to samples obtained during the PE (cd 6-8), with lower levels of both E2 and P4.

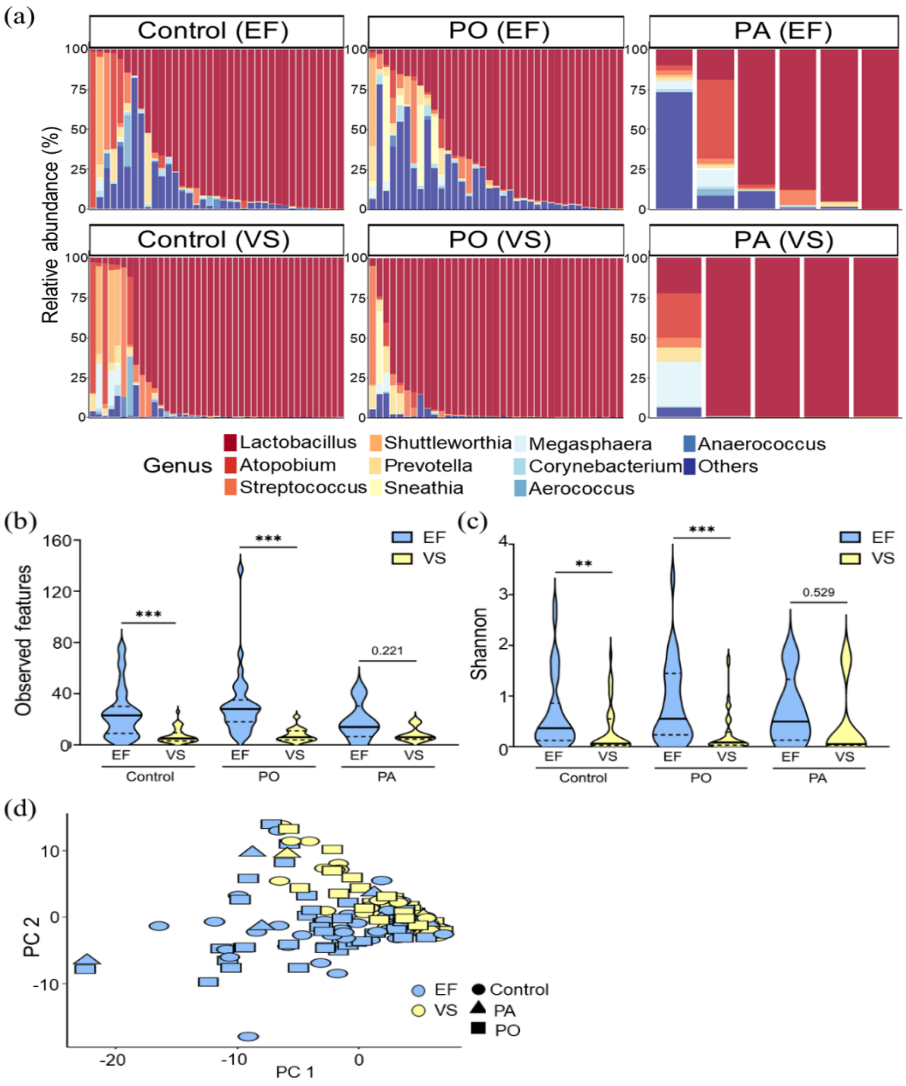

Fig. 9. Landscape of the endometrial and vaginal microbial communities based on PCOS diagnosis and PCOS ovulatory phenotypes. (a) Composition of the EF and VS microbial communities at the genus level. The top 10 most abundant genera are shown, while the remaining genera are grouped under "other." The community alpha diversity was represented by (b) the observed richness and (c) the Shannon index. The violin

plots display the distribution of the values, with the median values indicated by the middle line and the interquartile ranges represented by dashed lines. Blue represents EF samples, and yellow represents VS samples. A *p*-value was defined by Mann–Whitney U-test for comparing two groups and Kruskal–Wallis test for multiple groups. ***p*<0.01 and ****p*<0.001. (d) Beta diversity was calculated by PCA. Blue represents EF samples, and yellow represents VS samples. Each symbol corresponds to a single sample: Circles indicate control samples, triangles PA samples, and squares PO samples. A *p*-value was defined by PERMANOVA analysis. EF (endometrial fluid), VS (vaginal swab), PO (PCOS ovulatory), PA (PCOS anovulatory), PCA (principal component analysis)

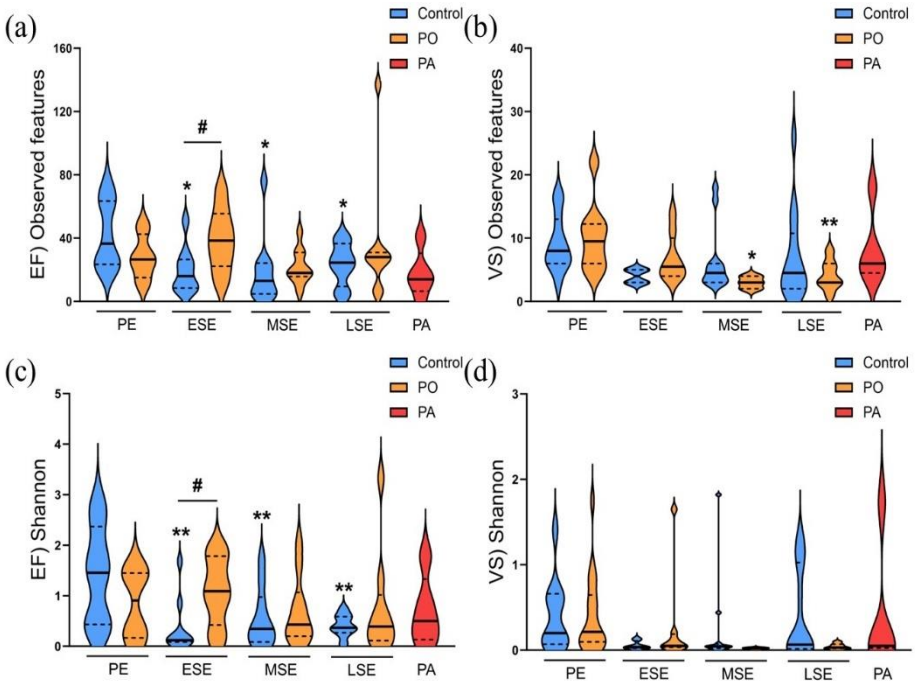

Fig. 10. Alpha diversity of the endometrial and vaginal microbial community across the menstrual cycle phases. Alpha richness was analyzed using observed features and the Shannon index: (a) EF observed features, (b) VS observed features, (c) EF Shannon, and (d) VS Shannon. The violin plots display the distribution of the values, with the median values indicated by the middle line and the interquartile ranges represented by dashed lines. Blue represents control, orange PO, and red PA. A *p*-value was defined by Mann–Whitney U-test for comparing two groups and Kruskal–Wallis test for multiple groups. **p*<0.05 and ***p*<0.01 when comparing the PE and other cycle phases, including the SE and PA, within control or PCOS samples, and #*p*<0.05 when comparing control and PO samples at the same phase. EF (endometrial fluid), VS (vaginal swab), PO (PCOS ovulatory), PA (PCOS anovulatory), PE (proliferative phase), ESE (early secretory phase), MSE (mid-secretory phase), LSE (late secretory phase), PCA (principal component analysis)

