

# BMJ Open Microbiome Characteristics in Early Threatened Miscarriage Study (MCETMS): a study protocol for a prospective cohort investigation in China

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## ABSTRACT

**Introduction** Studies have suggested that the vaginal microbiome and gut microbiome are involved in pregnancy-related diseases, but little exploration of the link with early miscarriage or threatened miscarriage (TM) has been done. Whether the characteristics of the vaginal microbiome and gut microbiome in early pregnancy are related to TM and early pregnancy outcomes remains unclear.

**Methods and analysis** The Microbiome Characteristics in Early Threatened Miscarriage Study (MCETMS) is a prospective investigation that will recruit 326 pregnant women with early TM. Pregnant women will be enrolled at 4–8 weeks of gestation, and their vaginal secretions, faecal samples, clinical data and sociodemographic characteristics will be collected prospectively. Pregnant women with TM will be followed up to 12 weeks of gestation to determine the early pregnancy outcomes (ongoing pregnancy or pregnancy loss). DNA will be extracted from the collected samples and will be analysed by 16S rRNA gene sequencing.

**Ethics and dissemination** The MCETMS study protocol has been approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Traditional Chinese Medical University (ZYYECK[2020]051). Dissemination of study findings will occur through peer-reviewed journals, conferences and presentations.

**Trial registration number** ChiCTR2000041172.

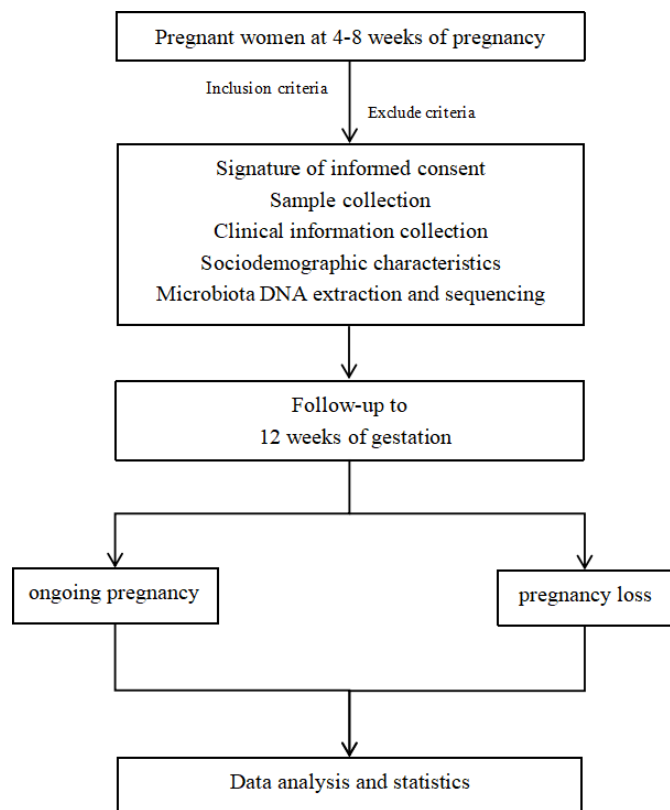
## INTRODUCTION

As the precursor to miscarriage, threatened miscarriage (TM) refers to the occurrence of vaginal bleeding before 20 weeks of gestation with a closed cervix. It is a common pregnancy complication with an incidence of 20%, and half of the cases of TM end in miscarriage.<sup>1 2</sup> Because most miscarriages occur in early pregnancy,<sup>3 4</sup> early detection, early diagnosis and early treatment in the early TM stage are very important for the clinical management of women with TM. Having an ideal clinical management strategy can effectively avoid the occurrence of adverse pregnancy outcomes.

## STRENGTHS AND LIMITATIONS OF THIS STUDY

- ⇒ The Microbiome Characteristics in Early Threatened Miscarriage Study is the first China cohort study to characterise the gut and vaginal microbiota in women with early threatened miscarriage.
- ⇒ Following up to document the early pregnancy outcomes at 12 weeks of gestation will allow us to explore the potential species from the gut and reproductive tract microbiota indicative of different early pregnancy outcomes.
- ⇒ A detailed covariate assessment will enable us to control confounding factors that may influence the microbiota.
- ⇒ The primary limitation of this study is that all subjects in this study will be recruited in China, requiring caution in extrapolating our results to populations with different demographic characteristics or in other areas.

