Human Reproduction Open, pp. 1–16, 2022
https://doi.org/10.1093/hropen/hoac015

human reproduction open

PROTOCOL

The microbiome in reproductive health: protocol for a systems biology approach using a prospective, observational study design

Maria Christine Krog [1,2,3,*,†], Mette Elkjær Madsen [1,4,†], Sofie Bliddal [5,6], Zahra Bashir [1,7], Laura Emilie Vexø[1,4], Dorthe Hartwell[4], Luisa W. Hugerth [8], Emma Fransson [8], Marica Hamsten[8], Fredrik Boulund [8], Kristin Wannerberger[9], Lars Engstrand [8], Ina Schuppe-Koistinen [8], and Henriette Svarre Nielsen [1,3,10]

[1]The Recurrent Pregnancy Loss Unit, The Capital Region, The Fertility Clinic, Copenhagen University Hospitals Rigshospitalet and Hvidovre, Copenhagen, Denmark [2]Department of Clinical Immunology, Rigshospitalet, Copenhagen University Hospital, Copenhagen Ø, Denmark [3]Department of Clinical Medicine, Copenhagen University, Copenhagen N, Denmark [4]Department of Gynecology, The Endometriosis Unit, Rigshospitalet, Copenhagen University Hospital, Copenhagen Ø, Denmark [5]The Department of Medical Endocrinology and Metabolism, Rigshospitalet, Copenhagen University Hospital, Copenhagen Ø, Denmark [6]Institute for Inflammation Research, Center for Rheumatology and Spine Diseases, Rigshospitalet, Copenhagen University Hospital, Copenhagen Ø, Denmark [7]Department of Obstetrics and Gynecology, Slagelse Hospital, Slagelse, Denmark [8]Centre for Translational Microbiome Research (CTMR), Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden [9]Ferring International Center SA, Saint-Prex, Switzerland [10]Department of Obstetrics and Gynecology, Copenhagen University Hospital, Hvidovre, Denmark

*Correspondence address. The Recurrent Pregnancy Loss Unit, The Fertility Clinic 4071, Rigshospitalet, Copenhagen University Hospital, Blegdamsvej 9, 2100 Copenhagen Ø, Denmark. E-mail: maria.christine.krog@regionh.dk https://orcid.org/0000-0002-2110-0479

Submitted on October 26, 2021; resubmitted on January 11, 2022; editorial decision on March 02, 2022

STUDY QUESTION: What is the microbiome profile across different body sites in relation to the normal menstrual cycle (with and without hormonal contraception), recurrent pregnancy loss (RPL) (before and during pregnancy, pregnancy loss or birth) and endometriosis (before, during and after surgery)? How do these profiles interact with genetics, environmental exposures, immunological and endocrine biomarkers?

WHAT IS KNOWN ALREADY: The microbiome is a key factor influencing human health and disease in areas as diverse as immune functioning, gastrointestinal disease and mental and metabolic disorders. There is mounting evidence to suggest that the reproductive microbiome may be influential in general and reproductive health, fertility and pregnancy outcomes.

STUDY DESIGN, SIZE, DURATION: This is a prospective, longitudinal, observational study using a systems biology approach in three cohorts totalling 920 participants. Since microbiome profiles by shot-gun sequencing have never been investigated in healthy controls during varying phases of the menstrual cycle, patients with RPL and patients with endometriosis, no formal sample size calculation can be performed. The study period is from 2017 to 2024 and allows for longitudinal profiling of study participants to enable deeper understanding of the role of the microbiome and of host–microbe interactions in reproductive health.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Participants in each cohort are as follows: Part 1 MiMens—150 healthy women with or without hormonal contraception; Part 2 MiRPL—200 couples with RPL, 50 healthy couples with prior uncomplicated pregnancy and 150 newborns; Part 3 MiEndo—120 patients with endometriosis requiring surgery with or without hormonal treatment. Microbiome profiles from saliva, faeces, rectal mucosa, vaginal fluid and endometrium will be studied, as well as the Omics profile, endocrine disrupting chemicals and endocrine and immune factors in blood, hair, saliva and urine. Pregnancy loss products, seminal microbiome, HLA types, endometriotic tissue and genetic risk and comprehensive questionnaire data will also be studied, where appropriate. Correlations with mental and physical health will be evaluated.

[†]The authors consider that the first two authors should be regarded as joint First Authors.

STUDY FUNDING/COMPETING INTEREST(S): This work is supported by funding from Ferring Pharmaceuticals ([#MiHSN01] to H.S.N., M.C.K., M.E.M., L.E.V., L.E., I.S.-K., F.B., L.W.H., E.F. and M.H.), Rigshospitalet's Research Funds ([#E-22614-01 and #E-22614-02] to M.C.K. and [#E-22222-06] to S.B.), Niels and Desiree Yde's Foundation (S.B., endocrine analyses [#2015-2784]), the Musikforlæggerne Agnes and Knut Mørk's Foundation (S.B., endocrine and immune analyses [#35108-001]) and Oda and Hans Svenningsen's Foundation ([#F-22614-08] to H.S.N.). Medical writing assistance with this manuscript was provided by Caroline Loat, PhD, and funded by Ferring Pharmaceuticals. H.S.N. reports personal fees from Ferring Pharmaceuticals, Merck Denmark A/S, Ibsa Nordic, Astra Zeneca and Cook Medical outside the submitted work. K.W. is a full-time employee of Ferring Pharmaceuticals. No other conflicts are reported.

TRIAL REGISTRATION NUMBER: N/A

TRIAL REGISTRATION DATE: N/A

DATE OF FIRST PATIENT'S ENROLMENT: N/A

Key words: fertility / endometriosis / pregnancy loss / recurrent pregnancy loss / immunology / endocrinology / menstrual cycle / contraception / microbiome

WHAT DOES THIS MEAN FOR PATIENTS?

The human body constitutes a small ecosystem with trillions of micro-organisms co-existing in the body. The composition of micro-organisms depends on many factors such as genetics, type of birth (vaginal delivery or caesarean section), eating habits, environment and age.

The micro-organisms interact with the body, especially with the immune system, and have great impact on human health. A disturbance in the micro-organisms in the gut has been connected to several chronic diseases, such as inflammatory bowel disease, allergy and diabetes, while a disturbance in the vaginal micro-organisms could increase the susceptibility to sexually transmitted diseases, affect the outcome of fertility treatment, or even increase the risk of pregnancy complications.

In this study, we will examine the micro-organisms throughout a menstrual cycle in a healthy group of women. Then, we will examine the micro-organisms before and during pregnancy in couples that have experienced recurrent pregnancy loss, and finally, in patients with a chronic gynaecological inflammatory condition called endometriosis. This will improve our understanding of the contribution of micro-organisms in female reproductive health and disease to optimize patient evaluation and care.

Introduction

It is now widely accepted by scientists that humans live in a symbiotic relationship with a vast community of micro-organisms that inhabit a number of niches within and on the body. These micro-organisms, the microbiota, outnumber their human host in terms of genes (~10 million in the human microbiome versus 20 000 in the human genome (Li *et al.*, 2014)), and the number of micro-organisms and human cells is roughly equal (Sender *et al.*, 2016). The microbiota and its host form a human–microbe hybrid, also termed a 'superorganism' (Gill *et al.*, 2006) or 'holobiont' (Simon *et al.*, 2019), a result of millions of years of co-evolution and mutually beneficial functional integration.

The microbiota is present in the greatest numbers in the human gut but is also found in other locations including the oral cavity, the reproductive tract and on the skin. In recent years, research has demonstrated the crucial role the microbiota plays in human health—bacteria in the gut have been associated with multiple essential physiological processes, including short-chain fatty acid production, anti-inflammatory actions and the development and maturation of the immune system (Singh *et al.*, 2017). In turn, perturbations of the microbiota (dysbiosis) have been associated with a wide range of diseases such as mental disorders (within the framework of the gut–brain axis) (Ouabbou *et al.*, 2020), metabolic disorders (Sonnenburg and Bäckhed, 2016), autoimmune disease (Costello *et al.*, 2015; Sprouse *et al.*, 2019) and gastrointestinal disease (Zhang *et al.*, 2015; Meng *et al.*, 2020).

To date, the microbiome of the reproductive tract has been the focus of less research than that of the gut, but there is mounting evidence to suggest that it may be influential in general and reproductive health, fertility and pregnancy outcomes (Al-Nasiry *et al.*, 2020). For example, studies have shown that the vaginal microbiota can influence vulnerability to sexually transmitted infections (van Houdt *et al.*, 2018), and that it may play a role in outcomes of ART (Koedooder *et al.*, 2019). The vaginal microbiota has also been reported to have a different composition in pregnant women who deliver preterm compared with those who do not (Kindinger *et al.*, 2017), although another study reported no difference (Romero *et al.*, 2014).

Relatively few studies have focused on the relevance of gut microbiota to reproductive health. However, it is thought that microbes in the large intestine may be associated with reproductive outcomes. This hypothesis was investigated in a Norwegian study of the faecal microbiota of 121 mothers, which found that low gut diversity and a distinct microbial composition were associated with spontaneous preterm delivery (Dahl *et al.*, 2017).

There is also increasing interest in the relevance of the oral microbiota to a range of health outcomes including diabetes (Brown *et al.*,

2020) and Alzheimer's disease (Olsen and Singhrao, 2021). A small number of studies have investigated the oral microbiota during pregnancy (Lin *et al.*, 2018; Ye *et al.*, 2020) and in relation to gestational diabetes (Crusell *et al.*, 2020; Xu *et al.*, 2020), but the potential role of the oral microbiota in reproductive and maternal health remains largely unexplored.