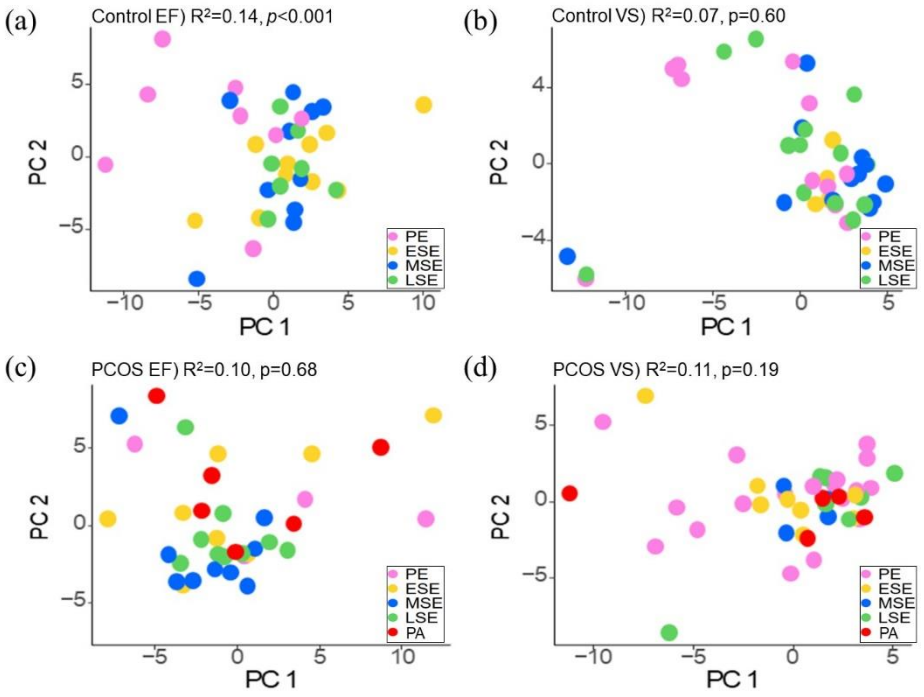

Fig. 11. Beta diversity of the endometrial and vaginal microbial community across the menstrual cycle phases. Beta diversity was calculated by PCA. (a) control EF, (b) control VS, (c) PCOS EF, and (d) PCOS VS. A single sample is presented as a dot. Pink represents PE samples, yellow ESE samples, blue MSE samples, green LSE samples, and red anovulatory samples. A *p*-value was defined by PERMANOVA analysis. EF (endometrial fluid), VS (vaginal swab), PO (PCOS ovulatory), PA (PCOS anovulatory), PE (proliferative phase), ESE (early secretory phase), MSE (mid-secretory phase), LSE (late secretory phase), PCA (principal component analysis)

Lastly, the microbial signatures associated with PCOS were explored. Since the PCOS microbial communities were not differentiated based on ovulatory status, all PCOS samples were merged for analysis. The centered log-ratio-transformed abundance of Acetobacteraceae uncultured, *Prevotella*_9, and *Rhodoferax* was significantly different based on the presence or absence of PCOS in the EF samples. Specifically, Acetobacteraceae uncultured and *Prevotella*_9 were more abundant in control samples, while *Rhodoferax* was more abundant in PCOS samples (Acetobacteraceae uncultured: beta=-0.08, FDR=0.09; *Prevotella*_9: beta=-0.70, FDR=0.09; *Rhodoferax*: beta=0.28, FDR=0.04) (**Fig. 12**). However, no taxa were differentially abundant according to the PCOS diagnosis in the VS samples (FDR≥0.1).

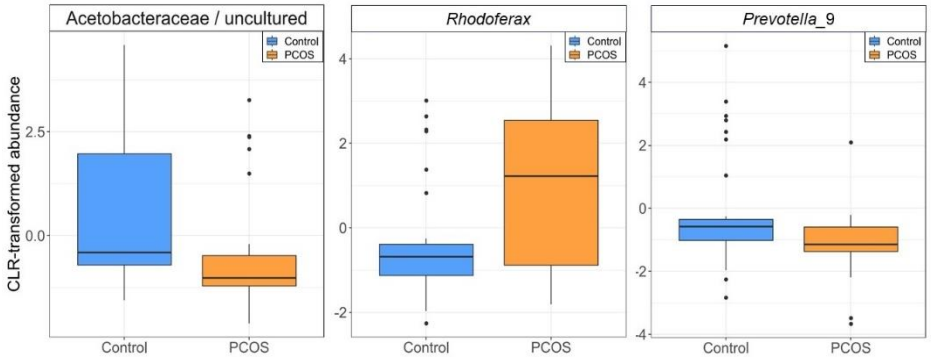

Fig. 12. Differential abundant taxa based on the PCOS diagnosis in the EF samples. The box plot shows the interquartile ranges, and the middle line represents the median values. Whiskers in the box plot denote minimum to maximum values. Blue represents control and orange PCOS samples. A *p*-value was calculated using linear mixed-effects models and adjusted using the Benjamini–Hochberg method. CLR (centered log-ratio)

The family Acetobacteraceae can suppress pathogen growth by releasing organic acids, which lowers the intracellular pH levels and disrupts the cellular processes of pathogens (Brown & Wernegreen, 2019; H. Chen et al., 2016; Sakakobara et al., 2010). Furthermore, the family Acetobacteraceae regulates blood glucose levels by reducing disaccharidase activity and facilitating the adenosine monophosphate-activated protein kinase pathway, leading to increased glucose uptake and its conversion to glycogen (Hlebowicz et al., 2007; Ogawa et al., 2000). A previous study on the endometrial microbiome among RIF patients demonstrated

a negative association between the relative abundance of *Rhodoferax* and the pregnancy success rate (P. Chen et al., 2022). While the role of Acetobacteraceae within the endometrium remains unexplored, these findings imply that an altered microbiome in the endometrium of women with PCOS may exacerbate pathogen infections, disrupt glucose metabolism, and potentially contribute to fertility complications. Further investigation is warranted to elucidate the role of these taxa in PCOS endometrium and their clinical implications for the pathophysiology of PCOS.

The potential beneficial roles of *Prevotella*_9 in women with PCOS have been suggested in the gut microbiome studies. *Prevotella*_9 is inversely correlated with 1-Linoleoylglycerophosphocholine, a metabolic product of the pyruvate carboxylase pathway, which itself shows a positive correlation with IR (Ruiz-Argüelles et al., 2018; Yu et al., 2022). This supports our findings, demonstrating higher HOMA-IR value and lower abundance of *Prevotella*_9 in women with PCOS compared to women without PCOS (**Table 1**, **Fig. 12**). Furthermore, *Prevotella* can enhance glucose and lipid metabolism, thereby reducing blood glucose and cholesterol and triglycerides levels (Gálvez et al., 2020; Guzior & Quinn, 2021; Kovatcheva-Datchary et al., 2015). Additionally, *Prevotella* can indirectly influence immune response through bile acids, which possess antimicrobial activities (Guzior & Quinn, 2021; Tian, Gui, et al., 2020). However, due to conflicting findings regarding the potential adverse effects of *Prevotella* on human health, further studies are required to gain a better understanding of its functions at species and strain levels. Furthermore, since the previous findings were derived from gut microbiome studies, more research on the RT microbiome is warranted.

5.2 Associations between gut microbial profile and MDs in women with PCOS (Study II)

Sections 2.3.1 (Gut microbiome) and 2.3.3 (The impact of microbiome on PCOS) discussed the crucial role of the gut microbiota in digesting nutrients, regulating immunity, metabolizing sex hormones, and influencing brain function (Devaraj et al., 2013; S. He et al., 2021; Peirce & Alviña, 2019; Simpson et al., 2021). However, the relationships between MDs and PCOS, with respect to the influence of the gut microbiome, remain unexplored despite existing evidence of links between the gut microbiome and both MDs and PCOS. The first aim of this study was to explore alterations in the gut microbiome associated with MDs in women with PCOS. Then, this study explored the correlations between the gut microbiome and common clinical features of MDs and PCOS to identify potential links between the gut microbiome and both conditions.

5.2.1 Changes in the landscape of the gut microbial community related to MDs in women with PCOS

The gut microbial community varied with MDs in women with PCOS. The PCOS MD cases exhibited a lower alpha diversity compared to the PCOS no-MD cases ($FDR_{Observed\ features}$=0.01, $FDR_{Shannon}$=0.01), although no distinctive clustering was shown in beta diversity (R^2=0.01, PERMANOVA=0.10) (**Fig. 13a,b,c**). On the contrary, controls did not show any MD-dependent alterations ($FDR_{Observed\ features}$=0.19, $FDR_{Shannon}$=0.14; R^2=0.01, PERMANOVA=0.39) (**Fig. 13a,b,d**). Additionally, PCOS did not affect community diversity when comparing MD cases with and without PCOS ($FDR_{Observed\ features}$=0.34, $FDR_{Shannon}$=0.42; R^2=0.02, PERMANOVA=0.96) (**Fig. 13a,b**). These results are consistent with the general understanding that higher diversity reflects higher stability and functionality within the microbial community, highlighting the advantageous ecological significance of the gut microbiota (Shade, 2017). However, due to conflicting results among studies regarding diversity in relation to MDs and PCOS, relying solely on community diversity may offer a simplified view of the gut microbiota and may not fully elucidate the intricate interactions with the host (Järbrink-Sehgal & Andreasson, 2020; Rizk & Thackray, 2021; Simpson et al., 2021; Sola-Leyva et al., 2023).

