Endometrial Origins of Stillbirth (EOS), a case–control study of menstrual fluid to understand and prevent preterm stillbirth and associated adverse pregnancy outcomes: study protocol

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ABSTRACT

Introduction Current research aimed at understanding and preventing stillbirth focuses almost exclusively on the role of the placenta. The underlying origins of poor placental function leading to stillbirth, however, remain poorly understood. There is evidence demonstrating that the endometrial environment in which the embryo implants impacts not only the establishment of pregnancy but also the development of some pregnancy outcomes. Menstrual fluid has recently been applied to the study of menstrual disorders such as heavy menstrual bleeding or endometriosis, however, it has great potential in the study of adverse pregnancy outcomes. This study aims to identify differences in menstrual fluid and menstrual cycle characteristics of women who have experienced preterm stillbirth and other associated adverse pregnancy outcomes, compared with those who have not. The association between menstrual fluid composition and menstrual cycle characteristics will also be determined.

Methods and analysis This is a case–control study of women who have experienced a late miscarriage, spontaneous preterm birth or preterm stillbirth or a pregnancy complicated by placental insufficiency (fetal growth restriction or pre-eclampsia), compared with those who have had a healthy term birth. Cases will be matched for maternal age, body mass index and gravidity. Participants will not currently be on hormonal therapy. Women will be provided with a menstrual cup and will collect their sample on day 2 of menstruation. Primary exposure measures include morphological and functional differences in decidualisation of the endometrium (cell types, immune cell subpopulations and protein composition secreted from the decidualised endometrium). Women will complete a menstrual history survey to capture menstrual cycle length, regularity, level of pain and heaviness of flow.

Ethics and dissemination Ethics approval was obtained from Monash University Human Research Ethics Committee (27900) on 14/07/2021 and will be conducted in accordance with these conditions. Findings from this study will be disseminated through peer-reviewed publications and conference presentations.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Collection of menstrual fluid is relatively non-invasive and simple for participants via a menstrual cup.
⇒ Use of validated menstrual survey will be correlated to both biological measures and clinical outcomes.
⇒ There is a small catchment area for recruitment due to time constraints related to sample transport and processing.
⇒ Classification into case groups may be challenging for some women who have experienced multiple adverse pregnancy events.

INTRODUCTION

The death of a baby is a devastating outcome of pregnancy. Current approaches to reduce stillbirth focus predominantly on care at the end of pregnancy1 and while gains are being made to reduce late stillbirth,2 85% of stillbirths actually occur in the preterm period (20–36 weeks’ gestation).3 Excluding congenital anomalies, the most common causes of preterm stillbirth are spontaneous preterm birth and placental insufficiency (eg, fetal growth restriction (FGR) and/or pre-eclampsia), accounting for over a third of all remaining preterm deaths.4 An additional 15% of stillbirths are considered unexplained.1 A better understanding of the causes and prevention of preterm birth and FGR, and the identification of other causes of stillbirth is therefore urgently needed. While the origins of both FGR and spontaneous preterm birth are multifactorial, they are largely considered to be placenta driven.5 6 7 The underlying origins of abnormal placental function however are poorly understood.

There is growing evidence to suggest that the endometrial environment, in which the
embryo implants, impacts not only on the establishment of pregnancy but also the development of pregnancy complications including spontaneous preterm birth, pre-eclampsia and FGR. It is well known that recurrent pregnancy loss is also associated with impaired decidualisation. More recently, endometrial biopsies from women who had developed severe pre-eclampsia in the past also demonstrated impaired structural and functional decidualisation of the endometrium.

Abnormalities in the decidua and genes that regulate decidual senescence have also been associated with spontaneous preterm birth. Uterine immaturity, characterised by endometrial progesterone resistance, altered estrogen-dependent growth and differences in cellular composition of the perivascular region is also thought to explain the increased rates of pre-eclampsia, FGR and preterm birth observed in pregnant adolescents. The role of decidualisation in stillbirth however has not been examined.