The current study will investigate the role of the microbiome in the following key areas.

The microbiota of the reproductive tract

One of the difficulties facing research into the microbiota and reproductive health is that there have been limited studies in healthy women of reproductive age to ascertain what can be considered a 'normal' microbiota profile. In contrast to the gut microbiota, the healthy vaginal microbiota displays fewer different bacteria, i.e. low taxonomic diversity and is typically dominated by *Lactobacillus* species (Ravel *et al.*, 2011). Commensal bacteria, including *Lactobacillus*, modulate the host immune system and may help to prevent colonization by pathogens. *Lactobacillus* feeds on oestrogen-dependent glycogen produced in the vaginal epithelium (Nunn and Forney, 2016). Lactic acid produced by *Lactobacillus* lowers the local pH and has bactericidal effects (Amabebe and Anumba, 2018). In addition to maintaining bacterial balance and preventing bacterial vaginosis and aerobic vaginitis (Donders *et al.*, 2017), the vaginal microbiome has been associated with reduced risk of viral infections such as human papilloma virus (Norenhag *et al.*, 2020), herpes simplex virus-2 (Cherpes *et al.*, 2003) and HIV (Farcasanu and Kwon, 2018). The vaginal microbiome might also play a role in protecting against adverse pregnancy outcomes such as early miscarriage (Eckert *et al.*, 2003) and preterm birth (Freitas *et al.*, 2018), as well as gynaecological cancers (Łaniewski *et al.*, 2020).

Menstrual cycle

The vaginal microbiome was found to be relatively stable throughout the menstrual cycle in a small sample of 27 women (Chaban *et al.*, 2014), while fluctuations have been noted in other studies (Gajer *et al.*, 2012). A study of 76 women using a copper intrauterine device or a levonorgestrel intrauterine system found no significant difference in vaginal microbiome based on type of contraception (Bassis *et al.*, 2017). Little is known, however, about how the vaginal, endometrial, oral and faecal microbiome may interact throughout the menstrual cycle in women using or not using hormonal contraception. It is crucial to ascertain these baseline data as many women of reproductive age are using hormonal contraception, and the data are therefore important as a starting point for further studies of the impact of the microbiome on reproductive health.

Recurrent pregnancy loss

Recurrent pregnancy loss (RPL)—defined in this protocol as three or more consecutive pregnancy losses—affects 1–2% of couples trying to conceive (Bender Atik *et al.*, 2018). The impact of the microbiome has never been investigated in women with RPL. However, it is thought that immunology may account for a large proportion of unexplained cases, with either a failure of the immune system to adapt to normal pregnancy, or a failure of the immune system to prevent the implantation of abnormal pregnancies (Odendaal *et al.*, 2019). It is well established that gut bacteria have a potent immune regulatory capacity that markedly affects systemic inflammatory cell responses (Rooks and Garrett, 2016).

Previous pregnancies could also influence the composition of microorganisms in the woman and may change the regulation of the immune system during future pregnancies owing to the persistent presence of foetal cells in the mother's circulation (microchimerism). Indeed, male-specific cells have been demonstrated in women who have given birth to a boy decades earlier (Bianchi *et al.*, 1996), and microchimerism is more common after pregnancy complications—including pregnancy loss, termination of pregnancy and obstetric complications (Yan *et al.*, 2005). A high proportion of births prior to secondary RPL (women trying to conceive a second child) are obstetrically complicated (Nielsen *et al.*, 2010) and microchimerism may therefore play a role in subsequent immunological reactions in women experiencing secondary RPL.

Aberrations in both thyroid function and cortisol production are also associated with adverse pregnancy outcomes, as well as with the function of the immune system and the interplay between various hormonal axes (Klecha *et al.*, 2008; Parker and Douglas, 2010; Twig *et al.*, 2012). Gut microbiota are likely highly intertwined with the function of the neuroendocrine system (de Weerth, 2017; Köhling *et al.*, 2017). Therefore, the investigation of endocrine systems, together with the microbiome, represents an important part of the current project and will contribute to the understanding of the mechanisms involved in reproductive health and failure.

Endometriosis

Endometriosis is a prevalent gynaecological disease characterized by proliferation and bleeding of endometrial-like lesions outside the uterus, primarily on the pelvic peritoneum, the ovaries, in the recto-vaginal septum, in the bladder and in the bowel, where they induce a chronic inflammatory response, adhesions and pain. It is known that proliferation of endometrial-like lesions is driven by oestrogen, and ectopic endometrial-like tissue recruits circulating stem and progenitor cells, leading to further growth (Wang *et al.*, 2020). However, the reason why some women develop endometrial-like lesions and others do not is still not clarified. The gut microbiota regulates a variety of inflammatory and proliferative conditions (Wang *et al.*, 2017) and is also important in oestrogen metabolism (Baker *et al.*, 2017) and stem cell homeostasis (Xiao *et al.*, 2017; Tan *et al.*, 2019). As such, the gut microbiota is an important candidate for investigation in the aetiology of endometriosis (Laschke and Menger, 2016). Approximately 50% of the risk for endometriosis is attributable to genetic factors (Montgomery *et al.*, 2020), and DNA methylation in lesions is likely to influence disease progression (Wang *et al.*, 2019). This study will therefore investigate the genetic, epigenetic and microbiome profiles in patients with endometriosis.

Therefore, there is much we do not yet know in this exciting research field. In the current study, we aim to augment the existing limited data on the microbiota at different body sites in relation to reproductive health, while also taking into account a broad range of other potentially contributing factors such as genetics, environmental exposures, immunological and endocrine biomarkers. We will evaluate the microbiota present in multiple niches in three different cohorts—healthy women of reproductive age (across the menstrual cycle, with

and without hormonal contraceptive use; Part 1 MiMens), couples who have experienced RPL (before and during pregnancy, pregnancy loss and birth; Part 2 MiRPL), and women with endometriosis requiring surgery with or without hormonal treatment (before, during and after surgery; Part 3 MiEndo). This study represents one of the first and largest studies to perform a comprehensive investigation of the role of the microbiome and other relevant environmental and physiological parameters in three important areas of reproductive health and dysfunction.

Outcomes

An overview of objectives for all three parts of the study is presented in Table I. Aims and primary and secondary outcome measurements are described in Table II. A study overview and sample collection schedules for MiMens (microbiome during menstrual cycle), MiRPL (microbiome in RPL), and MiEndo (microbiome in endometriosis) are shown in Figs 1–3, respectively.

Materials and methods

All three parts of the study will be collaborations between the Recurrent Pregnancy Loss Unit in the Capital Region of Denmark, Rigshospitalet/Hvidovre Hospital (Copenhagen), the Endometriosis Unit at Rigshospitalet, Copenhagen University Hospital and the Centre for Translational Microbiome Research (CTMR) at the Karolinska Institute (Stockholm). In addition, Erasmus University Medical Center (Rotterdam), and the Institute of Food, Nutrition and Health (Zürich) will collaborate on Part 2 (MiRPL). Sample collections and microbiome investigations are fully funded, but we have not yet obtained full funding for all the additional analyses.

Study participants

A summary of inclusion and exclusion criteria and planned sample sizes for each part of the study is shown in Table III. Since microbiome profiles have never been investigated in these populations, no formal sample size calculation can be performed.

The total number of participants in the three cohorts in this study will therefore be ~920 Part 1 MiMens (150 healthy women), Part 2 MiRPL (200 couples—400 men and women—with RPL, 50 healthy couples (100 men and women) with prior uncomplicated pregnancy and 150 newborns) and Part 3 MiEndo (120 patients with endometriosis requiring surgery).

The scientific setup (study setting, sample methods, questionnaires, analysis) will be the same for all three study parts, with some study-specific adaptations to questionnaires.

Biological material and research biobank

All samples planned to be analysed for the microbiome (faecal, rectal, vaginal, endometrial, peritoneal, endometriotic tissue, peritoneal liquid, saliva, semen and meconium) are collected sterile and stored at −80°C in tubes with DNA/RNA shield or lyophilized DNA stabilization buffer (from Zymo Research, Irvine, CA, USA and STRATEC Molecular GmbH, Germany).

All biological samples (faecal, rectal, vaginal, oral, endometrial, peritoneal, myometrial, endometriotic tissue, peritoneal liquid, semen, meconium, urine, blood and DNA) will be stored in a research biobank during the study period of 2017–2024. Residual samples for future research will, after patient approval, be stored at the Fertility Clinic 4071, Rigshospitalet and the biobank facility in the Capital Region of Denmark until 2 January 2042. After this date, samples will be destroyed according to the guidelines of the Data Protection Agency.

All samples will be allocated a project number and barcode. Except for urine, paraffin-embedded tissue and isolated mononuclear blood cells, all samples will be stored at −80°C in biobank freezers at The Fertility Clinic, Rigshospitalet. Urine will be stored at −20°C, paraffin-embedded tissue at room temperature and isolated mononuclear blood cells will be stored in liquid nitrogen. When shipped to collaborators abroad, a project number/barcode will be used for identification.

Table IV presents an overview of sample collection in the three parts of the study.

Analysis of samples

Microbiome

The tissues planned for microbiome analyses (faecal, rectal, vaginal, endometrial, peritoneal, endometriotic tissue, peritoneal liquid, saliva, semen and meconium) will be stored in a DNA/RNA shield after collection to stabilize and protect the microbiome. All planned microbiome analyses are described in general under 'Multi-omics' analyses.