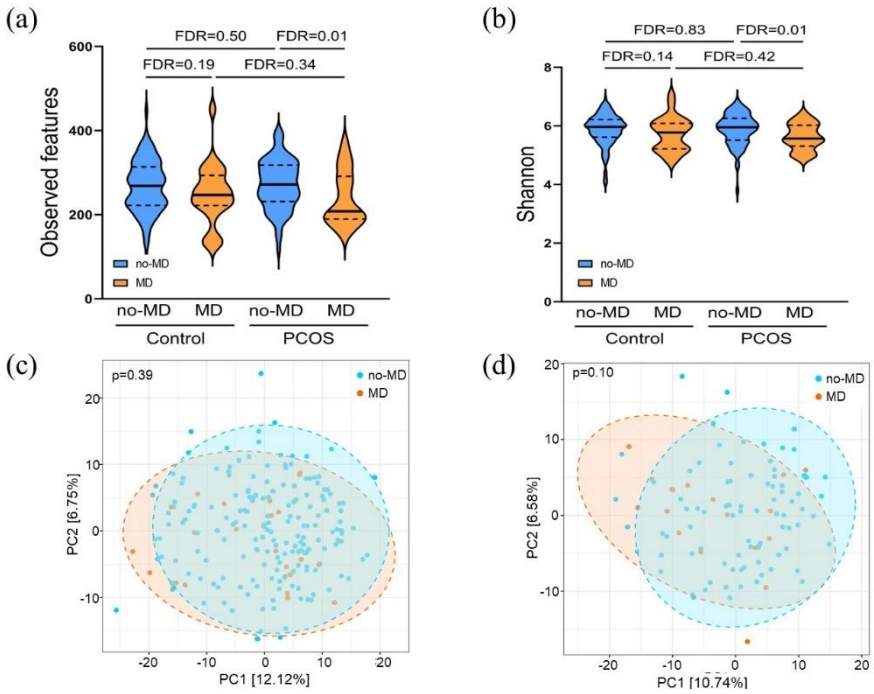

Fig. 13. The gut microbial community diversity. In (a) observed features and (b) the Shannon index. The violin plots are shown to display the distribution of the values. The median values are indicated by the middle line, while the interquartile ranges are represented by the dashed lines. Blue represents no-MD and orange represents MD cases. A *p*-value was defined using Kruskal–Wallis test adjusted with the Benjamini–Hochberg method. In the Bray–Curtis of (c) PCOS and (d) control groups, a single individual is presented as a dot, and the variation is shown by the percentages at the two axes. Blue represents no-MD and orange represents MD cases. A *p*-value was defined by PERMANOVA analysis. MD (mood disorder)

The median-based relative abundance of *Sutterella* was significantly higher in the PCOS MD cases compared to the PCOS no-MD cases (FDR<0.001), while no taxa showed statistically significant differences based on MDs in the control population (**Table 7**). Additionally, in the differential abundance analyses, the log transformation-based relative abundance of *Butyricicoccus* was significantly lower in the PCOS MD cases compared to the PCOS no-MD cases (ALDEx2, log fold change=-0.52, FDR=0.06; ANCOM-BC, log fold change=-0.90, FDR=0.04) (**Table 8**). Again, no taxa were differentially abundant based on MDs in the control

population (**Table 8**). Of note, no significant differences were found when comparing control MD and PCOS MD cases (**Table 9**). The lack of significance in community diversity and differential abundance analysis between MD cases with and without PCOS is likely attributable to the limited number of PCOS MD cases (*n*=18). Our results align with previous findings indicating decreased abundance of *Butyricicoccus* in a PCOS-like rat model, in generalized anxiety disorder patients, and in women with depressive symptoms (Chang et al., 2021; Jiang et al., 2018; Takeda et al., 2022; Y. Zhou et al., 2020). *Butyricicoccus*, a butyrate-producing bacterium, contributes to suppressing inflammation by modulating immune activation in the colon and enhancing brain function through brain-derived neurotrophic factor and neuroplasticity (Z. Li et al., 2018; Y. Zhou et al., 2020). Given the roles of SCFAs, such as anti-inflammatory mediators and neurotransmitters, the decreased abundance of *Butyricicoccus* and consequently reduced SCFA production can contribute to neuroinflammation, ultimately impacting mood in women with PCOS (Arneth, 2018; Simpson et al., 2021).

Table 7. Median-based relative abundance of the 10 most abundant genera in the PCOS population

	PCOS		Control		FDR[1a]		FDR[b]	
	no-MD[2] (*n*=84)	MD (*n*=18)	no-MD (*n*=180)	MD (*n*=25)	PCOS	Control	no-MD	MD
Bacteroides	21.1 [14.6;29.5]	27.1 [15.0;36.7]	27.6 [16.2;40.8]	24.7 [18.5;37.9]	0.30	0.75	**0.001**	0.51
Faecalibacterium	11.4 [6.4;18.6]	9.2 [5.1;18.2]	13.3 [7.6;19.4]	11.7 [8.2;15.3]	0.55	0.24	0.18	0.56
Alistipes	8.3 [3.6;13.5]	8.7 [4.1;12.8]	10.6 [6.2;16.6]	8.7 [4.6;14.7]	0.82	0.35	**0.02**	0.77
Parabacteroides	1.3 [0.0;3.8]	3.3 [0.4;6.0]	1.1 [0.1;4.5]	1.4 [0.3;4.1]	0.22	0.85	0.60	0.44
Ruminococcus	1.6 [0.9;4.1]	1.2 [0.6;5.0]	1.7 [0.7;3.4]	1.4 [0.5;2.5]	0.72	0.31	0.46	0.51
Subdoligranulum	2.8 [1.1;4.9]	1.6 [0.6;4.6]	3.2 [1.4;6.1]	2.5 [1.3;3.8]	0.26	0.14	0.27	0.58
Oscillospiraceae_UCG_002	1.7 [0.4;4.6]	1.8 [0.0;3.0]	2.4 [0.6;5.5]	1.7 [0.0;7.4]	0.12	0.56	0.27	0.30
Barnesiella	1.6 [0.0;4.6]	0.4 [0.0;4.7]	0.6 [0.0;3.0]	0.01 [0.0;1.5]	0.99	0.12	0.09	0.14
Sutterella	0.0 [0.0;0.0]	0.1 [0.1;1.4]	0.0 [0.0;0.5]	0.0 [0.0;0.6]	**<0.001**	0.95	**<0.001**	0.26
Prevotella	0.0 [0.0;0.2]	0.0 [0.0;0.0]	0.0 [0.0;0.2]	0.0 [0.0;3.6]	0.32	0.89	0.26	0.35

The 10 most abundant genera within the PCOS group are shown. The relative abundance of genera is presented as a median with an interquartile range [Q1;Q3]. A *p*-value was determined by Mann–Whitney U-test with the Benjamini–Hochberg FDR adjustment. FDR[a] is the statistical analysis between no-MD and MD cases in each group (PCOS or control), and FDR[b] is the statistical analysis between PCOS and control cases with the same MD status. Bold values represent FDR<0.05. [1]false discovery rate, [2]mood disorder

Table 8. Log transformed relative abundance of genera between no-MD and MD cases

	ALDEx2[1]			ANCOM-BC[2]		
	Taxa	Effect size	FDR[3]	Taxa	Effect size	FDR
Control	*Anaerotruncus*	-0.28	0.60	Lachnospiraceae_UCG-008	0.91	0.48
	Lachnospiraceae_UCG-008	0.33	0.57	*Streptococcus*	0.88	0.95
	Ruminococcaceae_DTU089	-0.24	0.62	Eubacterium_coprostanoligenes_group	1.01	0.95
	Oscillospiraceae_uncultured	-0.28	0.62	*Romboutsia*	0.85	0.95
	[Clostridium]_methylpentosum_group	-0.21	0.62	*Anaerotruncus*	-0.64	0.95
PCOS	*Butyricicoccus*	-0.52	0.06	*Butyricicoccus*	-0.90	**0.04**
	Oscillospiraceae_uncultured	-0.55	0.09	Oscillospiraceae_uncultured	-0.72	0.47
	Holdemania	-0.36	0.16	*Izemoplasmatales*	1.43	0.47
	Ruminococcaceae_DTU089	-0.37	0.20	*Holdemania*	-1.23	0.47
	Oscillibacter	-0.45	0.26	Ruminococcaceae_DTU089	-1.02	0.60
	Ruminococcus_torques_group	-0.39	0.53	*Oscillibacter*	-0.52	0.60
	Bacteroides	-0.37	0.54	Ruminococcus_torques_group	-0.54	0.60