Pregnancy is a complex and coordinated physiological phenomenon. During pregnancy, a mother's gut microbiome changes significantly.<sup>5</sup> Reproductive immunology contends that embryos, as semi-alloantigens, can survive and develop in the mother's uterus without being rejected by the immune system due to pregnancy immune tolerance, which develops both at the level of local immunity at the mother–fetus interface and at the level of maternal peripheral system immunity. The gut microbiota participates in the construction of the intestinal mucosal immune barrier and metabolic activities, thus playing a crucial role in shaping and regulating the immune system and immune response.<sup>6</sup> As previously reported, Proteobacteria and Actinobacteria as proinflammatory microbiota function to protect the mother and fetus from infections.<sup>7</sup> Liu *et al*<sup>8</sup> previously showed that the intestinal microbiota regulates related proinflammatory cytokines through its metabolites to have a biological impact on abortion.



**Figure 1** The flowchart of the study.

The key events during early pregnancy are accurate regulation of inflammation and tissue reorganisation of the upper genital tract,<sup>9–11</sup> which the vaginal microbiota may affect. Studies have found that the complex dynamic characteristics of the reproductive tract microbiota participate in the regulation of host immunity.<sup>12–13</sup> Previous studies have also revealed that the reproductive tract microbiome is involved in implantation failure,<sup>14–15</sup> and some of the major obstetrical syndromes, including spontaneous preterm birth<sup>16</sup> and preterm prelabour rupture,<sup>17</sup> have been covered in the literature, yet little exposition of early TM has been done.

According to what has been previously stated, we established the Microbiome Characteristics in Early Threatened Miscarriage Study (MCETMS) in 2020 to improve our understanding of the relationship between the vaginal microbiome as well as the gut microbiome and the development of early TM. Considering that most miscarriages occur in early pregnancy, the important stage of the whole pregnancy, we will prospectively collect vaginal secretions and faecal samples of women with TM in early pregnancy (4–8 weeks of gestation) and follow-up on the early pregnancy outcomes (ongoing pregnancy or pregnancy loss) at 12 weeks of gestation.

More specifically, the aims of this cohort study are as follows:

1. To characterise the gut and vaginal microbiota in women with early TM.

2. To characterise the gut and vaginal microbiota of women with ongoing pregnancy and pregnancy loss at 12 weeks.
3. To identify potential species from the vaginal microbiota and gut microbiota indicative of different early pregnancy outcomes among women with TM.

## MATERIALS AND ANALYSIS

### Study design and setting

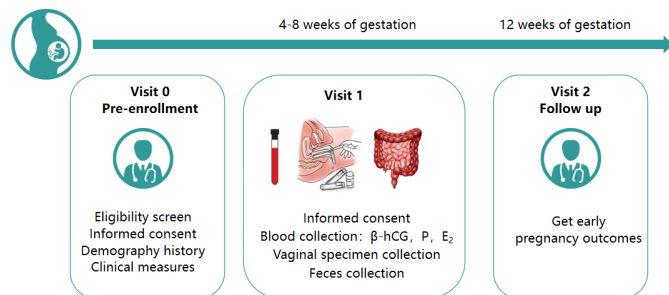
MCETMS is a prospective observational cohort study that will be conducted by members of the First Affiliated Hospital of Guangzhou Traditional Chinese Medical University and Shenzhen Hospital of Integrated Traditional and Western Medicine. Patient recruitment started in December 2020 and is expected to end in December 2022, with the goal of including 326 pregnant women with TM (4–8 weeks of gestation). After providing informed consent, subjects will be enrolled, and the sociodemographic characteristics, clinical data and biological samples of each subject will be prospectively collected. The study procedure is shown in figure 1.