Menstrual cycle abnormalities have been associated with some adverse pregnancy outcomes. For instance, early menarche has been linked with higher risks of pre-eclampsia and preterm birth and self-reported heavy/irregular periods have also been linked with preterm birth. While the associations between menstrual characteristics and components of menstrual fluid have not been examined, menstrual fluid has recently been employed as a biological tool to better understand the endometrial environment. Menstrual fluid can be collected non-invasively from women using a menstrual cup and contains many factors including tissue fragments, endometrial stem/progenitor cells, immune cells and secreted proteins derived from the endometrium. Cellular and protein parameters of menstrual fluid have minimal variability across multiple cycles, thus one sample may be representative of a woman’s usual cycle.

Menstrual fluid from women who have unexplained infertility has been identified to have altered immune cell proportions and differential secretion of decidualisation proteins compared with fertile women. Morphological and functional differences of endometrial stem cells derived from menstrual fluid have also been demonstrated in women with endometriosis, a condition associated with painful, heavy menstrual cycles and infertility. Menstrual fluid has not been examined in women who have had a preterm birth/stillbirth or placental insufficiency and thus offers a novel tool to assess the endometrium in women who have experienced later adverse pregnancy outcomes.

The aims of this study are:

1. Characterise differences in endometrial cells, immune cells and proteins that are associated with decidualisation and endometrial receptivity, assessed via menstrual fluid in women who have experienced a preterm birth or stillbirth, late miscarriage, placental insufficiency, compared with those who have not.
2. Validate the association between menstrual fluid composition and characteristics of a woman’s menstrual cycle and,

3. Compare differences in characteristics of a woman’s cycle, in women who have experienced a preterm birth or stillbirth, late miscarriage, placental insufficiency, compared with those who have not.

METHODS AND ANALYSIS

Study design

This is a case–control study.

Study setting

Women living in Victoria, Australia, will be invited to participate.

Participants

Women aged 18–40 years who are currently menstruating and who have had a singleton pregnancy between 12 and 40 weeks’ gestation in the previous 3 years with a pregnancy that meets either the case or control definitions are invited to participate. Women must be willing to wear a menstrual cup. Women will be excluded from participating if they are currently using hormonal contraception, have a clinically diagnosed endometrial pathology (eg, endometriosis, endometrial cancer or endometrial submucosal fibroid), have had an endometrial ablation or if the pregnancy was a result of assisted reproductive technology. Cases will also be excluded if their stillbirth was due to infection or chromosomal abnormalities, from a multiple pregnancy or if the gestation of stillbirth/miscarriage is unknown. Cases and controls will be matched according to maternal age group (within 5 years), body mass index (BMI) group and gravidity.

Case Definitions: There are four case groups.

Case group 1: preterm stillbirth, defined as antepartum or intrapartum stillbirth between 20 and 36+6 weeks’ gestation.

Case group 2: spontaneous preterm birth, defined as live birth of a baby 20–36+6 weeks’ gestation.

Case group 3: late miscarriage, defined as spontaneous loss of a fetus between 12 and 19+6 weeks’ gestation.

Case group 4: placental insufficiency defined as a pregnancy complicated by FGR (<3rd centile at birth according to population charts) and/or pre-eclampsia.

Control definition: term (37–42 weeks’ gestation) live birth of an appropriately grown infant. Appropriately grown is defined as above the 10th centile of weight for gestational age at birth according to population-based charts.

Recruitment and study procedures

Recruitment will be undertaken via social media and advertised through relevant advocacy organisations. After expressing interest via the study contact email, a patient information and consent form is provided. A screening phone call is then arranged, and participants are encouraged to ask any questions about the study while screened for eligibility.

After providing informed consent, participants are provided with a menstrual fluid collection kit including
a menstrual cup, a collection tube, a small freezer pack and the menstrual history survey. The kit will also contain instructions on menstrual cup use and how to contain the sample for transportation back to the laboratory. Participants are requested to provide their completed survey with their first sample. They retain the same menstrual cup for use and are provided with a new collection kit prior to subsequent collections.

On day 1 of menses, the participant contacts the researcher to arrange a convenient collection location and time. On day 2 of menses, participants are asked to insert a menstrual cup an hour after waking and undertaking activities (eg, breakfast, shower) in the morning to collect their menstrual fluid for 4–6 hours and transfer the sample to a collection tube. The sample will then be collected and transported on ice to be processed in the laboratory for each of the respective exposure measures according to standard protocols. Up to five menstrual fluid samples from different menstrual cycles will be collected per participant. This recruitment and participation process is summarised in figure 1.