The five and seven differentially abundant genera between no-MD and MD cases within the control and PCOS population are shown. A positive effect size value indicates a higher taxon abundance in the MD cases, while a negative value indicates a higher abundance in the no-MD cases. A *p*-value was adjusted using the Benjamini–Hochberg method. [1]ANOVA-like differential expression 2, [2]analysis of compositions of microbiomes with bias correction, [3]false discovery rate

Table 9. Log transformed relative abundance of genera between control MD and PCOS MD cases

	ALDEx2[1]			ANCOM-BC[2]		
	Taxa	Effect size	FDR[3]	Taxa	Effect size	FDR
MD	*Dialister*	0.11	0.98	*Peptococcus*	0.01	0.99
	Erysipelotrichaceae_UCG-003	0.13	0.98	*Agathobacter*	0.05	0.99

The two most differentially abundant genera between control MD and PCOS MD cases are shown. A positive effect size value indicates a higher taxon abundance in the PCOS MD cases, while a negative value indicates a higher abundance in the control MD cases. A *p*-value was adjusted using the Benjamini–Hochberg method. [1]ANOVA-like differential expression 2, [2]analysis of compositions of microbiomes with bias correction, [3]false discovery rate

5.2.2 Associations between the gut microbiome and common clinical features of PCOS and MDs

Partial correlation analyses were performed to investigate the potential involvement of the gut microbiome in MDs and PCOS, aiming to reduce the impact of confounding factors. Genera were selected based on their abundance in each group, while the chosen clinical features were those commonly found in both MDs and PCOS. In the correlation analysis between the gut microbiome and PCOS, in which the presence or absence of MDs was controlled, *Sutterella* showed correlations with BMI (R^2=0.31, FDR=0.01), waist circumference (R^2=0.29, FDR=0.02), fasting glucose level (R^2=0.46, FDR<0.001), fasting insulin level (R^2=0.24, FDR=0.045), and zonulin (rR^2=0.25, FDR=0.03) (**Fig. 14a**). *Sutterella* adheres to host intestinal mucosal layer and has pro-inflammatory properties (Hiippala et al., 2016). Previous studies have demonstrated a higher abundance of *Sutterella* and a decrease in SCFA-producing bacteria in women with PCOS and PCOS-like rodent models (Arroyo et al., 2019; W. Chu et al., 2020; G. Li et al., 2022; Torres et al., 2018; Zhang et al., 2019; L. Zhou et al., 2020). Additionally, given a reverse correlation between neurotransmitters and serum testosterone levels, an increase in the relative abundance of *Sutterella* may influence not only inflammation but also the progression of both MDs and PCOS (F. He & Li, 2020; Lal et al., 2001; R. Liu et al., 2017; Silva et al., 2020). However, the virulence and function of *Sutterella* vary depending on the species, and conflicting findings exist regarding its abundance in MDs across studies (Dong et al., 2021; Hiippala et al., 2016; Simpson et al., 2021; C. Wang et al., 2020; Y. Zhou et al., 2023).

On the other hand, two correlations were observed, including *Parabacteroides* and BMI (R^2=0.44, FDR=0.02) and *Bifidobacterium* and zonulin level (R^2=0.51, FDR=0.01), in the correlation analysis between the gut microbiome and MDs, in which the presence or absence of PCOS was controlled (**Fig. 14b**). However, these results did not confirm previous findings that demonstrated the negative correlations between the abundance of *Parabacteroides* and human disease, such as obesity and inflammatory bowel diseases, as well as the beneficial roles of *Bifidobacterium* in gut homeostasis, MDs, and even PCOS (Cui et al., 2022; C.-S. Kim et al., 2021; Tojo, 2014).

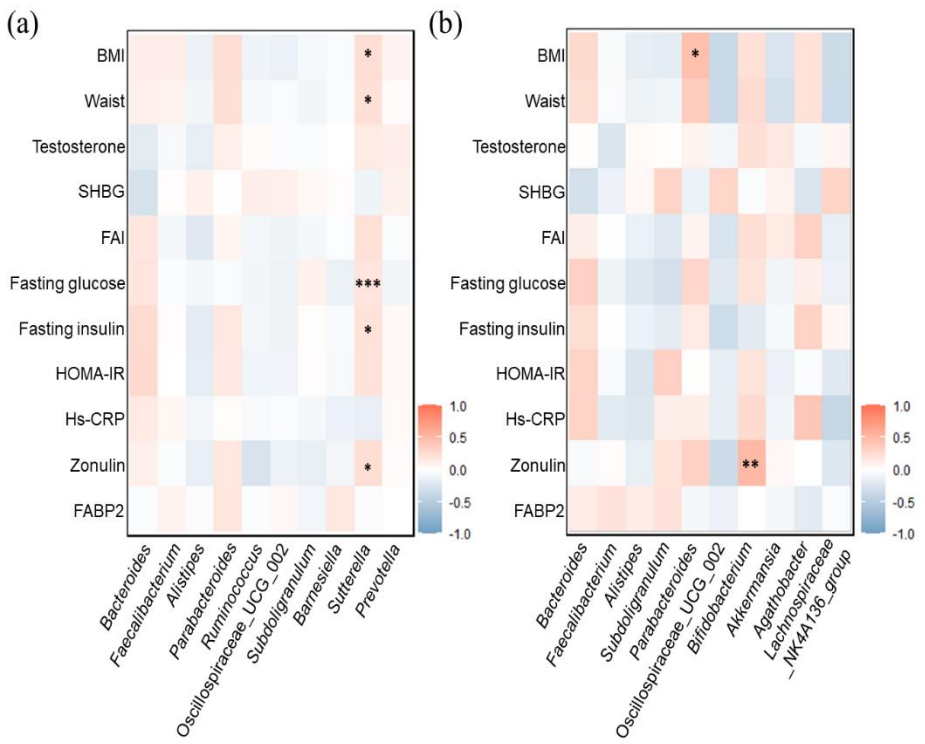

Fig. 14. Partial correlation analyses between the top 10 most abundant genera and common clinical features of both PCOS and MDs. (a) PCOS population (i.e., PCOS no-MD + PCOS MD cases, *n*=102), (b) MD population (i.e., control MD + PCOS MD, *n*=43). A negative correlation is indicated as blue, and a positive correlation is indicated as red, in the color key. A *p*-value was determined by Kruskal–Wallis test adjusted with the Benjamini–Hochberg method. *FDR<0.05, **FDR<0.01, and ***FDR<0.001. BMI (body mass index), SHBG (sex hormone-binding globulin), FAI (free androgen index), HOMA-IR (Homeostatic Model Assessment for Insulin Resistance), Hs-CRP (high sensitive C-reactive protein), FABP2 (fatty acid binding protein 2)

5.3 AI analysis on histological traits in infertility-associated endometrium (Studies III, IV)

The PCOS and RIF endometrium exhibit altered receptivity during the window of implantation and immune profiles as described earlier in sections 2.2 (Female infertility-associated conditions) (Cimadomo et al., 2023; Stener-Victorin et al., 2024).

In Study III, epithelial percentages were analyzed to uncover patterns of epithelial gland development in women with PCOS and women with RIF. In Study IV, CD138+ cell percentages were calculated to evaluate the local inflammatory status in PCOS and RIF endometrium. For PCOS, menstrual cycle phases and ovulatory status were considered. Meanwhile, in the case of RIF, endometrial receptivity was considered. Additionally, correlations were examined between menstrual cycle-related physiological features and both epithelial percentages and CD138+ cell percentages to affirm the reliability of the AI model and to elucidate factors influencing the histological traits across the menstrual cycle or in the context of the two infertility-related conditions.