### Eligibility criteria

The inclusion criteria for TM cases are as follows: (1) aged 18–45 years, (2) 4–8 weeks of gestation, (3) the blood  $\beta$ -human chorionic gonadotropin (hCG) result is positive, (4) the level of  $\beta$ -hCG is not consistent with the gestational age, (5) vaginal bleeding occurs with a closed cervix or abdominal pain, (6) ultrasonography suggests endometrial cavity fluid and (7) ultrasonography suggests a weak embryonic heartbeat. Of the above, the first three items all must be met, while  $\geq 1$  of items 4–7 must be met.

The study entry exclusion criteria for participants are as follows: (1) chromosomal abnormalities in either or both spouses; (2) suffering from endocrine diseases, autoimmune diseases, infectious diseases, inflammatory diseases of the gastrointestinal tract, inflammatory diseases of the reproductive tract, systemic inflammatory diseases or serious diseases of the vital organs (eg, brain, liver, kidney or blood system) or being diagnosed with anxiety and depression, anorexia nervosa, or mental disorders that leave the individual unable to cooperate; (3) the use of antibiotics, antifungal drugs, probiotic supplements, drugs that impair embryonic development or an enema within 3 months; (4) the occurrence of vaginal sex, vaginal or cervical topical medication, or vaginal irrigation within 48 hours; (5) current intrauterine device placement; (6) still in the last pregnancy or lactation period; (7) experiencing excessive vaginal bleeding (vaginal bleeding so much that women need to use sanitary pads (230 mm or more)) that may affect the quality of the sample; (8) refusal to be followed up and (9) refusal to sign the informed consent form.

Finally, the elimination criteria are as follows: (1) diagnosed with a type of vaginitis, such as vulvovaginal candidiasis and trichomonas vaginitis, via a vaginal discharge examination; (2) diagnosed with gestational trophoblastic



**Figure 2** Microbiome Characteristics in Early Threatened Miscarriage Study timeline overview of visits and sample collection. During visit 0 (pre-enrolment), we will inquire about demographic and medical history and perform physical examinations. Visit 1 will occur at 4–8 weeks of gestation and visit 2 will occur at 12 weeks of gestation. E2, estradiol, hCG, human chorionic gonadotropin; P, progesterone.

disease through pathological examination; (3) diagnosed with documented investigator-identified diseases that fall within the exclusion criteria after being included in the study and (4) loss of follow-up with an unclear pregnancy outcome.

## Power calculation and sample size

Determining the minimum number of participants required for clinical microbiome research is still a largely unresolved subject, and some scholars<sup>18</sup> have suggested performing the selection of a suitable metric of  $\alpha$ -diversity or  $\beta$ -diversity to estimate the sample size. Based on a statistical power of 0.8 and an  $\alpha$ -error of 0.025 to demonstrate a moderate effect size of 1.67<sup>12</sup> on vaginal microbiota  $\alpha$ -diversity, a sample size of 326 is required as calculated using EmpowerStats (X&Y Solutions, Inc., Boston, Massachusetts, USA). Further, based on a statistical power of 0.8 and an  $\alpha$ -error of 0.025 to demonstrate a moderate effect size of 0.48<sup>19</sup> on gut microbiota  $\alpha$ -diversity, a sample size of 236 is required as calculated via EmpowerStats. Therefore, the minimum calculated sample size ( $n=326$ ) was determined as the sample size for this study.

## Data collection

### Data collection timeline

Data time points and samples and the data collected at each time point are summarised in [figure 2](#) and [table 1](#). At each point of sample collection, participants will be given oral, written and graphical instructions.

**Table 1** Study design schedule

Timepoint	Study period		
	Visit 0	Visit 1	Visit 2
	Pre-enrolment	4–8 weeks of gestation	12 weeks of gestation
Enrolment			
Eligibility screen	X		
Informed consent	X		
Demography and medical history	X	X	X
Clinical measures			
Physical examination	X	X	X
Record concomitant medications	X	X	X
Ultrasonic measurement	X	X	X
$\beta$ -HCG, progesterone and estradiol testing	X	X	X
Pathological examination of the aborted material for miscarriages		X	X
Specimen collection			
Blood specimen collection		X	
Vaginal specimen collection		X	
Instruct subjects on stool collection and provide stool collection kit		X	
Obtain stool specimen		X	
Follow-up			
Follow-up to know early pregnancy outcomes			X
Laboratory processes			
Microbiota DNA extraction and quantification			X
16S rRNA gene sequencing			X
Data analysis			X

### Biological samples

Specimen collection methods will include blood sampling performed by medical professionals, vaginal swabbing performed by trained medical professionals, and self-collection of faeces samples.