Blood samples

Blood samples (full blood, DNA, RNA, plasma, serum and isolated peripheral blood mononuclear cells) will be biobanked and all blood analyses will be performed using standard methods. Blood samples (and cord blood from live-born children in Part 2: MiRPL) will be used to analyse biomarkers of inflammation, immune regulators, markers of low-grade inflammation and markers of the innate immune system, factors and activators of the complement system, cytokine profiles and other immune markers such as thyroid peroxidase antibodies, thyroglobulin-antibodies and HLA antibodies. Endocrine factors (such as thyroid hormones, sex hormones etc.) will be analysed. DNA will be used for HLA typing and other genetic analyses (Part 3: MiEndo). DNA methylation patterns will be investigated in women and men, before and after pregnancy (Part 2: MiRPL) and surgery (Part 3: MiEndo). Mother's blood and endometrium samples will be analysed for the presence of foetal cells.

Urine samples

Urine and blood samples will be investigated by isotope dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS) for a screening panel of endocrine disrupting chemicals (EDCs) and other environmental factors. Levels in urine will be correlated with levels in the blood. Furthermore, iodine concentration will be measured.

Hair samples

Hair samples (~100 strands) will be cut from the posterior vertex as close to the scalp as possible. Steroids in the hair samples will be quantified on a Xevo TQS LC-MS/MS (Waters Chromatography, Waters Corporation, Milford, MA, USA) and analysed at the Erasmus University Medical Center in Rotterdam.

Table 1 Summary of objectives for each part of the study to investigate the microbiome in reproductive health.

Objectives	Part 1 MiMens	Part 2 MiRPL	Part 3 MiEndo
Describe and compare the microbiome in the saliva, faeces, rectal mucosa, vaginal fluid and endometrium and relate it to secondary outcomes	X	X + semen + meconium	X + peritoneum + endometriotic tissue + peritoneal liquid + cyst liquid
Compare immune factors and endocrine biomarkers in the blood to microbiome in the individual	X	X + cortisol in saliva and hair + iodine in urine	X
Describe the Omics profile (transcriptomics, proteomics, metabolomics and lipidomics) in relation to the microbiome and biomarkers	X	X	X
Investigate the level of EDCs and immune factors in blood and urine in women and correlate to the microbiome, reproductive history, pregnancy outcome, immune and endocrine biomarkers	X	X + in men	X
Investigate the sperm quality and DNA-fragmentation level in relation to the seminal microbiome and pregnancy outcome		X	
Investigate the transfer of micro-organisms and EDCs from mother to a live-born child and correlate to the reproductive history, pregnancy outcome (including birth complications), sex of child, immune and endocrine biomarkers		X	
Investigate the HLA types of the mother, father, prior firstborn child (if applicable), circulating foetal cells in the mother's blood and possible live-born children in the study and correlate to the immune parameters in the mother, microbiome profile and reproductive history		X	
Investigate pregnancy loss products for structural chromosomal abnormalities in the foetus, pathological and histological signs of placenta insufficiency and molecular immune reactions		X	
Compare the immune factors in blood with the immune cells present in endometriotic tissue			X
Explore the histological characteristics of endometriotic tissue and compare with unaffected peritoneal tissue and myometrial tissue			X
Examine genetic (e.g. genomics and epigenomics) and molecular connections to endometriosis			X

EDC, endocrine disrupting chemicals; MiEndo, microbiome in endometriosis, 120 patients with endometriosis requiring surgery with or without hormonal treatment; MiMens, microbiome during menstrual cycle, 150 healthy women with or without hormonal contraception; MiRPL, microbiome in recurrent pregnancy loss, 200 couples with recurrent pregnancy loss, 50 healthy couples with prior uncomplicated pregnancy and 150 newborns.

Table II Primary and secondary outcome measures.

Study	Aim	Primary outcome measure	Secondary outcome measure		
Part 1. Microbiome during menstrual cycle (MiMens)	To investigate the microbiome during the menstrual cycle in healthy women of reproductive age with and without hormonal contraception.	Microbiome profile throughout the menstrual cycle	Determine the level of: • endocrine biomarkers • inflammatory biomarkers • EDCs • stress and depression scores The above levels will be correlated with the microbiome profile.		
Part 2. Microbiome in Recurrent Pregnancy Loss (MiRPL)	To explore the microbiome in couples with RPL.	Microbiome profile in association with pregnancy outcome (minimum follow-up time: 12 months)	<u>For female participants:</u> • inflammatory biomarkers • HLA antibodies • immune markers (incl. B- and T cells) • endocrine biomarkers and EDCs • stress and depression scores The above will be compared with the microbiome profile and with healthy controls with prior uncomplicated pregnancies.	<u>For male partner:</u> • semen analysis (incl. DNA fragmentation) and the seminal microbiome profile • inflammatory biomarkers • endocrine biomarkers and EDCs The above will be compared with pregnancy outcome.	<u>For newborns:</u> • inflammatory biomarkers • endocrine biomarkers and EDC's • microbiome profile in meconium and in association with parents
Part 3. Microbiome in Endometriosis (MiEndo)	To investigate the microbiome in endometriosis patients and compare with the healthy control group (MiMens).	Microbiome profile in patients with moderate to severe endometriosis	• symptom questionnaires (incl. pain score, stress and depression score, quality of life) • clinical data (grade of disease defined by rASRM classification of endometriosis, phenotype, surgical outcome, clinical history) Determine level of: • inflammatory biomarkers • immune markers (incl. B- and T cells in blood and tissue) • HLA antibodies • endocrine biomarkers and EDCs		

EDC, endocrine disrupting chemicals; rASRM, Revised American Society for Reproductive Medicine.

Saliva samples

Saliva samples will be collected at time of visit and at home, with sample collection three times over a 24-h period. Samples from hospital visits will be analysed for the microbiome profile and steroids.

Semen samples

Semen quality will be evaluated following standard procedures including assessment of sperm concentration and sperm morphology. Sperm DNA fragmentation will be evaluated and compared by SDI® (sperm

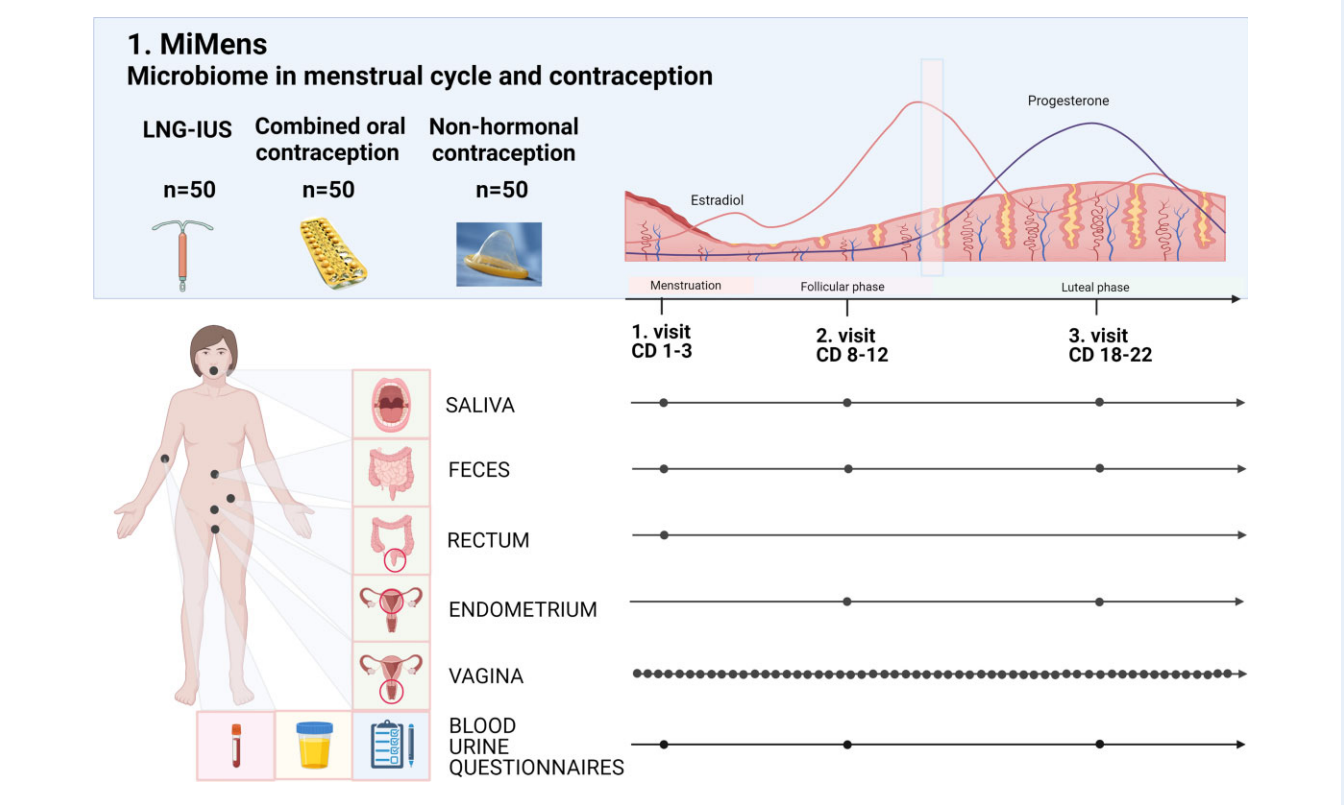

Figure 1. Study overview of participants and timeline of sample collections in MiMens. Created with BioRender.com. CD, cycle day; LNG-IUS, levonorgestrel intrauterine system; MiMens, microbiome during menstrual cycle.