5.3.1 The high functionality of the AI model

The AI model, developed for the first time to target endometrial CD138+ plasma cells, comprised two distinct CNNs: CNN 1, operating at the regional layer, was trained to segment both the epithelium and stroma, while CNN2, functioning at the object layer, was trained to discern cells in the stroma based on the presence or absence of CD138 staining (**Fig. 15a**). The AI model demonstrated high accuracy in recognizing endometrial compartments, including the epithelium and stroma as well as CD138+ cells (**Fig. 15b-g**, **Table 10**). The presence of pre-decidual structures in the stroma led to higher error rates in identifying the epithelium. This occurred because the AI model categorized these regions as the epithelium, leading to an oversight of stromal cells in those areas. Additionally, the smaller training area of the epithelium (2.56 mm^2) may have contributed to a higher training error than the stroma, which was trained with 24.87 mm^2 (**Table 10**). However, despite these challenges, the training results of the AI model exhibited better performance compared to previous studies using the same AI platform (Mäkelä et al., 2021; Penttinen et al., 2018; Sjöblom et al., 2021).

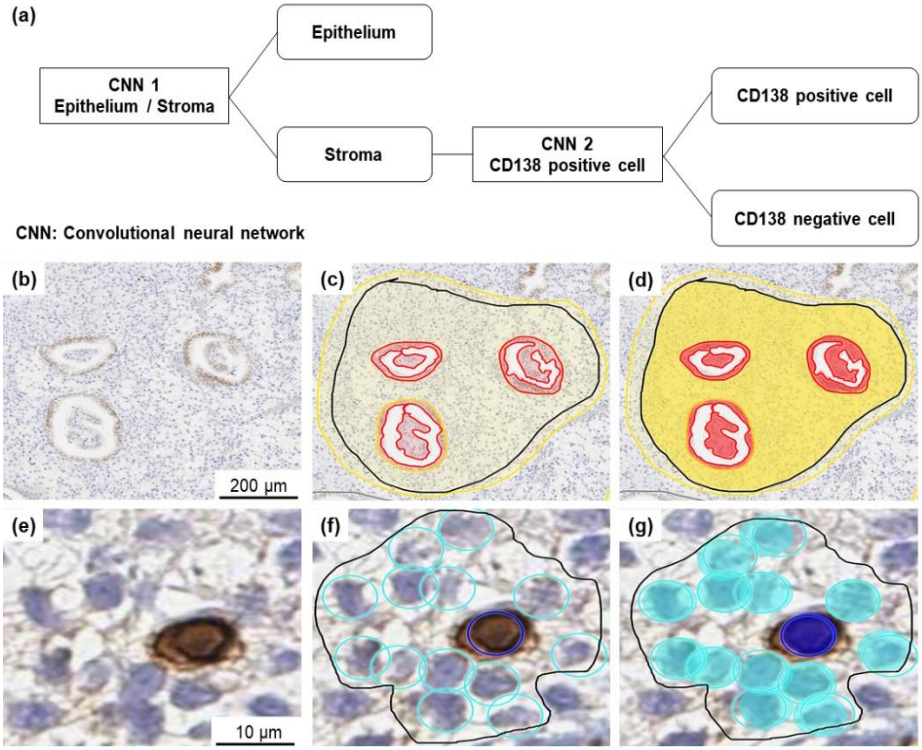

Fig. 15. Structure of the AI model and examples of CNN training. (a) A structure of the AI model. Examples of (b)–(d) CNN1 and (e)–(g) CNN2 training. The training only focused on the areas delineated by solid black lines. (b) and (e) original images, (c) and (f) manually annotated regions and objects, (d) and (g) the AI model training result. The epithelium is highlighted in red, the stroma in yellow, CD138+ cells in dark blue, and CD138- cells in cyan. AI (artificial intelligence), CNN (convolutional neural networks)

Table 10. Training results of the AI model

(%)	Total error	Precision	Sensitivity	F1 score	Specificity	Accuracy
CNN[1]1	2.28	99.17	98.46	98.81	99.6	99.23
Epithelium	15.2	92.86	91.86	92.36	92.94	92.4
Stroma	1.04	99.82	99.14	99.48	99.82	99.23
CNN2	6.15	99.45	94.36	96.84	99.48	96.92
CD138+ cells	3.23	100	96.77	98.36	100	98.39
CD138- cells	6.32	99.42	94.22	96.75	99.45	96.84

Total error (FP^2 + FN^3), precision (TP^4/[TP + FP]), sensitivity (TP/[TP + FN]), F1 score (2 x Precision x Sensitivity/[Precision + Sensitivity]), specificity (TN^5/[TN + FP]), and accuracy ([TP + TN]/[TP + TN + FP + FN]) percentages were calculated to assess the training performance of the AI[6] model. [1]convolutional neural network, [2]false positives, [3]false negatives, [4]true positives, [5]true negatives, [6]artificial intelligence

To ensure the reliability of the AI model, external pathologists who were not involved in the training process assessed its training and performance (**Fig. 16**). Two pathologists conducted the training validation, demonstrating complete agreement between the pathologists and the AI model in identifying CD138+ cells (**Fig. 16a-j**, **Table 11**). During the performance validation, three pathologists manually counted CD138+ cells, and these counts were then compared to the automated cell counting performed by the AI model. The results demonstrated an excellent level of accuracy (ICC: 0.76, 95% confidence interval: 0.36 to 0.93, p=0.002) and revealed a significant positive correlation (Spearman's rank correlation coefficient: 0.79, p<0.01). Moreover, the interobserver variability between pathologists revealed excellent reliability for the compartments in CNN1 and good reliability for the cells in CNN2 (**Table 12**).

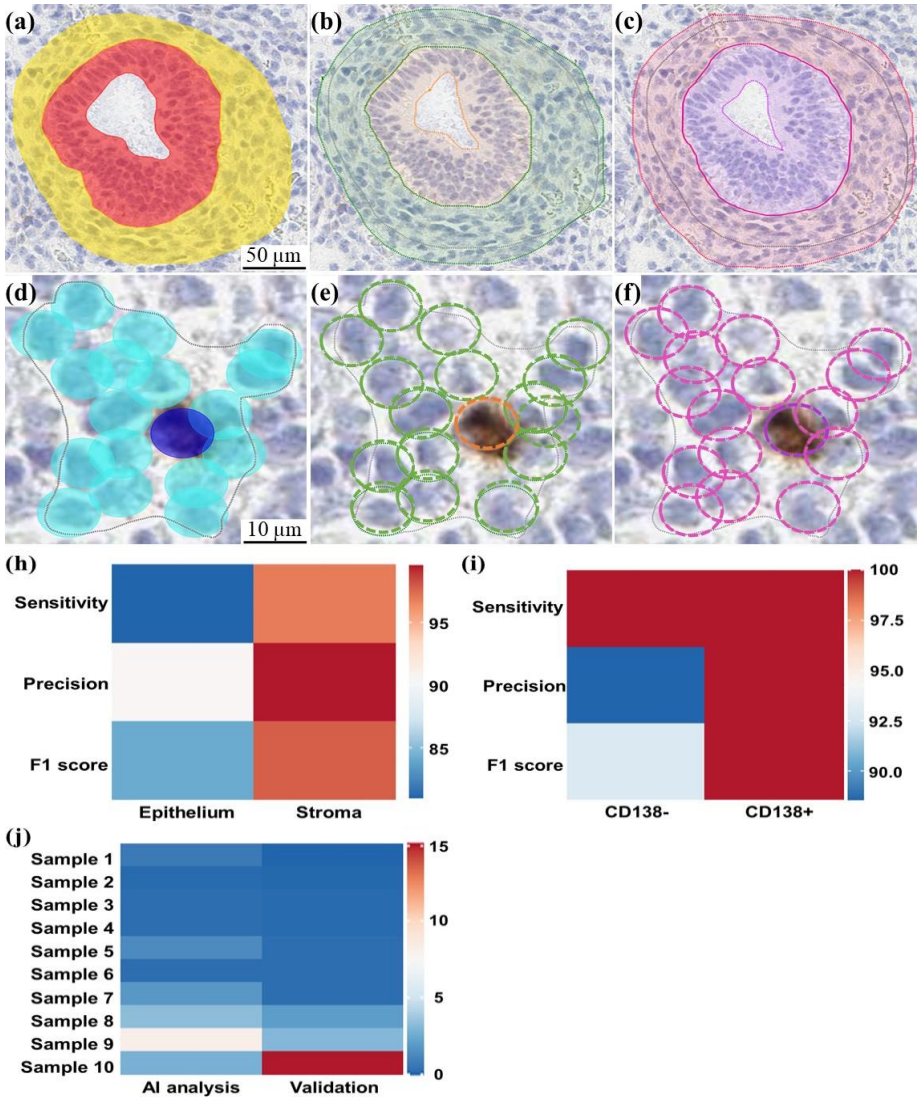

Fig. 16. The AI model validation results. Examples of the training validation for (a)–(c) CNN1 and (d)–(f) CNN2. (a) and (c) show the AI analysis for each CNN, (b) and (e) are the annotations from pathologist 1, and (c) and (f) are the annotations from pathologist 2. The results of the training validation are for (h) CNN1 and (i) CNN2. The heatmap, generated by calculating the median between the assessments of the two pathologists,