Whole venous blood specimen sampling will be completed following the patient's outpatient visits or admission to the hospital, before the patient is prescribed clinical treatment by medical professionals. Ten millilitres of fresh whole venous blood will be collected in ethylenediaminetetraacetic acid-treated tubes for  $\beta$ -hCG, progesterone and estradiol testing. The blood samples will be delivered to the hospital laboratory and analysed by an automatic haematology analyzer (BM831, Baolingman Sunshine Technology Co. Ltd., Shenzhen, China).

Vaginal secretions will be sampled from the posterior fornix of the vagina by a trained gynaecologist using a transport medium (eSwab; Copan Italia Spa, Brescia, Italy). The collected samples will be delivered to the laboratory and placed in a refrigerator at  $-80^{\circ}\text{C}$ ; then, researchers will complete the sample collection registration form.

Faeces will be self-collected, and each subject will be offered a collection kit at their baseline visit for faeces collection, as well as oral, written and graphical instructions detailing the steps and precautions for sampling. For subjects from outpatient clinics, the sampling should be done within 24 hours preceding a re-visit and kept cool in the offered polystyrene box with ice packs (see details in online supplemental file 1: Instructions for self-collection of stool samples). For subjects recruited from the inpatient department, the sampling should be done within 24 hours after hospital admission. The collected samples will be handed over to the researchers, who will bring them back to the laboratory, store them at  $-80^{\circ}\text{C}$  in the refrigerator and complete the sample collection registration form.

### Baseline information and clinical data

Maternal demography and medical history will be recorded at each visit, including age (years), parity, education level, marital status, household income, smoking and drinking history, coffee intake during pregnancy, tea intake during pregnancy, pet exposure, medication exposure, pregnancy and delivery history, prior history, menstrual conditions, current medications and accompanying symptoms during pregnancy (eg, vaginal bleeding or abdominal pain).

### Physical measures

Physical measures of interest include height (m), weight (kg), body mass index, weight gain (kg) during pregnancy (assessed at 12 weeks of gestation), waist circumference (cm) and blood pressure (mm Hg).

### Exposure assessment, outcome variable definition and derived covariate assessment

Our primary exposure variables are the gut and vaginal microbiota (4–8 weeks of gestation). The primary

exposure measure is the  $\alpha$ -diversity. The secondary diversity measure is the  $\beta$ -diversity. The third exposure measure is OTU relative abundance. Pregnancy loss will be considered as the outcome variable. According to the American College of Obstetricians and Gynecologists guidelines 95 (1), pregnancy loss is defined as an intrauterine pregnancy loss occurring spontaneously at a time when the embryo or fetus cannot survive independently.<sup>20</sup>

Potential confounding factors that will be assessed as covariates when modelling associations between microbiota from the gut and reproductive tract and pregnancy outcomes include age,<sup>21</sup> body mass index,<sup>22–23</sup> parity,<sup>24</sup> education level,<sup>25</sup> marital status, household income,<sup>26</sup> pregnancy and delivery history,<sup>24</sup> prior history, menstrual conditions,<sup>27–28</sup> abdominal and vaginal bleeding, baseline level of  $\beta$ -hCG, baseline level of progesterone,<sup>29–30</sup> baseline level of estradiol,<sup>30–31</sup> smoking and drinking history,<sup>21</sup> coffee intake during pregnancy,<sup>32</sup> tea intake during pregnancy,<sup>33</sup> pet exposure,<sup>34</sup> medication exposure 3 months before pregnancy, embryonic chromosomal abnormalities (for pregnancy loss group), weeks of gestation and different treatments for miscarriage prevention (including Chinese medicine, progestogens and Chinese medicine combined with progesterone).