DNA integrity based on the sperm chromatin structure assay) test (Blomberg Jensen *et al.*, 2018) and the COMET (microgel electrophoresis technique) assay or TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick-end labelling). Microbiome analysis of raw semen samples will be performed, and raw semen, semen plasma and semen pellet will be stored to investigate immunological products/reactions, proteins, microRNAs and other relevant biomarkers for quality of sperm/fertilization potential. Semen samples will also be used to investigate a possible immunological reaction in the mother's immune cells.

Mouth swabs

Mouth swabs will be collected from firstborn children to extract DNA and perform HLA typing (Part 2: MiRPL). If the male partner is unable to provide a blood sample, he can provide a mouth swab for DNA extraction and HLA typing.

Pregnancy loss product

If a woman has a pregnancy loss (Part 2: MiRPL) at home, she will collect the pregnancy loss product or, alternatively, if she is undergoing a surgical evacuation procedure, the pregnancy loss product will be collected by the treating doctor in the gynaecological department. The foetal DNA will be analysed by karyotyping at the Center for Chromosome Stability, University of Copenhagen. If karyotyping is not possible because of low input of foetal cells, the foetal DNA will also be sequenced by whole-genome sequencing by PicoPLEX® single-cell WGA, Multiple Displacement Amplification (MDA), or Linear Amplification via Transposon Insertion (LIANTI) protocols resulting in the same resolution as karyotyping.

Endometrial samples

The microbiome will be analysed, and endometrial samples will be examined for the same immune factors and regulators as the blood samples. The Omics profile will also be examined, and in Part 3 (MiEndo), the endometrium will be fixed in formalin and paraffin embedded for histology with immunohistochemical coloring. The collection, processing and storage of endometrial tissue for Part 3 (MiEndo) follows the standard operating procedure (SOP) recommended by the World Endometriosis Research Foundation (WERF) (EPHect | World Endometriosis Research Foundation, n.d.).

Peritoneal liquid and cyst liquid

After analysis of the microbiome, samples will be examined for immune factors, regulators and Omics profile. The collection, processing and storage of peritoneal liquid follows the SOP recommended by WERF (EPHect | World Endometriosis Research Foundation, n.d.).

Endometriotic tissue, cyst wall, peritoneal biopsy and myometrium

Endometriotic tissue excised during surgery will be cut into ~1 cm pieces, with two pieces for microbiome analysis and two pieces to be snap frozen in a tube on dry ice.

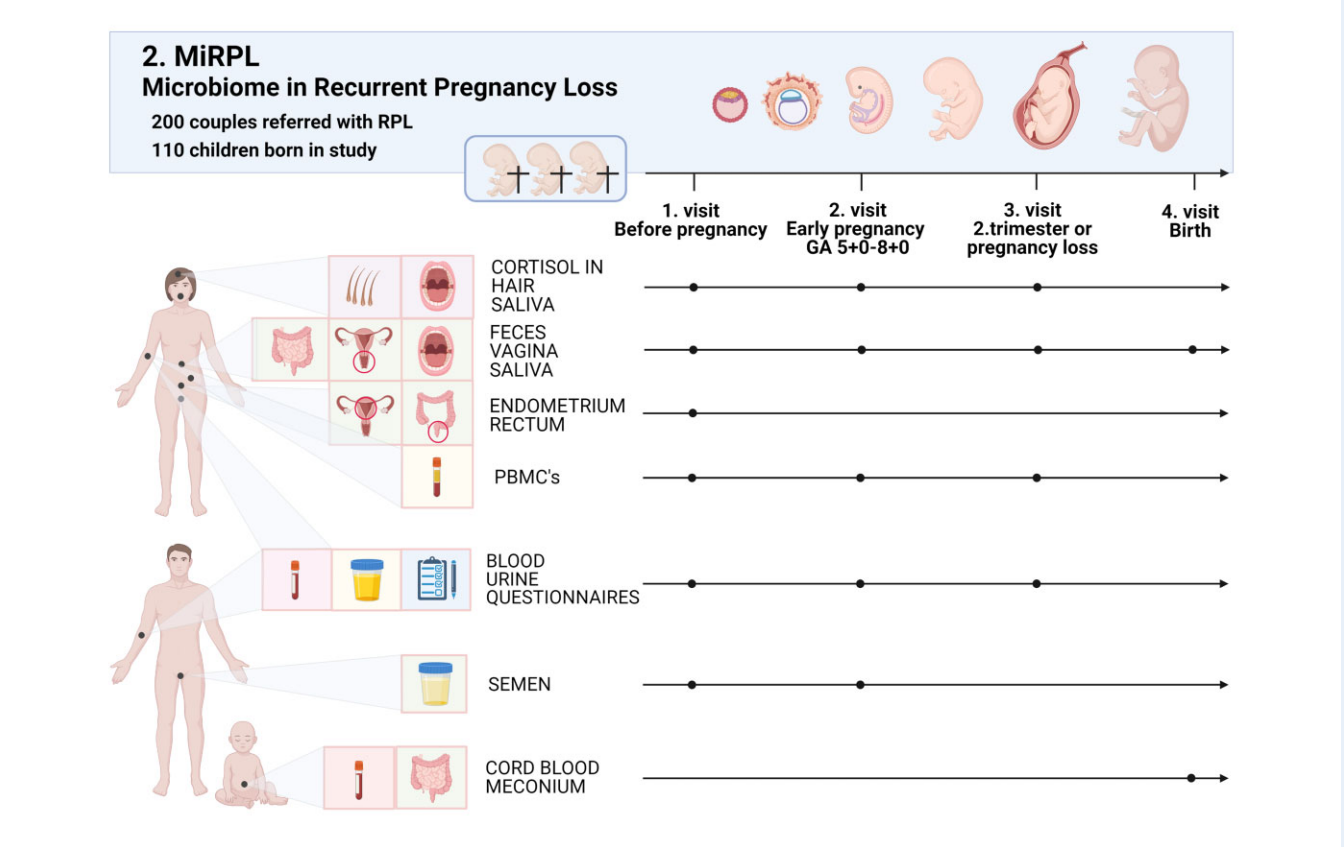

Figure 2. Study overview of patients and timeline of sample collections in MiRPL. Created with BioRender.com. GA, gestational age; MiRPL, microbiome in recurrent pregnancy loss; PBMC, peripheral blood mononuclear cell.

One sample of resected bowel, endometriotic plaque, cyst wall, myometrium and adenomyosis will be formalin fixated and embedded in paraffin. The paraffin-embedded tissue will be histologically examined after immunohistochemical staining and compared with the immune markers in the blood, the liquids of peritoneum and cysts, and the microbiome profile. The unaffected tissues are controls for the affected tissue. The Omics profile and immune factors/markers will be examined in snap frozen tissues. The collection, processing and storage of the endometriotic tissue follows the SOP recommended by WERF (EPHect | World Endometriosis Research Foundation, n.d.).

'Multi-omics' analyses

Longitudinal deep profiling of blood, tissue and microbiome samples will be performed to elucidate the role of the microbiome and host–microbe interactions in reproductive health. Molecular profiles associated with reproductive high-risk patients will be based on the analyses described below.

Microbiome analysis

Microbiome analysis will be performed at CTMR (Karolinska Institutet) and will focus on quantification of known microbial diversity and how relative abundances of taxonomic groups within samples correlate with external factors. Taxonomical profiling will be based on shotgun sequencing or 16S rRNA gene profiling, as appropriate for the sample type (i.e. percentage of human DNA in the sample). Deep metagenomic sequencing will be performed in a subset of samples to identify important organisms that are distantly related to organisms in public databases, as well as to characterize their functional potential and obtain strain-level information.

Bioinformatics

The 16S gene sequences will be subjected to error correction and annotation on the DADA2 suite (Callahan *et al.*, 2016). Shotgun reads will be submitted to quality-based trimming with BBTools, followed by host removal and taxonomic annotation through mapping to appropriate databases, such as HOMD (Chen *et al.*, 2010) for saliva samples and OptiVagDB (https://github.com/ctmrbio/optivag/tree/master/database) (Hugerth *et al.*, 2020) for vaginal samples. Faecal and rectal samples, as well as samples with no available dedicated database, will be annotated against the National Center for Biotechnology Information (US) (NCBI) RefSeq. Functional annotation will be based on TIGRFam (Haft *et al.*, 2013) as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata *et al.*, 1999).