illustrates the level of agreement between the AI model and the pathologists. (j) The results of the performance validation. The median values of the cell counts determined by the three pathologists were compared with the results from the AI analysis. AI (artificial intelligence), CNN (convolutional neural networks)

Table 11. Training validation results of the AI model

(%)	Validators	Total error	Precision	Sensitivity	F1 score	Specificity	Accuracy
CNN[1]1	1	5.92	95.69	91.25	93.42	91.24	95.83
	2	5.57	96.85	91.04	93.85	91.01	96.09
Epithelium	1	7.66	85.13	80.46	82.73	80.47	81.38
	2	8.24	91.32	75.54	82.69	75.69	82.09
Stroma	1	4.18	99.12	94.8	96.91	94.79	96.90
	2	2.9	98.65	97.03	97.83	97.03	97.82
CNN2	1	15.05	88.93	97.03	92.81	43.00	87.59
	2	19.25	85.44	97.35	91.00	45.65	85.25
CD138+ cells	1	15.99	88.30	96.85	92.37	43.00	86.95
	2	20.52	84.60	97.17	90.45	45.65	84.52
CD138- cells	1	0.00	100	100	100	-	100
	2	0.00	100	100	100	-	100

Total error (FP[2] + FN[3]), precision (TP[4]/[TP + FP]), sensitivity (TP/[TP + FN]), F1 score (2 x Precision x Sensitivity/[Precision + Sensitivity]), specificity (TN[5]/[TN + FP]), and accuracy ([TP + TN]/[TP + TN + FP + FN]) percentages were calculated to assess the training performance of the AI[6] model. [1]convolutional neural network, [2]false positives, [3]false negatives, [4]true positives, [5]true negatives, [6]artificial intelligence

Table 12. Interobserver variability in the validation process

ICC[1] (95% confidence interval)	CNN[2]1		CNN2	
	Epithelium	Stroma	CD138- cells	CD138+ cells
Training validation between two pathologists	0.93*** (0.87,0.97)	0.93*** (0.86,0.96)	0.86*** (0.72,0.93)	
Performance validation between three pathologists				0.82** (0.49,0.95)

An ICC estimate was defined using a two-way mixed-effects model with an absolute agreement model. Poor reliability: ICC<0.5; moderate reliability: 0.5-0.75; good reliability: 0.75-0.9; excellent reliability: ICC>0.9. * p<0.05, ** p<0.01, *** p<0.001. [1]intraclass correlation coefficient, [2]convolutional neural network

Remarkably, the AI model demonstrated its effectiveness by surpassing current limitations in histopathological analysis. Our model was able to analyze a whole scanned slide within one minute without the need for additional staining to differentiate between epithelium and stroma and further image processing to assess staining intensity (Camacho Benítez et al., 2021; Hull et al., 2012; Liang et al., 2007; Miwa et al., 2009; Surov et al., 2017). Furthermore, the model delivered results with high accuracy and consistency, eliminating the issues of intra- and inter-observer biases. Beyond the application of AI in clinical research for disease prediction and progression, AI technology can also enhance biological research.

5.3.2 Epithelium-to-stroma proportions in women with PCOS across the menstrual cycle phases and in women with RIF concerning endometrial receptivity

The epithelium percentages increased from the PE toward the SE, peaking at the MSE (**Fig. 17a**). The presence or absence of PCOS did not significantly affect the epithelium percentages (control vs. PCOS: p_{PE}=0.09, p_{ESE}=0.67, p_{MSE}=0.62, p_{LSE}=0.58). Based on this finding, women with PCOS are likely to exhibit normal epithelial development once they achieve natural ovulation. As expected, PA endometrium showed epithelium percentages similar to the PCOS PE endometrium (p=1.00). The lack of P4 resulting from the absence of the corpus luteum in the anovulatory endometrium leads to a stagnant state, preventing proper development of the GE and stromal decidualization (Giudice, 2006; Hood et al., 2015; Hosseinzadeh et al., 2021; Koot et al., 2012). Additionally, the epithelium percentages did not vary across PCOS phenotypes (**Fig. 17b**). Similar testosterone and P4 levels between phenotype A and D samples may contribute to the absence of significant differences (**Table 5**). The epithelium percentages in the control SE endometrium positively correlated with P4 (R^2=0.64, FDR<0.001) and negatively correlated with free androgen index (FAI) (R^2=-0.34, FDR=0.01). Similarly, the epithelium percentages in the PCOS SE endometrium showed a positive correlation with serum P4 level (R^2=0.52, FDR<0.001) (**Table 13**). These findings demonstrated the reliability of the AI analysis in reflecting physiological changes. The absence of correlations between the epithelium percentages in the PA samples and hormonal features may be attributed to the limited sample size (n=12).

The receptivity status on day 5 of hormone replacement treatment did not affect the epithelium percentages in RIF patients (p=0.88) (**Fig. 17c**). The lack of significance might be due to the limited number of RIF samples. Nevertheless, the AI model can be utilized to assess the epithelial gland development in RIF patients. While the epithelial proportions cannot directly reflect endometrial thickness, they can still offer valuable insights that align with the investigation recommended by ESHRE for RIF patients, specifically the 'Re-assessment of endometrial thickness' (Cimadomo et al., 2023).

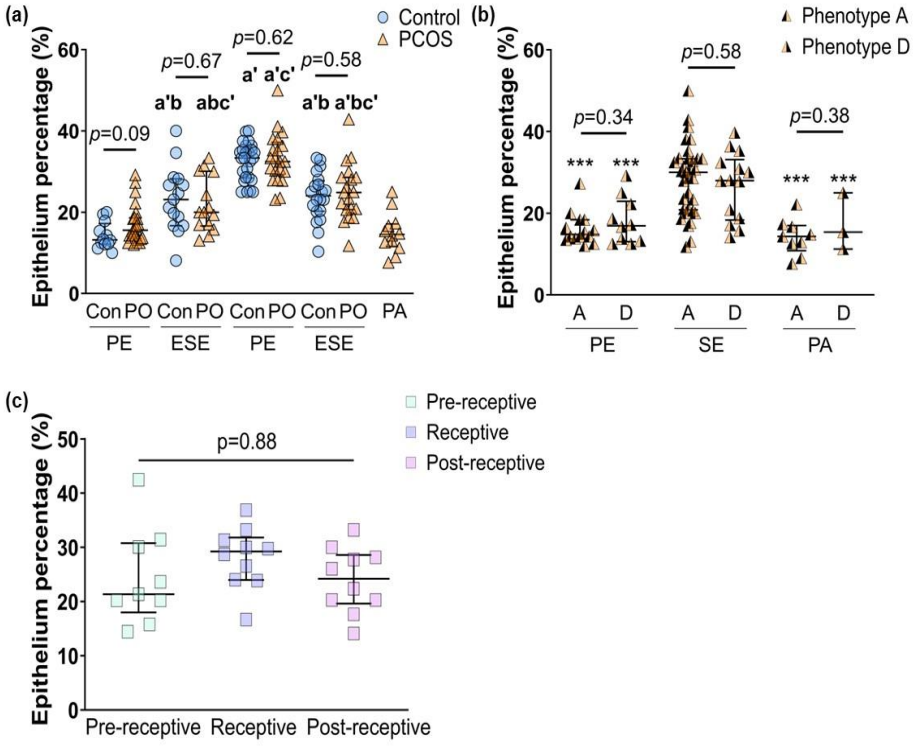

Fig. 17. Epithelium percentages in the PCOS and RIF endometrium. (a) Epithelium percentages in controls and in women with PCOS. Epithelium percentages in (b) different PCOS phenotypes. $^{a}p<0.05$ and $^{a'}p<0.001$ when compared to the PE samples, $^{b}p<0.001$ when compared to the MSE samples, and $^{c}p<0.01$ and $^{c'}p<0.001$ when compared to the anovulatory samples, $^{***}p<0.001$ when compared to the SE samples by the mixed-model analysis. Epithelium percentages in (c) different endometrial