### Microbiota DNA extraction, quantification and sequencing

Microbiota DNA will be extracted from the faecal and vaginal samples using the QIAamp DNA microbiome kit (Qiagen, Hilden, Germany). The whole genomic DNA of each sample will be extracted by the centrifugal adsorption column method, and the quality of DNA will be assessed by agarose gel electrophoresis (agarose concentration, 1.0%; voltage, 150 V; electrophoresis time, 20 min). We will select samples with clear gene banding, total DNA > 200 ng, DNA concentration > 10 ng/ $\mu\text{L}$ , OD<sub>260/280</sub> > 1 and OD<sub>260/230</sub> > 2 for sequencing library construction and sequencing.

The V3–V4 hypervariable region of the 16S rRNA gene will be used for PCR amplification.<sup>35</sup> Using a multiplex PCR reaction, we will first carry out PCR amplification with inner linker-specific primers, then carry out PCR amplification and purification with outer linker-specific primers for library construction. We will use Qubit V.2.0 to detect the library concentration and detect the main peak and fragment distribution of the library. We will follow the MiSeq user guide for on-board sample preparation using the Illumina MiSeq platform (Illumina, San Diego, California, USA) and MiSeq reagent kit V.3 (600 cycles PE) kit (Illumina). pair end flow cell and run pair end (2×300) standard sequencing procedures.

### Quality assurance and quality control

Quality assurance and quality-control systems will be implemented in accordance with standard operating procedures. The clinical collection site will establish internal quality-control systems to ensure that the protocols are implemented consistently.

### Data handling and record-keeping

All the clinical data will be recorded on case report forms and will be entered into an electronic database, YiEr DataWeb (<http://www.empowerstats.com/dataweb/>), which is an electronic record system responsible for the management, quality audit and reporting of research data.

### Bioinformatics and statistical analysis

#### Statistical analysis

Statistical analysis will be conducted at the end of the study using the EmpowerStats and R (The R Foundation for Statistical Computing, Vienna, Austria) statistical software programmes. We plan to compare variables between the ongoing pregnancy group and pregnancy loss group. For descriptive data analysis, standard distribution parameters, such as mean, median, range, count, proportion, SD, IQR and CI values, will be used to describe patients' characteristics. Continuous data will be analysed by t-tests/analyses of variance for normally distributed data but the Mann-Whitney U test for non-normally distributed data. For categorical variables, Pearson's  $\chi^2$  test or Fisher's exact test ( $n < 5$ ) will be applied. Moreover, univariate analysis will be performed to assess the association between  $\alpha$ -diversity (or  $\beta$ -diversity or OTU relative abundance) and miscarriage. In addition, non-adjusted and multivariate-adjusted models will be performed simultaneously to assess the association between  $\alpha$ -diversity (or  $\beta$ -diversity or OTU relative abundance) and miscarriage. Furthermore, subgroup analysis will be performed using hierarchical linear regression models. Interactions and modifications of subgroups will be assessed by the likelihood ratio test.

#### Bioinformatics analyses

The  $\alpha$ -diversity refers to the diversity of the microorganisms in the sample, indicating the richness and uniformity of the microbial species contained in the sample. The more microbial species, the more balanced the distribution of species and the higher the  $\alpha$ -diversity. The algorithm description of  $\alpha$ -diversity can be divided into three types: richness index, evenness index and diversity index, commonly including the Chao1 index, Observed\_OTUs, Shannon index, PD\_whole\_tree, Ace index and Simpson index. In this study, the Illumina MiSeq platform will be used to analyse the  $\alpha$ -diversity of the microbiome and generate the Shannon index, Observed\_OTUs, Chao1 index and PD\_whole\_tree results. Because the  $\alpha$ -diversity index does not conform to normality and groups are independent of each other, the Wilcoxon rank-sum test as a non-parametric statistical method will be used to analyse the difference in  $\alpha$ -diversity indices between groups.