Transcriptomics

RNA- and single-cell RNA-sequencing will be performed for global expression profiling of all genes including risk genes, splice variants of

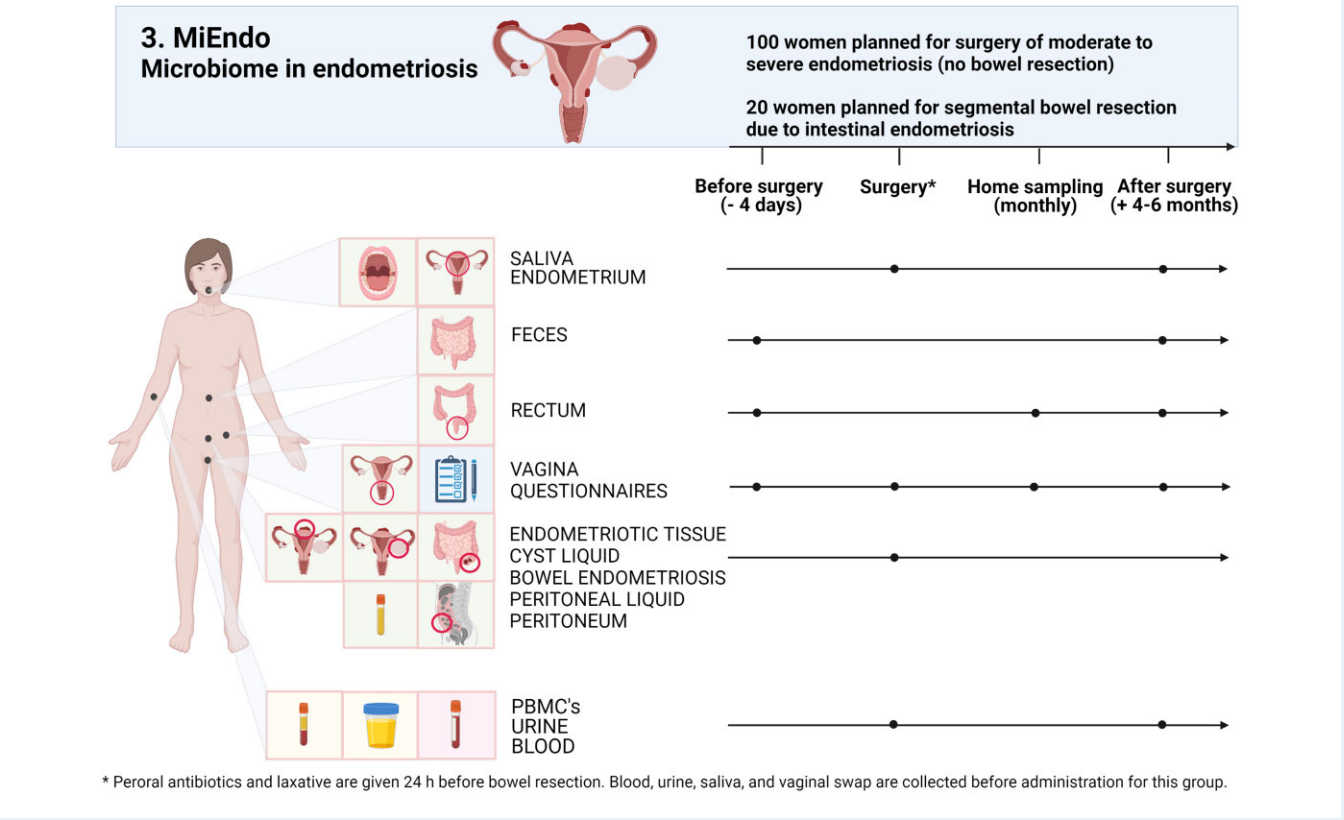

Figure 3. Study overview of patients and timeline of sample collections in MiEndo. Created with BioRender.com. MiEndo, microbiome in endometriosis; PBMC, peripheral blood mononuclear cell.

known genes and non-coding RNA. RNA is to be sequenced from blood cells, tissues and microbiome samples.

Proteomics

Large-scale protein analysis will be used to detect expression patterns in blood cells, tissues and microbiome samples. Both MS-based technologies and targeted assays based on proximity extension technology will be used.

Metabolomics and lipidomics

Small molecule metabolic products, including lipids, will be analysed in blood, tissues and microbiome samples using MS. Both untargeted methods for general profiling and targeted assays for the quantification of metabolites known to be involved in microbial metabolism and mediators of inflammation, such as short-chain fatty acids, will be applied.

Epigenomics

Global analysis of DNA methylation and hydroxymethylation will be used to identify if there are any associations with active or inactive gene expression. Chromatin structure and histone modifications will also be investigated.

Genomics

In Part 3 (MiEndo), for identification of genetic variants, genomic DNA or amplified DNA-products will be analysed with whole-exome sequencing (200-fold exome coverage) followed by ultra-deep sequencing of 30–100 relevant molecule gene mutations (500-fold coverage) identified by whole-exome sequencing (Illumina platform and reagents).

Mate-pair whole-genome analysis (15–30-fold coverage) will be used to identify structural/chromosomal deviations.

Multi-omics data integration

Different human datasets can be integrated using appropriate metabolic atlases (Robinson *et al.*, 2020). This will increase the strength of concordant findings and reduce the size of the dataset. Further data reduction and integration with microbiome data will rely on traditional data reduction techniques, such as principal component analysis and redundancy analysis. Finally, this streamlined dataset will be submitted to appropriate machine learning techniques, such as random forests or artificial neural networks, to separate cases from controls or the severity of cases, as appropriate.

Questionnaires and data recording

Information about previous pregnancies, births, current evaluations and treatments, including current medication, results from

Table III Overview of participants and the inclusion/exclusion criteria.3. MiEndo

Study part	Participants	Age (years)	Key inclusion criteria	Key exclusion criteria	Recruitment and remuneration (if relevant)
1. MiMens	150 healthy women of reproductive age, including: • 50 with regular menstrual cycle and no hormonal contraception • 50 using combined oral oestrogen/progesterone contraception • 50 using levonorgestrel intrauterine system (LNG-IUS)	18–40	• Regular menstrual cycle for ≥6 months prior to initiation of hormonal contraception	• Antibiotics, antimycotic and antiviral medication within past 2 weeks from inclusion • Currently pregnant or planning to become pregnant in study period	Advertisements at University of Copenhagen; 3000 DKK for 6 weeks
2. MiRPL	200 couples with unexplained RPL referred for evaluation (a total of 400 women and men) • The participants will only be sampled during the first pregnancy after referral (except biochemical pregnancies, which will only be recorded)	18–40	• ≥3 consecutive pregnancy losses (spontaneous early/late miscarriages, prior to gestational week 22) or biochemical pregnancy losses OR • ≥2 consecutive late pregnancy losses (>12 weeks), with pregnancy documented by nuchal translucency scan	• Antibiotics, antimycotics and antiviral medication within past 2 weeks • Pregnant at referral • Fertility treatment with preimplantation genetic testing • Uterine malformations • Chromosomal aberrations that can explain RPL	Patients at Rigshospitalet and Hvidovre Hospital. No remuneration. Minimum follow-up time: 12 months.
	Approximately 110 newborns born during study	–			
	Approximately 40 firstborn children (mouth swab only)	–			
	50 healthy couples (control group; 50 women, 50 men)	18–40	• Given birth to one shared child	• History of reproductive failure (fertility treatment, induced abortions or pregnancy loss) • Pregnancy within the last 3 months • Antibiotics within the last 2 weeks	Advertisements at hospitals, social media, www.forsoegsperson.dk
3. MiEndo	100 women with moderate to severe endometriosis without bowel resection The rASRM score is used for classification of endometriosis	18–45	Planned surgery of expected moderate to severe endometriosis	• Systemic antibiotics, antimycotics or antiviral drugs within 2 weeks before faecal sampling • Pregnancy or fertility treatment (only IVF or ICSI) within 3 months before surgery • Active cancer	Patients at Rigshospitalet. No remuneration. Follow-up time: 6 months.
	20 patients with intestinal involvement of endometriosis planned	18–45	• Endometriosis patients planned for segmental bowel resection		

(continued)

Table III Continued

Study part	Participants	Age (years)	Key inclusion criteria	Key exclusion criteria	Recruitment and remuneration (if relevant)
	for segmental bowel resection The rASRM score is used for classification of endometriosis			• Intraabdominal surgery in the last month due to suspected infection • No histological confirmed endometriosis after surgery	

MiEndo, microbiome in endometriosis; MiMens, microbiome during menstrual cycle; MiRPL, microbiome in recurrent pregnancy loss; rASRM, Revised American Society for Reproductive Medicine; RPL, recurrent pregnancy loss.

histology, microbiology, blood samples, MR and ultrasound, objective findings during gynaecological examination and surgery and finally findings during previous relevant gynaecological surgical procedures, will be retrieved from the patient medical records with patient consent.

A number of questionnaires will be completed (Table V).

All data, including surveys, will be stored in REDCap (https://www.project-redcap.org/) (Harris *et al.*, 2009, 2019), hosted by the Capital Region of Denmark. REDCap (Research Electronic Data Capture) is a secure, web-based software platform designed to support data capture for research studies, providing: an intuitive interface for validated data capture; audit trails for tracking data manipulation and export procedures; automated export procedures for seamless data downloads to common statistical packages; and procedures for data integration and interoperability with external sources.

Statistical analyses

We will report separately the findings regarding the microbiome, the immune and endocrine performance, levels of EDCs and Omics investigations for each cohort. We will apply a systems biology approach combining all obtained data to search for disease trajectories and disease-specific patterns.

Descriptive statistics will be presented as numbers and percentages for categorical data, means and SDs for normally distributed data or medians and interquartile ranges for non-normally distributed quantitative data. Differences will be assessed by use of the Chi-square test, Student's *t*-test or Mann–Whitney *U*-test, as appropriate. *P*-values <0.05 will be considered statistically significant. Multiple testing correction will be performed with the Benjamini–Hochberg procedure when appropriate.

The women in Part 1 (MiMens) will subsequently be used as control group in relation to questionnaire and clinical data to which the results from Part 2 (MiRPL) and 3 (MiEndo) will be compared. The groups will be compared using standard statistical software to analyse microbiome compositions with bioinformatic techniques. Taxonomic analyses are based on computational techniques from microbial ecology, including diversity measures within the samples (alpha diversity; Simpson's index) and between samples (beta-diversity; Bray-Curtis distance). Samples may also be divided into *a posteriori* categories based on their taxonomic profiles; these can be defined quantitatively or qualitatively (presence/absence).