receptivity in RIF patients. A *p*-value was calculated by Kruskal–Wallis test. The lines represent the upper quartiles, medians, and lower quartiles. Each symbol represents an individual sample: controls (blue dot), PCOS samples (orange triangle), pre-receptive samples (light green rectangle), receptive samples (light blue rectangle), and post-receptive samples (light purple rectangle). Con (control), PO (PCOS ovulatory), PA (PCOS anovulatory), PE (proliferative phase), ESE (early secretory phase), MSE (mid-secretory phase), LSE (late secretory phase), SE (secretory phase)

Table 13. Correlations with epithelium percentage and CD138+ cells with menstrual cycle-related physiological changes

	Epithelium %	Endometrial thickness (mm)	P4[1] (nmol/L)	Testosterone (nmol/L)	FAI[2]	FSH[3] (IU/L)	LH[4] (IU/L)	AMH[5] (ng/ml)
Control PE[6] (*n*=12)	13.19	5.25 (0.41)	0.22 (0.01)	1.01 (0.23)	2.20 (-0.52)	7.41 (0.04)	7.69 (0.09)	2.28 (0.10)
Control SE[7] (*n*=61)	27.73	9.85 (-0.06)	31.00 (**0.64*****)	1.04 (0.03)	2.00 (**-0.34****)	3.62 (-0.15)	7.47 (0.04)	2.23 (-0.06)
PO[8] PE (*n*=24)	15.56	5.25 (-0.07)	0.25 (-0.04)	1.36 (0.12)	2.74 (-0.17)	6.82 (-0.16)	8.72 (-0.13)	5.45 (0.07)
PO SE (*n*=54)	28.00	9.70 (-0.18)	28.49 (**0.52*****)	1.33 (0.05)	2.38 (-0.06)	3.73 (-0.13)	8.95 (0.10)	3.57 (0.00)
PA[9] (*n*=12)	14.54	5.30 (0.16)	0.34 (0.07)	2.48 (0.09)	7.67 (-0.25)	6.33 (0.09)	15.71 (0.50)	8.59 (0.15)
	CD138+ %	Endometrial thickness (mm)	P4 (nmol/L)	Testosterone (nmol/L)	FAI	FSH (IU/L)	LH (IU/L)	AMH (ng/ml)
Control PE (*n*=12)	0.04	5.25 (-0.16)	14.60 (-0.04)	1.01 (0.06)	2.20 (0.13)	7.41 (0.33)	7.69 (0.19)	2.28 (-0.40)
Control SE (*n*=59)	0.00	9.80 (0.21)	25.35 (**-0.43****)	1.17 (-0.11)	2.00 (**0.45*****)	3.68 (0.15)	7.64 (**0.41****)	2.25 (0.06)
PO PE (*n*=21)	0.06	5.35 (0.02)	11.21 (0.33)	1.00 (-0.02)	2.64 (0.30)	6.79 (**-0.52***)	9.72 (-0.03)	5.27 (0.24)
PO SE (*n*=50)	0.00	9.70 (-0.05)	22.49 (-0.24)	1.33 (**0.32***)	2.51 (0.09)	3.57 (-0.03)	8.97 (0.14)	3.68 (**0.30***)
PA (*n*=11)	0.01	5.60 (0.05)	0.29 (-0.17)	1.65 (-0.47)	6.42 (-0.05)	6.59 (0.36)	16.46 (0.03)	9.08 (0.02)

All values are presented as median. The standardized coefficient was calculated using linear mixed-effects models and is presented in brackets. A *p*-value was adjusted using the Bonferroni FDR method. * FDR<0.05, ** FDR <0.01, and *** FDR <0.001. [1]progesterone, [2]free androgen index, [3]follicle-stimulating hormone, [4]luteinizing hormone, [5]anti-Müllerian hormone, [6]proliferative phase, [7]secretory phase, [8]PCOS ovulatory, [9]PCOS anovulatory

5.3.3 Occurrence of CD138+ cells in women with PCOS across menstrual cycle phases and in women with RIF concerning endometrial receptivity

Menstrual cycle phases had a more significant impact on CD138+ cell percentages (p<0.001), indicating higher occurrence of CD138+ cells in the PE endometrium compared to the SE endometrium (**Fig. 18a**). These results align with previous findings, supporting the reliability of the AI model (Y. Li et al., 2021; D. Song et al., 2018). The presence of PCOS did not result in any significant alterations in cell percentages, with no significant differences between women with and without PCOS at each phase (p_{PE}=0.83, p_{ESE}=0.22, p_{MSE}=0.92, p_{LSE}=0.98) (**Fig. 18a**). CD138+ cell percentages in the PA endometrium were comparable to those in the PO endometrium during the SE but significantly lower during the PE (p<0.001) (**Fig. 18a**). This study represents the first comprehensive investigation of the occurrence of CD138+ cells in the endometrium of women with PCOS across different menstrual cycle phases. E2 seems to attract plasma cell progenitors from the systemic circulation into the endometrial tissue (McDermott et al., 1980). Nevertheless, even though the PA endometrium exhibits an altered expression of estrogen receptors and increased sensitivity to E2, we did not detect any elevation in CD138+ cell percentages within the PA cases (Palomba et al., 2021; X.-L. Xu et al., 2021). This may be attributable to the limited size of the PA samples (n=12). Additionally, serum estrogen levels were not measurable as they fell below the detection threshold by liquid chromatography-mass spectrometry during a single run processing for a panel of steroids in Studies III and IV. Therefore, further studies with larger sample sets are needed to investigate how E2 influences the presence of CD138+ cells across different menstrual cycle phases and specifically in the endometrium of those with anovulatory PCOS.

CD138+ cell percentages varied according to the PCOS phenotypes (**Fig. 18b**). Phenotype A, representing the most severe phenotype, showed significantly higher CD138+ cell percentages compared to phenotype D, representing the mildest phenotype, in the PE (p=0.03) (**Fig. 18b**). Additionally, during the SE in PCOS cases, CD138+ cell percentages showed positive correlations with testosterone (R^2=0.32, FDR=0.02) and AMH (R^2=0.30, FDR=0.03) (**Table 13**). To sum up, HA may facilitate the recruitment of CD138+ cells. However, the phenotype-dependent

difference was only observed in the PE, which warrants further investigation in the SE.

Receptivity status did not influence CD138+ cell accumulation in RIF endometrium (p=0.81) (**Fig. 18c**). Again, taking into account ESHRE good practice recommendations, the AI model could serve as a useful tool for screening CE in RIF patients, while also having the potential to establish a consistent diagnostic standard for CE (Cimadomo et al., 2023). Additionally, future research should validate previous findings indicating a higher prevalence of endometrial inflammation diagnosed by CD138+ plasma cell aggregation among IVF patients by incorporating RIF patients and healthy controls with natural or stimulated menstrual cycles (Bashiri et al., 2018; Y. Li et al., 2021).

