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mSep: investigating physiological and immune-metabolic biomarkers in septic and healthy pregnant women to predict feto-maternal immune health – a prospective observational cohort study protocol

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ABSTRACT

Introduction Maternal sepsis remains a leading cause of death in pregnancy. Physiological adaptations to pregnancy obscure early signs of sepsis and can result in delays in recognition and treatment. Identifying biomarkers that can reliably diagnose sepsis will reduce morbidity and mortality and antibiotic overuse. We have previously identified an immune-metabolic biomarker network comprising three pathways with a >99% accuracy for detecting bacterial neonatal sepsis. In this prospective study, we will describe physiological parameters and novel biomarkers in two cohorts—healthy pregnant women and pregnant women with suspected sepsis—with the aim of mapping pathophysiological drivers and evaluating predictive biomarkers for diagnosing maternal sepsis.

Methods and analysis Women aged over 18 with an ultrasound-confirmed pregnancy will be recruited to a pilot and two main study cohorts. The pilot will involve blood sample collection from 30 pregnant women undergoing an elective caesarean section. Cohort A will follow 100 healthy pregnant women throughout their pregnancy journey, with collection of blood samples from participants at routine time points in their pregnancy: week 12 'booking', week 28 and during labour. Cohort B will follow 100 pregnant women who present with suspected sepsis in pregnancy or labour and will have at least two blood samples taken during their care pathway. Study blood samples will be collected during routine clinical blood sampling. Detailed medical history and physiological parameters at the time of blood sampling will be recorded, along with the results of routine biochemical tests, including C reactive protein, lactate and white blood cell count. In addition, study blood samples will be processed and analysed for transcriptomic, lipidomic and metabolomic analyses and both qualitative and functional immunophenotyping.

Ethics and dissemination Ethical approval has been obtained from the Wales Research Ethics Committee 2 (SPON1752-19, 30 October 2019).

Trial registration number NCT05023954.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Changes in systemic immune health that occur in an uncomplicated pregnancy will be extensively investigated.
⇒ Physiological and alternative biomarkers in women with clinical feto-maternal sepsis will be evaluated to identify early markers of infection (including Sep3).
⇒ Immunophenotyping, immunoassays, integrated transcriptomics, lipidomics and functional analysis will be performed during the pregnancy journey and in cases of suspected maternal sepsis to identify host response patterns for discriminating various infections (bacterial, viral, fungal) and to identify new avenues for treatment.
⇒ This is a simultaneous study with neonatal sepsis (ongoing, nSep, NCT03777670), with linking of data between the mother–infant dyad.
⇒ All patients with suspected sepsis are identified early and immediately treated with antibiotics, so most cases of sepsis are expected to be mild.
⇒ There is no follow-up plan to investigate convalescence.

INTRODUCTION

Maternal sepsis is a life-threatening condition defined as dysregulated host response and organ dysfunction resulting from infection during pregnancy, childbirth, postabortion

or postpartum period.[1] Due to the physiological, immunological and mechanical changes during pregnancy, women are particularly predisposed to developing infection and sepsis, particularly during the peripartum period.[2] Physiological adaptations to pregnancy obscure signs and symptoms of infection and sepsis,[3] which may delay recognition and treatment.[1] Maternal sepsis remains an important cause of maternal death and morbidity in the UK, even after improvements made by the introduction of the Modified Early Obstetric Warning Score[4] and the Royal College of Obstetricians and Gynaecologists' Maternal, Newborn and Infant Clinical Outcome Review Programme report. Additionally, the impact of maternal sepsis is not only on the mother but also on the fetus, with a proportion of early neonatal sepsis originating from intrauterine and antepartum maternal infections.[5] Consequently, feto-maternal infection can result in sepsis before parturition, where early diagnosis is crucial for a successful management. The definition of sepsis from the Sepsis-3 convention[6] (Sequential Organ Failure Assessment (SOFA) score >2) cannot be applied to feto-maternal sepsis because the fetus may have an infection which is not reflected in maternal SOFA scores. Feto-maternal infection may lead to severe sepsis in the mother or the infant; therefore, when studying sepsis in pregnancy, all aspects of feto-maternal health and early treatment of both the mother and the child must be considered.