**Primary exposure measures**

1. Cellular composition: Cell types present will be determined using a multicolour Flow Cytometry panel. Analysis will focus on endometrial cell subpopulations (epithelial progenitors, mesenchymal stem cells and mature epithelial and stromal cells) which have a role in endometrial receptivity and quality.

2. Immune cell composition: endometrial tissue leukocyte subpopulations (uterine natural killer cells, mast cells, T cells, macrophages, neutrophils and dendritic cells) are essential to decidual transformation of the endometrium and will also be determined using multicolour flow cytometry.

3. High-throughput proteomics: composition of proteins secreted from the decidualised endometrium will be determined using standard non-targeted proteomic liquid chromatography–mass spectrometry (LC-MS/MS) approaches.

Menstrual fluid samples will also be banked and future experiments may be performed on freshly collected or banked samples based on the findings of these experiments.

**Secondary exposure measures**

Data about menstrual history will be determined via self-report. A study-specific survey has been developed drawing on questions from the Menstrual Disorders Questionnaire at the University of California, Los Angeles’ Department of Obstetrics and Gynaecology Patient History Questionnaire and the Growing Up Today Study Questionnaire. The parameters captured will include: age at menarche (years), last menstrual period (date), regularity (how often and predictability within 1 week), cycle length (days), period length (days), spotting, heaviness of flow, period pain, symptoms during menstruation, characteristics of period and interference of periods on lifestyle.

**Covariates/confounders**

Potential covariates and confounders will also be captured within the study-specific survey. Variables captured include:

1. Contraception history: Most recent means of contraception and contraceptive history timeline including duration (years), method of contraception and reason ceased.

2. Obstetric history: Obstetric history including gravidity, parity, diagnosis/cause of death (if stillborn) gestation at birth (weeks), sex of baby, pregnancy complications (such as gestational diabetes, pre-eclampsia), birth weight of baby (grams).

3. Reproductive health: reproductive conditions (polycystic ovary syndrome, fibroids, polyps, pelvic inflammatory disease, adenomyosis, blocked tubes, irregular ovulation, chlamydia/gonorrhoea, heavy menstrual/abnormal uterine bleeding, retroverted uterus, other), gynaecological surgeries which may alter the endometrium (hysteroscopy, ovarian cyst removal, dilation and curettage, other), iron infusion/tablets.

4. Medical history: family reproductive history (first-degree female relatives), other significant medical conditions (asthma, epilepsy, multiple sclerosis, thyroid disorder, cardiovascular disease, diabetes, cystic fibrosis, autoimmune disease, chronic renal conditions, thalassaemia, cancer, chronic infections, other), current medications and other investigations related to the period.

5. Sociodemographic data: maternal age (years), BMI, place of residence (metro/regional), country of birth, ethnicity, English proficiency, indigenous status and smoking status.

**Potential bias and limitations**

There is the potential that a sample collected several years after a pregnancy outcome does not reflect the endometrial environment in the past. However, previous studies assessing endometrial biopsies from women who developed pre-eclampsia up to 5 years prior to assessment demonstrated differences in endometrial composition when compared with women with past healthy pregnancies. As reverse causation is also possible, our menstrual survey will capture whether the characteristics of the woman’s menstrual cycle changed after pregnancy. We have chosen to restrict this study to a pregnancy up to 3 years to allow for the resumption of normal menstrual cycles and will match controls based on age, BMI and gravidity to ensure similarity in factors that may influence the endometrium. There may be other uncaptured confounders, which are outside the scope of this study, such as consideration of the vaginal microbiome. We have also shown consistency in menstrual fluid composition across five cycles. There is the possibility that women may have an undiagnosed endometrial pathology,
EOS Recruitment and Participation Process

**Social Media Advertisement**
Social tiles shared to social media platforms to advertise EOS study eligibility and information.

**Expression of Interest**
Potential participant from population contacts researchers.

**Screening and Consent**
Researcher provides participation information and consent form and completes screening call to confirm eligibility.

**Menstrual Fluid Collection Kit**
Participant provided with kit: menstrual cup, instructions, survey and collection tube.