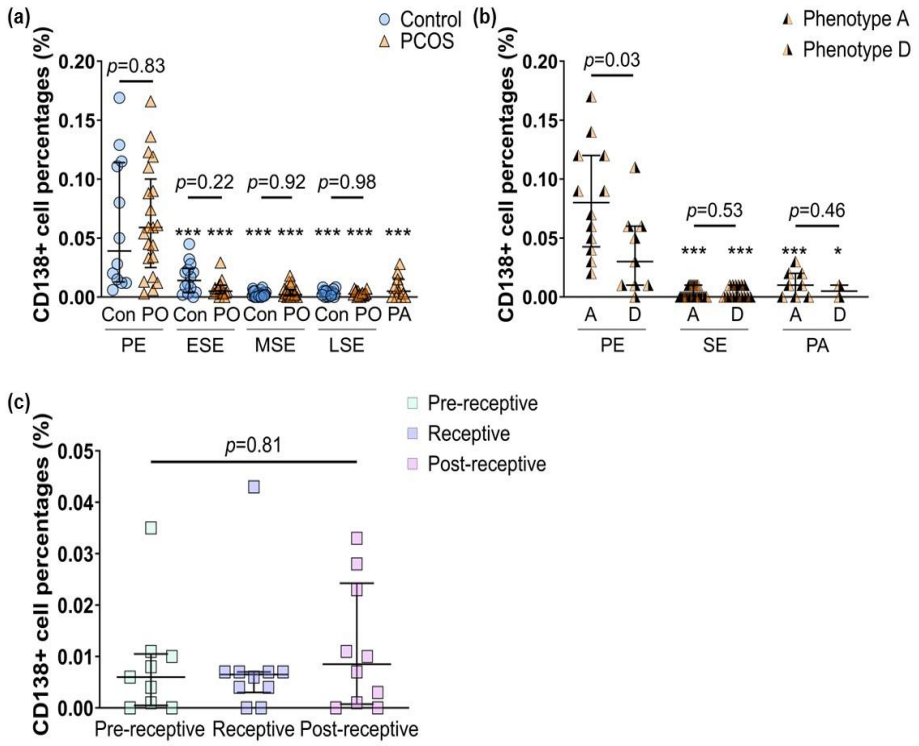

Fig. 18. CD138+ plasma cell percentages in PCOS and RIF endometrium. CD138+ cell percentages were analyzed by the AI model for (a) controls and PCOS cases and (b) different PCOS phenotypes. ***p<0.001 compared to PE. The statistical differences were

calculated using the mixed-model ANOVA. CD138+ cell percentages in (c) different endometrial receptivity in RIF patients. A *P*-value was calculated by Kruskal–Wallis test. The lines represent the upper quartiles, medians, and lower quartiles. Each symbol represents an individual sample: controls (blue dot), PCOS samples (orange triangle), pre-receptive samples (light green rectangle), receptive samples (light blue rectangle), and post-receptive samples (light purple rectangle). Con (control), PO (PCOS ovulatory), PA (PCOS anovulatory), PE (proliferative phase), ESE (early secretory phase), MSE (mid-secretory phase), LSE (late secretory phase), SE (secretory phase)

6 Strengths and limitations

The overall strength of this thesis lies in its use of both rare and ample human samples associated with infertility, including endometrial tissue biopsy, EF, VS, and fecal samples. Age and BMI were carefully adjusted between women with and without PCOS to mitigate the potential effects of the confounding factors on PCOS phenotypes and microbiome. The study population had low ethnic variation, although this may also be considered a limitation. Samples were collected systematically, considering the menstrual cycle phase confirmed by experienced gynecologists and pathologists. Furthermore, Studies I and II represent the largest number of sample sets to date in RT and gut microbiome studies in women with PCOS, respectively. In terms of novelty, Study I pioneered the investigation of the endometrial and vaginal microbiome in women with PCOS across menstrual cycle phases. The involvement of the gut microbiome in the MDs in women with PCOS had yet to be explored prior to Study II. Lastly, the AI model utilized in Studies III and IV was introduced for the first time to target endometrial CD138+ plasma cells.

The main limitation of this thesis stemmed from sample categorization. While the number of samples from women with PCOS was sufficient for reliable statistical analysis and represented one of the largest studies in the field, stratifying samples based on ovulatory or MD status in women with PCOS posed challenges in achieving robust statistical power. Additionally, in Studies I, III, and IV, serum estrogen levels were not attainable as they fell below the detection limit by liquid chromatography-mass spectrometry.

In Study I, the use of transvaginal ultrasonography for identifying the corpus luteum may affect the vaginal microbiome during the insertion and withdrawal of the probe, which should be taken into consideration in future studies.

Study III lacked confirmed cases of CE to serve as positive controls during the AI model training process, although the primary focus was not on diagnosing overt endometritis. Furthermore, the limited number of RIF samples may lead to an underestimation of histological differences based on receptivity status. Biochemical data from serum samples for RIF patients were also unavailable. This lack of information limits the exploration of possible associations between epithelial proportions and clinical characteristics as well as between CD138+ cell occurrence and clinical characteristics. Lastly, our analysis was further constrained by the lack of non-RIF controls.

7 Conclusions and future perspectives

As outlined in Aim 1, Study I revealed a distinct microbiome profile dependent on the collection site, whether the endometrium or vagina. This was evidenced by significantly different levels of the relative abundance of *Lactobacillus* and microbial community diversity. Additionally, the relative abundance of *Lactobacillus* increased, and alpha diversity decreased across the menstrual cycle from the PE to the SE, regardless of PCOS diagnosis. In contrast, the presence of PCOS is associated with increased alpha diversity during the ESE compared to controls in EF samples. Furthermore, PCOS cases showed significantly lower abundances of Acetobacteraceae uncultured and *Prevotella*_9 and a higher abundance of *Rhodoferax* compared to controls. These findings may provide valuable insights into the composition and function of the RT microbiome in women with PCOS throughout the menstrual cycle. Moreover, the findings lay the groundwork for future investigations, particularly in understanding the role of RT microbiota in the pathophysiology of PCOS and in developing novel treatment approaches. To advance this field, larger study populations, particularly focusing on PA cases, are necessary to elucidate the associations between the RT microbiome and anovulatory status in women with PCOS. Furthermore, given the significant impact of E2 on the RT microbiome, future research should consider variations in E2 levels.

Study II addressed aim 2 by highlighting the notable differences in the gut microbiome between PCOS no-MD and PCOS MD cases, characterized by reduced alpha diversity and elevated abundance of *Sutterella* in PCOS MD cases. Additionally, *Butyricicoccus* was significantly less abundant in PCOS MD cases compared to PCOS no-MD cases. Correlation analyses revealed that in the PCOS population, the abundance of *Sutterella* was positively associated with obesity, glucose metabolism, and inflammation-related clinical features. Meanwhile, the abundances of *Parabacteroides* and *Bifidobacterium* were positively correlated with BMI and zonulin, respectively, in the MD populations. These findings hint at a possible link between MDs and PCOS through alterations in the gut microbiome, potentially impacting factors, such as obesity, inflammation, and glucose metabolism. This could pave the way for novel treatment strategies aimed at ameliorating these conditions by restoring gut microbiota balance. Furthermore, future mechanistic studies with sufficient sample sizes and high-resolution analysis

of the gut microbiome—utilizing advanced techniques, such as metagenomic sequencing—are necessary to elucidate the roles of *Sutterella*, *Parabacteroides*, and *Bifidobacterium* in both MDs and PCOS.

The AI model utilized in Studies III and IV demonstrated reliable performance in segmenting the epithelium and stroma and in identifying CD138+ cells within the endometrium. The precision and capabilities of the AI model provide several distinctive advantages, including rapid examination of whole slide images, decreased reliance on trained pathologists, and improved consistency in results. The AI analysis covered aims 3 and 4 by revealing an increase in epithelium proportion and a decrease in the occurrence of endometrial CD138+ cells from the PE to the SE, irrespective of PCOS diagnosis. Additionally, CD138+ percentages were higher in more severe phenotypes among PCOS cases. On the contrary, endometrial receptivity did not affect two histological features—epithelium percentages or CD138+ percentages among the RIF patients. These findings may suggest the potential integration of AI technology into clinical practice, offering promise for large-scale sample analysis due to quick and accurate diagnostic potential.

In summary, the findings of the current study support the idea that (i) women with PCOS have altered RT microbiome compared to women without PCOS, and (ii) MDs in women with PCOS is associated with altered gut microbiome. Additionally, (iii) neither the diagnosis of PCOS nor endometrial receptivity among RIF patients appears to impact epithelial development or occurrence of endometrial CD138+ cells. A more extensive mechanistic investigation employing metagenomic approaches like whole shotgun sequencing for microbiome studies and incorporating a diverse range of histology images alongside clinical data of the subjects for AI studies should be performed to confirm and extend the current findings.

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