$\beta$ -diversity analysis compares the differences in microbial communities between samples or groups. The matrix distance between samples is calculated using related software, and the  $\beta$ -diversity between samples is measured by the distance index. The higher the  $\beta$ -diversity, the greater the difference in the composition, abundance or

phylogeny of the microbial community among samples. The community structure index is generally used when performing  $\beta$ -diversity analysis, commonly including the Bray\_Curtis distance, Binaary\_jaccard distance, weighted Unifrac distance and unweighted Unifrac distance. This study intends to use the unweighted Unifrac distance and Binaary\_jaccard distance matrix to analyse the principal coordinates and select the most important eigenvalues with the greatest influence and contribution to show in the coordinates and create a map.

For a significance analysis of differences between groups, after marking the characteristic species between groups, we will detect their species abundance and obtain statistically different species through a series of statistical calculations, then perform a significant analysis of differences between groups to obtain the species with significant differences at each classification level, which will be displayed in various forms, such as an LDA value distribution histogram or species cladogram.

### Patient and public involvement

No patients were or will be involved in the design of this study.

### Ethics and dissemination

The authors are accountable for all aspects of the present work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study will be conducted in accordance with the amended Declaration of Helsinki (as revised in 2013), and the study protocol has been approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Traditional Chinese Medical University (ZYYECK[2020]051). The ability to provide oral and written informed consent will be made available to each included participant. To ensure privacy protection, all subjects will be assigned a unique anonymised identification code to be used for data and samples. Personally identifiable information will not be recorded during data exportation nor made public at any point. Dissemination of findings will occur through peer-reviewed journals, conferences and presentations.

### DISCUSSION

To our knowledge, the MCETMS is the first study to investigate the composition of the reproductive tract microbiome and gut microbiome in women with early TM. The goals of this cohort study are to identify specific microbial signatures of the gut and reproductive tract associated with early TM and explore the potential species from the gut and reproductive tract microbiota indicative of different early pregnancy outcomes. The expected results will increase clinicians' recognition of early TM, providing a new perspective for the clinical management of early TM.

New gene-sequencing techniques, such as metagenomic and culturomic analyses, have made it possible to characterise the human microbiota. Understanding the

physiological and healthy microbiological characteristics of pregnancy will contribute to recognising women at risk of obstetric and pregnancy complications and may correct imbalances in their microbiota and help to regulate the microbiota before or during pregnancy, such as by the use of targeted antibiotics, probiotics, phages, microbiota transplantation<sup>36 37</sup> and other new therapies. It may also be possible to achieve individualised treatment according to specific individual ecological disorders. The integrity of the ecosystem is suggested to be an important therapeutic target, and exploring the optimal composition and structure of micro-organisms in the human body has been a target for future research.

Limitations should be considered when carrying out this study protocol. First, all subjects in this study will be recruited in China, necessitating caution in the extrapolation of our results to populations with different demographic characteristics or in other areas. As such, we believe that further research should be performed to verify whether the results of our study are applicable to women outside of China. Further large-scale studies that include different ethnic groups are needed in the future. Second, the reproductive tract microbiome and gut microbiome can be affected by many different factors (eg, heredity, diet, obesity, number of sexual partners, medication exposure). However, we will offer a lifestyle questionnaire to each subject and collect their sociodemographic information to ensure homogeneity of the participants to reduce bias. Furthermore, we have also established a set of strict inclusion and exclusion criteria to control these disturbance factors. Third, our study will be conducted at only two centres. However, the First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine, a gynaecology clinic centre in the south of China, receives a large number of pregnant women every year, and the data have certain representation and advantages. Moreover, our study population spans 4–8 weeks of gestation, and an influence of differences in the levels of  $\beta$ -hCG, progesterone and estradiol at different gestational weeks on the gut and vaginal microbiota cannot be ruled out. Therefore, we need to conduct a stratified analysis of gestational age,  $\beta$ -hCG, progesterone and estradiol to identify whether there is an effect of each on the gut and vaginal microbiota.

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and record-keeping: SC, YG, XC, HZ and XH. Sample management, processing and analysis: YZ, SC, YG and XC. All authors have read and approved the final manuscript.

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**Competing interests** None declared.

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