Potential predictors of a live birth, success of operation and pain reduction will be evaluated in simple logistic regression analyses. Significant predictors will then be analysed by multiple logistic regression. Time to live birth will be illustrated in a Kaplan–Meier plot (survival analysis) according to the microbiome profile and compared using the log-rank test.

Ethics approval

This study is approved by the Danish Data Protection Agency (Protocol No. 2012-58-0004). Ethics approval has been obtained from the Regional Ethics Committee of the Capital Region of Denmark before undertaking this study (Protocol No. H-17017580).

All patients/volunteers will be given written information and invited (with a companion if desired) to a personal meeting where further information will be given orally and consent forms signed (or participants may request additional time to consider, with a private consultation a week later). Participants can withdraw their consent at any time and with immediate effect. The participants in Part 1 (MiMens) will be remunerated with 3000 DKK before taxes when they complete 6 weeks' participation. Clinical and questionnaire data will be collected using REDCap electronic data capture tools (Harris *et al.*, 2019), hosted at the Capital Region of Denmark.

When patients become pregnant and are to be discharged from the RPL Unit (Part 2: MiRPL), the parents will receive written and oral information about research concerning children in the project. Both legal parents will be asked to give consent on behalf of their participating child for meconium and cord blood to be collected from the newborn baby and mouth swabs from possible first-born children—there are no risks or discomfort for the participating children.

All genome data are saved according to guidelines from the Danish Data Protection Agency and The General Data Protection Regulation.

Table IV Study visits and details on sample collections at each visit.

	Part 1. MiMens			Part 2. MiRPL				Part 3. MiEndo			
Study stage	**1. Initial consultation**	**2. Follicular phase**	**3. Luteal phase**	**1. Baseline**	**2. Early pregnancy**	**3. Second trimester/loss**	**4. Birth**	**1. Baseline**	**2. Operation**	**3. Home sampling**	**4. Follow up**
Time specification	**Cycle days 1–3**	**Cycle days 8–12**	**Cycle days 18–22**	**Pre-pregnancy**	**Gestational age 6–8 weeks**	**Second trimester or time of pregnancy loss**	**Time of delivery**	**4 days before surgery**	**Day of surgery/day before surgery**	**Monthly after surgery until follow-up**	**4–6 months after surgery**
Sample pack 1, 2 or 3*	1	1	1	2[b]	2	2	2		3[d]		3
Endometrium (2 ml)		X	X	X					X		X
Pregnancy loss product						X					
Semen sample (5 ml)				X	X						
Urine sample (30 ml)	X	X	X	X	X	X			X		X
Home sampling	X[a]	X[a]	X[a]					X[c]		X[e]	
Cord blood (10–20 ml), meconium (2 ml)							X				
Endometriotic tissue + unaffected tissue**									X		
Male partner sample pack***				X[b]	X						
Male partner or firstborn child mouth swab				X							

*Sample pack 1:

- Blood samples 3× EDTA tubes 9 ml, 3× serum clot activator tubes 9 ml, 1× PAX gene tube 2.5 ml, 56.5 ml blood in total pr. visit for DNA, RNA, serum and plasma storage.
- Faecal sample: ∼2 ml faeces.
- Rectal sample: 1 swab.
- Vaginal swabs: 2 swabs.
- Oral sample: ∼2 ml saliva.

Sample pack 2:

- Blood samples 8× EDTA tubes 9 ml, 3× serum clot activator tubes 9 ml, 1× PAX gene tube 2.5 ml, 101.5 ml blood in total pr. visit for DNA, RNA, peripheral blood mononuclear cell (PBMC), serum and plasma storage.
- Faecal sample: ∼2 ml faeces (first 100 women only).
- Rectal samples 1 swab (first 100 women only).
- Vaginal swabs: 2 swabs (first 100 women only).
- Oral samples: ∼6 ml saliva (2 ml three times over the course of 24 h on day of visit).
- Hair samples: ∼100 hair strands from the posterior vertex cranii (∼50 women). Only if >4 weeks since last sample.

Sample pack 3:

- Blood samples 4× EDTA tubes 9 ml, 1× EDTA tube 6 ml, 2× serum clot activator tubes 9 ml, 1× PAX gene tube 2.5 ml, 2× Sodium-Heparin tubes 9 ml, 80.5 ml blood in total pr. visit for DNA, RNA, PBMC, serum and plasma storage.
- Faecal sample: ∼2 ml faeces.
- Rectal sample: 1 swab.
- Vaginal swabs: 2 swabs.
- Oral sample: ∼2 ml saliva.

**Endometriosis operation sample pack; plaques: five samples of ∼1 cm; endometriotic cyst liquid: ∼10 ml; resected bowel: five samples of ∼1 cm; peritoneal liquid: ∼10 ml; unaffected peritoneal biopsy: three biopsies of ∼0.5 cm; myometrium: three biopsies of ∼1 cm (if hysterectomized); adenomyosis: three biopsies of ∼1 cm (if hysterectomized); cyst wall: three biopsies of ∼1 cm.

***Male partner sample pack: blood samples 1× EDTA tubes 9 ml, 1× 6 ml EDTA tube, 1× serum clot activator tubes 9 ml, 24 ml blood in total. Sample pack includes semen and urine; 50 male partners also give hair samples.

[a]Daily vaginal home sampling for 6 weeks.

[b]Healthy control couples will only deliver a sample corresponding to the baseline visit, which for the men corresponds to male partner sample pack and for the women only includes 6 ml saliva (3 × 2 ml), one urine sample (∼30 ml), blood samples (68 ml) and hair samples (∼100 hair strand).

[c]Home sampling 4 days before surgery: vaginal, rectal and faecal samples.

[d]Sample collection on the day of endometriosis surgery is without faecal and rectal samples.

[e]Home sampling after surgery: monthly vaginal and rectal sampling until follow up 4–6 months after surgery.

MiMens, microbiome during menstrual cycle, 150 healthy women with or without hormonal contraception; MiRPL, microbiome in recurrent pregnancy loss, 200 couples with recurrent pregnancy loss, 50 healthy couples with prior uncomplicated pregnancy and 150 newborns. MiEndo, microbiome in endometriosis, 120 patients with endometriosis requiring surgery with or without hormonal treatment.

Table V Questionnaires completed during the study.

Questionnaires	Description	Part 1. MiMens	Part 2. MiRPL	Part. 3. MiEndo
Background information for all participants (both women and men) after first visit	General and reproductive health, family history, use of antibiotics and medication, lifestyle factors such as detailed dietary questions, smoking, alcohol, exercise etc.	X	X	X
Perceived Stress Scale (PSS) and Major Depression Inventory (MDI) for all participants (both women and men)	Measurement of emotional stress measured by the PSS (Cohen *et al.*, 1983) and depressive symptoms by the MDI (Bech *et al.*, 2015).	X	X	X
Food-recall	The diet of the participants will be recorded using a 24- to 48-h food-recall questionnaire after faecal sampling at home.	X	X	X
Gynaecological symptoms	Bleedings and sexual intercourse. Current gynaecological health issues will be noted at every visit (also when home sampling in Part 1: MiMens and Part 3: MiEndo).	X	X	X
Short Form 36v2 (SF-36v2)	Quality of life questionnaire—measures social functioning, mental and physical health (Ware and Sherbourne, 1992; Maruish, 2011).			X
Bowel Endometriosis Syndrome (BENS) score and Low Anterior Resection Syndrome (LARS) score	Questionnaires on intestinal symptoms (Emmertsen and Laurberg, 2012; Riiskjær *et al.*, 2017).			X
Endometriosis Patient Questionnaire-Minimum (EPQ-M)	A questionnaire on clinical history and anamnestic responses. The EPQ questionnaire is previously validated in English by the WERF and has been translated to Danish and validated on endometriosis patients in the outpatient clinic at Rigshospitalet before being used in this study.			X
Standard Surgical Form (SSF)	A detailed questionnaire designed by WERF for clinicians to register data on findings and procedures done during endometriosis surgery.			X

MiEndo, microbiome in endometriosis, 120 patients with endometriosis requiring surgery with or without hormonal treatment; MiMens, microbiome during menstrual cycle, 150 healthy women with or without hormonal contraception; MiRPL, microbiome in recurrent pregnancy loss, 200 couples with recurrent pregnancy loss, 50 healthy couples with prior uncomplicated pregnancy and 150 newborns; WERF, World Endometriosis Research Foundation.

All results—positive, negative and inconclusive—will be submitted for publication in international peer-reviewed journals. If the results are not published, they will be announced on our websites (Centre for Translational Microbiome Research (CTMR) | Karolinska Institutet, n.d.; Enheden for Gentagne Graviditetstab, n.d.).

Discussion

This large-scale observational study of reproductive high-risk patient groups and healthy controls represents a major contribution to an important and emerging area of research into the role of the microbiome in reproductive health and disease. The study includes a large number of patients, comprehensive biomaterial, clinical and questionnaire data combined with longitudinal sampling and follow-up data. A potential weakness is that only patients with severe disease (i.e. RPL or moderate to severe endometriosis) and healthy women and couples are included, and as such the role of the microbiome in mild degrees of reproductive failure or mild endometriosis is not explored.

Data availability

As this is a study protocol, no new data have been generated or analysed in support of this publication.