**Sample Collection**
Menstrual fluid collected 4-6 hours on day 2 of menses and transported on ice to laboratory. Repeat for up to 5 samples per participant.

Figure 1  Overview of EOS Study recruitment and participation process. EOS, Endometrial Origins of Stillbirth.
such as an endometrial polyp or fibroids. We will collect information from women about their normal menstrual cycle. It is likely that volumes collected from women will differ, however our current methods are able to accommodate small volumes and our human research ethics approval allows us to collect up to five cycles. The use of a menstrual cup may deter some women, although its popularity has markedly increased in recent years and with various models available, our team are skilled at assessing cup suitability for participants. As this is the first study of its kind, we are unsure of the power required to detect statistical significance, however, this study is hypothesis-generating to provide the rationale for future research.

Sample size and statistical analysis
This will be the first case–control study of menstrual fluid as an indicator for endometrial differences in women who have experienced poor pregnancy outcomes compared with those who have not. No formal power calculation has been performed, however, the study aims to recruit up to 15 participants from each of the five groups (ie, a maximum of 75). This sample size has been derived based on past recruitment and sample processing time feasibility. In 2020 alone in Victoria, there were approximately 232 preterm stillbirths (excluding congenital anomaly, termination of pregnancy and infections) thus there should be sufficient cases available to recruit 15 women who have experienced a preterm stillbirth in the past 3 years. Preterm birth, late miscarriage and FGR rates are more common than stillbirths, therefore the sample size should be achievable. The findings from the Endometrial Origins of Stillbirth Study will assist to inform and power future studies.

Characteristics of the women will be tabulated and compared using standard approaches. Differences in each of the exposure measures and covariates will be determined between the groups and between controls and all cases pooled using standard statistical approaches after testing assumptions. Benjamini-Hochberg false discovery corrections will be applied to address multiple testing. The association between menstrual history and menstrual fluid composition and with the pregnancy outcomes will be determined using regression approaches, adjusting for potential confounders. All statistical analysis will be undertaken using Stata/IC 17 for Mac.

Patient and public involvement
Participants were not asked or offered the opportunity to participate in the study design, however, one of the study investigators has a lived experience of preterm stillbirth. The researchers did consider the study requirements in relation to participant convenience and scheduled collection of all menstrual fluid samples at a location that was most convenient for the participants.

ETHICS AND DISSEMINATION
Ethical approval was obtained for this study on 14/07/2021 and will be conducted in accordance with the conditions of Monash University Human Research Ethics Committee (MUHREC, Project ID: 27900). The confidentiality of all the participant’s data will be strictly maintained by all researchers in line with national and local guidelines. Future use of samples will be in line with the ethics approval and if participants have consented to future use of their samples. A new ethics application or amendment would be made prior to any future studies reusing samples. Recruiting participants who have experienced a stillbirth or adverse pregnancy outcome may cause some distress; our consent materials also provide links to access support should women need it. Study outcomes will be disseminated at international conferences and published in peer-reviewed scientific journals. Lay reports will be made available to study participants on request at the completion of the project.

DISCUSSION
The majority of stillbirths and two-thirds of preterm births occur in the absence of any evident risk factors. Additionally, where stillbirths are due to poor placental function, the origins of that poor placental function are unclear. Using novel, minimally invasive sampling of menstrual fluid, differences in cellular and soluble protein composition in decidualised tissue fragments shed in the menstrual fluid of women who have experienced a stillbirth, preterm birth, late miscarriage or placental insufficiency will be characterised for the first time and associated to their menstrual cycle characteristics. This study will uncover new mechanisms underlying adverse pregnancy outcomes. It may also reveal the foundation of placental issues that lead to these adverse outcomes of pregnancy.

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Contributors MD-T, CEG and CEF conceptualised the study. All authors (KT, CEF, CEG, FC, KRP, BV, MD-T) were involved in study design. KT drafted the protocol manuscript for publication. All authors contributed to the editing and approval of the final manuscript. Participant recruitment and screening will be performed by KT. MD-T will oversee all ethical considerations. KT, under supervision of MD-T, CEG and FC will collect and analyse all data. All authors will be involved in data interpretation and manuscript preparation.

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