Current knowledge of physiological and immune parameters in pregnancy

It is understood that physiological changes of pregnancy overlap with haemodynamic changes associated with the initial presentation of sepsis.[7] An example of this is tachycardia, which can occur due to pain and effort in the second stage of labour as well as the host response to infection.[3] Systematic reviews looking at physiological parameters have found that the normal ranges for physiological parameters during pregnancy and immediately post partum substantially overlap with the systemic inflammatory response syndrome (SIRS) criteria.[1] SIRS consists of physiological changes that are associated with underlying infection,[8] but heart rate and respiratory rate during normal pregnancy can meet the criteria for SIRS, thus reducing its specificity and use in the diagnosis of sepsis. Temperature is also an SIRS criterion which can be complex to interpret during labour, particularly in women who have epidural analgesia due to an associated maternal hyperpyrexia.[9] The presence of a raised fetal heart rate can be multifactorial but is also a recognised feature of sepsis and is therefore incorporated into maternal sepsis pathways.

Our knowledge of immunology in pregnancy has been largely shaped by concepts extrapolated from studies in non-pregnant models.[10] It is understood that in pregnancy there are subtle but functionally significant changes in maternal immunity and metabolism which allow the body to accept and support the pregnancy while still retaining the ability to protect the mother and the fetus against pathogens.[11] Previously it was thought the maternal immune system changes towards a state of tolerance or suppression, but more current understanding describes a more complex modulation of the immune system and its functional links with metabolism.[12] Importantly, we do not know how these changes affect sepsis risk and identification.

Current approach to diagnosing and treating sepsis

Currently sepsis is diagnosed by incorporating clinical features, examination, physiological parameters and later investigations. The presence of one or more clinical features suggestive of sepsis (online supplemental appendix 1) elicits prompt clinical attention,[13] and a thorough history and examination is obtained from the woman and the local clinical algorithm followed. There is no rapid, accurate diagnostic bedside test that can be used to accurately identify maternal sepsis and therefore the diagnosis is predominantly clinical, with the addition of laboratory blood tests (white blood cell count (WBC) and C reactive protein (CRP)) to support clinical diagnosis[14 15] (online supplemental appendix 1). The only point-of-care test currently available in routine clinical practice is serum lactate.[16] All other blood samples have a delay of at least an hour and a raised lactate may occur in non-infective situations, including dehydration, maternal effort during labour and reduced clearance.[17] WBC is used as a biomarker of sepsis outside of pregnancy; however, elevation in WBC is a normal finding during pregnancy, making this parameter less useful.[18 19] CRP is used to help in the diagnosis of sepsis, but this also has limitations including a marked rise postsurgery (caesarean sections) and in non-infective inflammation, making infection hard to distinguish and reducing its specificity as a biomarker. Procalcitonin (PCT) is another host response biomarker used for detecting inflammatory reactions and may have clinical use in identifying maternal sepsis,[20] although baseline levels of PCT are elevated in pregnancy and are notably higher post partum.[21] A raised fetal heart rate may indicate feto-maternal infection[22 23]; however, this can be a non-specific change that requires further investigation.

If there is suspicion of sepsis, administration of intravenous broad-spectrum antibiotics is recommended within 1 hour[24] according to local microbiology policy[25] (online supplemental appendix 2). In addition, the source of sepsis should be sought and treated as soon as possible. In cases of chorioamnionitis (infection of the membranes that surround the fetus), the delivery of the infant will remove the source of infection.[26] A major problem with commencing antibiotics on suspicion of sepsis is the potential for feto-maternal exposure to unnecessary antibiotics. The rate of inappropriate antibiotic prescriptions in the hospital setting is estimated at 30%–50%.[27] Antibiotics have been associated with allergic reactions, gastrointestinal disturbances, cardiac arrhythmia and death,[28] and have been linked to an increased rate of necrotising enterocolitis observed in preterm infants whose mothers

were given co-amoxiclav in pregnancy.[29] Another major problem associated with antibiotic overuse is the development of multiresistant bacteria, as evident in the treatment and prophylaxis of group B streptococcus.[30]

The final diagnosis of sepsis in the maternal unit is decided once microbiological and histological data are analysed alongside the clinical parameters. Microbial growth on placental/vaginal swabs is assessed for potential pathogens; however, this is time-consuming, taking days to weeks for results to be available. Pathogens may be missed by this method; however, 'culture positive' microbiological samples provide good evidence of infection and are used to diagnose sepsis in other scenarios. In addition, conditions such as chorioamnionitis can be confirmed with tissue pathology in placental histology samples. It is unlikely that pathology such as chorioamnionitis is aseptic and therefore this can be considered evidence of an infection; however, this process can also take weeks or even months.