Acknowledgements

The following have made substantial contributions to the project so far: Anne Louise Lunøe, Karen H. Kirchheiner Jensen, Marie L.T. Chonovitsch (MiMens and MiRPL: care for study patients, data collection); Anders Nyboe Andersen (MiMens: endometrial biopsies); Anna Slot, Josefine Reinhardt Nielsen and Kathrine Hviid (MiRPL: inclusion, data collection and monitoring); Margarita F. Salcedo and Laura O.M. Marchard (MiRPL: data collection and monitoring); Janne Gasseholm Bentzen, Birgitte Oxlund-Mariegaard, Lene Hee Christensen and Astrid Marie Kolte (MiRPL: endometrial biopsies); Lise K.A. Kähler, Sofie E. Thomasson, Liv Dyrved, Benedikte H. Ejsing (MiEndo: inclusion, data collection and monitoring). Medical writing assistance with this manuscript was provided by Caroline Loat, PhD.

Authors' roles

H.S.N. conceptualized the project together with L.E. and I.S.-K.. H.S.N., I.S.-K., M.C.K., M.E.M., S.B., D.H., K.W., L.E.V., Z.B., L.W.H., F.B., M.H. and E.F. made substantial contributions to the design of the study. M.C.K. and H.S.N. obtained all approvals to initiate the study and ensured the clinical and sample infrastructure. M.C.K., M.E.M. and S.B. are primary study coordinators. Z.B. is primary study assistant on MiMens and L.E.V. is primary study assistant on MiEndo. M.C.K. and M.E.M. contributed equally to this paper and drafted the first version of the protocol under the supervision of H.S.N. S.B. drafted or revised the parts of the protocol related to endocrinology and immunology. All of the authors contributed to a critical discussion and revision of this protocol, and all authors approved the final version. All authors agree to be accountable for all aspects of the work.

Funding

The project described in this article is supported by a Centre grant provided by Ferring Pharmaceuticals for the infrastructure to obtain the clinical samples at Copenhagen University Hospital ([#MiHSN01] to M.C.K., M.E.M., S.B., Z.B., L.E.V. and H.S.N.), and for the establishment of the Centre for Translational Microbiome Research at the Karolinska Institutet (to L.W.H., E.F., M.H., F.B., L.E. and I.S.-K.). This work was also supported by funding from Rigshospitalet's Research Funds ([#E-22614-01 and #E-22614-02] to M.C.K. and [#E-22222-06] to S.B.), Oda and Hans Svenningsen's Foundation ([#F-22614-08] to H.S.N.), Niels and Desiree Yde's Foundation (S.B., endocrine analyses [#2015-2784]) and the Musikforlæggerne Agnes and Knut Mørk's Foundation (S.B., endocrine and immune analyses [#35108-001]). Medical writing assistance was funded by Ferring Pharmaceuticals.

Conflict of interest

H.S.N. reports personal fees from Ferring Pharmaceuticals, Merck Denmark A/S, Ibsa Nordic, Astra Zeneca and Cook Medical outside the submitted work. K.W. is a full-time employee of Ferring Pharmaceuticals.

No other conflicts are reported.

References

Al-Nasiry S, Ambrosino E, Schlaepfer M, Morré SA, Wieten L, Voncken JW, Spinelli M, Mueller M, Kramer BW. The interplay between reproductive tract microbiota and immunological system in human reproduction. *Front Immunol* 2020;**11**:378.

Amabebe E, Anumba DOC. The vaginal microenvironment: the physiologic role of *Lactobacilli*. *Front Med (Lausanne)* 2018;**5**:181.

Baker JM, Al-Nakkash L, Herbst-Kralovetz MM. Estrogen-gut microbiome axis: physiological and clinical implications. *Maturitas* 2017;**103**:45–53.

Bassis CM, Allsworth JE, Wahl HN, Sack DE, Young VB, Bell JD. Effects of intrauterine contraception on the vaginal microbiota. *Contraception* 2017;**96**:189–195.

Bech P, Timmerby N, Martiny K, Lunde M, Soendergaard S. Psychometric evaluation of the Major Depression Inventory (MDI) as depression severity scale using the LEAD (Longitudinal Expert Assessment of All Data) as index of validity. *BMC Psychiatry* 2015;**15**:190.

Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, Middeldorp S, Nelen W, Peramo B, Quenby S, Vermeulen N *et al*. ESHRE guideline: recurrent pregnancy loss. *Hum Reprod Open* 2018;**2018**:hoy004. Available at https://academic.oup.com/hropen/article/2018/2/hoy004/4963604 (30 May 2020, date last accessed).

Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci USA* 1996;**93**:705–708.

Blomberg Jensen M, Lawaetz JG, Petersen JH, Juul A, Jørgensen N. Effects of vitamin D supplementation on semen quality, reproductive hormones, and live birth rate: a randomized clinical trial. *J Clin Endocrinol Metab* 2018;**103**:870–881.

Brown EL, Essigmann HT, Hoffman KL, Palm NW, Gunter SM, Sederstrom JM, Petrosino JF, Jun G, Aguilar D, Perkison WB *et al*. Impact of diabetes on the gut and salivary IgA microbiomes. *Infect Immun* 2020;**88**:e00301-20.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;**13**:581–583.

Centre for Translational Microbiome Research (CTMR) | Karolinska Institutet. Available at https://ki.se/en/research/centre-for-translational-microbiome-research-ctmr (1 September 2020, date last accessed).

Chaban B, Links MG, Jayaprakash TP, Wagner EC, Bourque DK, Lohn Z, Albert AY, van Schalkwyk J, Reid G, Hemmingsen SM *et al*. Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle. *Microbiome* 2014;**2**:23.

Chen T, Yu W-H, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database (Oxford)* 2010;**2010**:baq013.Available at https://academic.oup.com/database/article/doi/10.1093/database/baq013/405450 (23 August 2020, date last accessed).

Cherpes TL, Meyn LA, Krohn MA, Lurie JG, Hillier SL. Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. *Clin Infect Dis* 2003;**37**:319–325.

Cohen S, Kamarck T, Mermelstein R. A global measure of perceived stress. *J Health Soc Behav* 1983;**24**:385–396.

Costello M-E, Robinson PC, Benham H, Brown MA. The intestinal microbiome in human disease and how it relates to arthritis and spondyloarthritis. *Best Pract Res Clin Rheumatol* 2015;**29**:202–212.

Crusell MKW, Brink LR, Nielsen T, Allin KH, Hansen T, Damm P, Lauenborg J, Hansen TH, Pedersen O. Gestational diabetes and the human salivary microbiota: a longitudinal study during pregnancy and postpartum. *BMC Pregnancy Childbirth* 2020;**20**:69.

Dahl C, Stanislawski M, Iszatt N, Mandal S, Lozupone C, Clemente JC, Knight R, Stigum H, Eggesbø M. Gut microbiome of mothers delivering prematurely shows reduced diversity and lower relative abundance of *Bifidobacterium* and *Streptococcus*. *PLoS One* 2017;**12**:e0184336.

de Weerth C. Do bacteria shape our development? Crosstalk between intestinal microbiota and HPA axis. *Neurosci Biobehav Rev* 2017;**83**:458–471.

Donders GGG, Bellen G, Grinceviciene S, Ruban K, Vieira-Baptista P. Aerobic vaginitis: no longer a stranger. *Res Microbiol* 2017;**168**: 845–858.

Eckert LO, Moore DE, Patton DL, Agnew KJ, Eschenbach DA. Relationship of vaginal bacteria and inflammation with conception and early pregnancy loss following in-vitro fertilization. *Infect Dis Obstet Gynecol* 2003;**11**:11–17.

Emmertsen KJ, Laurberg S. Low anterior resection syndrome score: development and validation of a symptom-based scoring system for bowel dysfunction after low anterior resection for rectal cancer. *Ann Surg* 2012;**255**:922–928.

Enheden for Gentagne Graviditetstab. Available at https://www.hvidovrehospital.dk/afdelinger-og-klinikker/enheden-for-gentagne-graviditetstab/Sider/default.aspx (1 September 2020a, date last accessed).

Enheden for Gentagne Graviditetstab. Available at https://www.rigshospitalet.dk/afdelinger-og-klinikker/julianemarie/fertilitetsafdelingen/specialfunktioner-i-afdelingen/enheden-for-gentagne-graviditetstab/Sider/default.aspx (1 September 2020b, date last accessed).

EPHect | World Endometriosis Research Foundation. Available at http://endometriosisfoundation.org/ephect/#2 (22 July 2020, date last accessed).

Farcasanu M, Kwon DS. The influence of cervicovaginal microbiota on mucosal immunity and prophylaxis in the battle against HIV. *Curr HIV/AIDS Rep* 2018;**15**:30–38.

Freitas AC, Bocking A, Hill JE, Money DM; the VOGUE Research Group. Increased richness and diversity of the vaginal microbiota and spontaneous preterm birth. *Microbiome* 2018;**6**:117.

Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UME, Zhong X, Koenig SSK, Fu L, Ma Z, Zhou X *et al.* Temporal dynamics of the human vaginal microbiota. *Sci Transl Med* 2012;**4**:132ra52.

Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE. Metagenomic analysis of the human distal gut microbiome. *Science* 2006;**312**:1355–1359.

Haft DH, Selengut JD, Richter RA, Harkins D, Basu MK, Beck E. TIGRFAMs and genome properties in 2013. *Nucleic Acids Res* 2013;**41**:D387–D395.

Harris PA, Taylor R, Minor BL, Elliott V, Fernandez M, O'Neal L, McLeod L, Delacqua G, Delacqua F, Kirby J *et al.*; REDCap Consortium. The REDCap consortium: building an international community of software platform partners. *J Biomed Inform* 2019; **95**:103208.

Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 2009;**42**: 377–381.

Hugerth LW, Pereira M, Zha Y, Seifert M, Kaldhusdal V, Boulund F, Krog MC, Bashir Z, Hamsten M, Fransson E *et al.* Assessment of *In Vitro* and *In Silico* Protocols for Sequence-Based Characterization of the Human Vaginal Microbiome. mSphere 2020;**5**:e00448–20.

Kindinger LM, Bennett PR, Lee YS, Marchesi JR, Smith A, Cacciatore S, Holmes E, Nicholson JK, Teoh TG, MacIntyre DA. The interaction between vaginal microbiota, cervical length, and vaginal progesterone treatment for preterm birth risk. *Microbiome* 2017;**5**:6.

Klecha AJ, Barreiro Arcos ML, Frick L, Genaro AM, Cremaschi G. Immune-endocrine interactions in autoimmune thyroid diseases. *Neuroimmunomodulation* 2008;**15**:68–75.

Koedooder R, Singer M, Schoenmakers S, Savelkoul PHM, Morré SA, de Jonge JD, Poort L, Cuypers WJSS, Beckers NGM, Broekmans FJM *et al.* The vaginal microbiome as a predictor for outcome of in vitro fertilization with or without intracytoplasmic sperm injection: a prospective study. *Hum Reprod* 2019;**34**:1042–1054.

Köhling HL, Plummer SF, Marchesi JR, Davidge KS, Ludgate M. The microbiota and autoimmunity: their role in thyroid autoimmune diseases. *Clin Immunol* 2017;**183**:63–74.

Łaniewski P, Ilhan ZE, Herbst-Kralovetz MM. The microbiome and gynaecological cancer development, prevention and therapy. *Nat Rev Urol* 2020;**17**:232–250.

Laschke MW, Menger MD. The gut microbiota: a puppet master in the pathogenesis of endometriosis? *Am J Obstet Gynecol* 2016;**215**: 68.e1–68.e4.

Li J, Jia H, Cai X, Zhong H, Feng Q, Sunagawa S, Arumugam M, Kultima JR, Prifti E, Nielsen T *et al.*; MetaHIT Consortium. An integrated catalog of reference genes in the human gut microbiome. *Nat Biotechnol* 2014;**32**:834–841.

Lin W, Jiang W, Hu X, Gao L, Ai D, Pan H, Niu C, Yuan K, Zhou X, Xu C *et al.* Ecological shifts of supragingival microbiota in association with pregnancy. *Front Cell Infect Microbiol* 2018;**8**:24.

Maruish, M.E.(Ed.) *User's Manual for the SF-36v2 Health Survey* (3rd ed.). Lincoln, RI: QualityMetric Incorporated. 2011.

Meng X, Zhang G, Cao H, Yu D, Fang X, de Vos WM, Wu H. Gut dysbacteriosis and intestinal disease: mechanism and treatment. *J Appl Microbiol* 2020;**129**:787–805.

Montgomery GW, Mortlock S, Giudice LC. Should genetics now be considered the pre-eminent etiologic factor in endometriosis? *J Minim Invasive Gynecol* 2020;**27**:280–286.

Nielsen HS, Steffensen R, Lund M, Egestad L, Mortensen LH, Andersen A-MN, Lidegaard Ø, Christiansen OB. Frequency and impact of obstetric complications prior and subsequent to unexplained secondary recurrent miscarriage. *Hum Reprod* 2010;**25**: 1543–1552.

Norenhag J, Du J, Olovsson M, Verstraelen H, Engstrand L, Brusselaers N. The vaginal microbiota, human papillomavirus and cervical dysplasia: a systematic review and network meta-analysis. *BJOG* 2020;**127**:171–180.

Nunn KL, Forney LJ. Unraveling the dynamics of the human vaginal microbiome. *Yale J Biol Med* 2016;**89**:331–337.

Odendaal J, Quenby S, Sammaritano L, Macklon N, Branch DW, Rosenwaks Z. Immunologic and rheumatologic causes and treatment of recurrent pregnancy loss: what is the evidence? *Fertil Steril* 2019;**112**:1002–1012.

Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 1999; **27**:29–34.

Olsen I, Singhrao SK. Low levels of salivary lactoferrin may affect oral dysbiosis and contribute to Alzheimer's disease: a hypothesis. *Med Hypotheses* 2021;**146**:110393.

Ouabbou S, He Y, Butler K, Tsuang M. Inflammation in mental disorders: is the microbiota the missing link? *Neurosci Bull* 2020;**36**: 1071–1084.

Parker VJ, Douglas AJ. Stress in early pregnancy: maternal neuroendocrine-immune responses and effects. *J Reprod Immunol* 2010; **85**:86–92.

Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO *et al*. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci USA* 2011; **108**:4680–4687.

Riiskjær M, Egekvist AG, Hartwell D, Forman A, Seyer-Hansen M, Kesmodel US. Bowel endometriosis syndrome: a new scoring system for pelvic organ dysfunction and quality of life. *Hum Reprod* 2017;**32**:1812–1818.

Robinson JL, Kocabaş P, Wang H, Cholley P-E, Cook D, Nilsson A, Anton M, Ferreira R, Domenzain I, Billa V *et al*. An atlas of human metabolism. *Sci Signal* 2020;**13**:eaaz1482.

Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Bieda J, Chaemsaithong P, Miranda J, Chaiworapongsa T, Ravel J. The vaginal microbiota of pregnant women who subsequently have spontaneous preterm labor and delivery and those with a normal delivery at term. *Microbiome* 2014;**2**:18.

Rooks MG, Garrett WS. Gut microbiota, metabolites and host immunity. *Nat Rev Immunol* 2016;**16**:341–352.

Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. *PLoS Biol* 2016;**14**: e1002533.Available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4991899/ (22 July 2018, date last accessed).

Simon J-C, Marchesi JR, Mougel C, Selosse M-A. Host-microbiota interactions: from holobiont theory to analysis. *Microbiome* 2019;**7**: 5.

Singh RK, Chang H-W, Yan D, Lee KM, Ucmak D, Wong K, Abrouk M, Farahnik B, Nakamura M, Zhu TH *et al*. Influence of diet on the gut microbiome and implications for human health. *J Transl Med* 2017;**15**:73.

Sonnenburg J, Bäckhed F. Diet-microbiota interactions as moderators of human metabolism. *Nature* 2016;**535**:56–64. Available at https://pubmed.ncbi.nlm.nih.gov/27383980/?from_term=microbiome+metabolic+disorders+backhed&from_filter=pubt.review&from_sort=pubdate&from_pos=8 (30 May 2020, date last accessed).

Sprouse ML, Bates NA, Felix KM, Wu H-JJ. Impact of gut microbiota on gut-distal autoimmunity: a focus on T cells. *Immunology* 2019; **156**:305–318.

Tan Y, Wei Z, Chen J, An J, Li M, Zhou L, Men Y, Zhao S. Save your gut save your age: the role of the microbiome in stem cell ageing. *J Cell Mol Med* 2019;**23**:4866–4875.

Twig G, Shina A, Amital H, Shoenfeld Y. Pathogenesis of infertility and recurrent pregnancy loss in thyroid autoimmunity. *J Autoimmun* 2012;**38**:J275–281.

van Houdt R, Ma B, Bruisten SM, Speksnijder AGCL, Ravel J, de Vries HJC. *Lactobacillus iners*-dominated vaginal microbiota is associated with increased susceptibility to *Chlamydia trachomatis* infection in Dutch women: a case-control study. *Sex Transm Infect* 2018;**94**:117–123.

Wang B, Yao M, Lv L, Ling Z, Li L. The human microbiota in health and disease. *Engineering* 2017;**3**:71–82.

Wang L, Zhao J, Li Y, Wang Z, Kang S. Genome-wide analysis of DNA methylation in endometriosis using Illumina Human Methylation 450 K BeadChips. *Mol Reprod Dev* 2019;**86**:491–501.

Wang Y, Nicholes K, Shih I-M. The origin and pathogenesis of endometriosis. *Annu Rev Pathol* 2020;**15**:71–95.

Ware JE, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. *Med Care* 1992;**30**:473–483.

Xiao E, He L, Wu Q, Li J, He Y, Zhao L, Chen S, An J, Liu Y, Chen C *et al*. Microbiota regulates bone marrow mesenchymal stem cell lineage differentiation and immunomodulation. *Stem Cell Res Ther* 2017;**8**:213.

Xu Y, Zhang M, Zhang J, Sun Z, Ran L, Ban Y, Wang B, Hou X, Zhai S, Ren L *et al*. Differential intestinal and oral microbiota features associated with gestational diabetes and maternal inflammation. *Am J Physiol Endocrinol Metab* 2020;**319**:E247–E253.

Yan Z, Lambert NC, Guthrie KA, Porter AJ, Loubiere LS, Madeleine MM, Stevens AM, Hermes HM, Nelson JL. Male microchimerism in women without sons: quantitative assessment and correlation with pregnancy history. *Am J Med* 2005;**118**:899–906.

Ye C, Xia Z, Tang J, Khemwong T, Kapila Y, Kuraji R, Huang P, Wu Y, Kobayashi H. Unculturable and culturable periodontal-related bacteria are associated with periodontal inflammation during pregnancy and with preterm low birth weight delivery. *Sci Rep* 2020; **10**:15807.

Zhang Y-J, Li S, Gan R-Y, Zhou T, Xu D-P, Li H-B. Impacts of gut bacteria on human health and diseases. *Int J Mol Sci* 2015;**16**: 7493–7519.