Using host gene expression networks as a biomarker of sepsis

Changes in systemic host gene expression can occur presymptomatically in response to infection, with the continuous interaction between blood and tissue allowing blood cells to act as biosensors for the changes.[31 32] Our group performed the first feasibility studies of using genome-wide RNA analysis as a methodological approach to identifying host biomarkers of sepsis in early life.[33 34] Despite patient heterogeneity, we identified a 52-node dual biomarker network comprising three pathophysiological immune and metabolic pathways (Sep3) that had greater than 99% accuracy for detecting bacterial infection, with 100% sensitivity achieved when including metabolic biomarkers, showing superior performance to previously characterised markers.[35] Furthermore, the specific combination of biomarkers allowed the detection of neonatal sepsis in samples which had displayed blood culture-negative microbiology results, illustrating the specific diagnostic benefits of biomarker combinations. Our more recent investigations have indicated that Sep3 has predictive power in detecting sepsis in children and adults. It is not known whether Sep3 has a predictive value in maternal sepsis. mSep was designed to prospectively measure such biomarkers in a healthy pregnant population and women thought to be at high risk of sepsis from clinical features, microbiological data and histological conclusions.

METHODS AND ANALYSIS
Hypothesis and specific aims

We hypothesise that integrative analytical measurements of networked immune and metabolic pathways that selectively change in infection can accurately identify feto-maternal sepsis and discriminate between bacterial, viral and fungal infections. Our specific aims include the following:

► Evaluation of the effectiveness of physiological parameters in predicting maternal sepsis.
► Evaluation of the effectiveness of routine laboratory and alternative biomarkers in diagnosing maternal sepsis, including Sep3.
► Investigation of the systemic immune health of women undergoing an uncomplicated pregnancy and labour.

Study design

Figure 1 graphically outlines the study design, showing the inclusion criteria, number and sample collection. Exclusion criteria include women under 18 years and those who have not consented or withdraw consent.

Pilot study

A pilot study will assess the feasibility of patient enrolment, sample storage and processing and investigate the physiological and transcriptomic parameters, which may include the use of these samples for refined power calculations and as baseline controls. We aim to recruit 30 women over 18 years old undergoing an elective caesarean section at the University Hospital of Wales and will obtain data including physiological parameters and study blood tests. This will require no additional venepuncture, and study blood samples will be taken at the time of routine sampling. The pilot study data collection period will run for 2 months. Data will be used to provide a source of control samples for cohort B, where sepsis occurs in late third trimester (but not in labour). Data can additionally be used to evaluate blood transcriptome differences between elective caesarean section and labour.

Main study

Following the successful completion of the pilot study, two cohorts of women will then be studied:

► Cohort A will follow 100 women who consented at their first routine booking appointment and will continue in the study until 24 hours after delivery of the infant or until the end of a sepsis episode, whichever was the longest. Maternal physiological observations will be recorded and 5 mL of blood will be taken in addition to their routine booking blood tests (at 10–12 weeks) and 28-week blood tests. In this study, we will ask the participants to visit the University Hospital of Wales for these blood tests. During labour admission, a blood sample is routinely obtained for FBC (Full blood count) and blood group at our institution. An additional 5 mL blood sample will be obtained at this stage, as well as maternal physiological observations. In total each participant will provide three blood samples over the course of the study, resulting in 300 samples in total being collected for cohort A. If a woman in cohort A is admitted and placed on the sepsis pathway, additional sepsis study samples will be taken.
► Cohort B will study 100 women who are over 12 weeks gestation and present to the maternity unit with
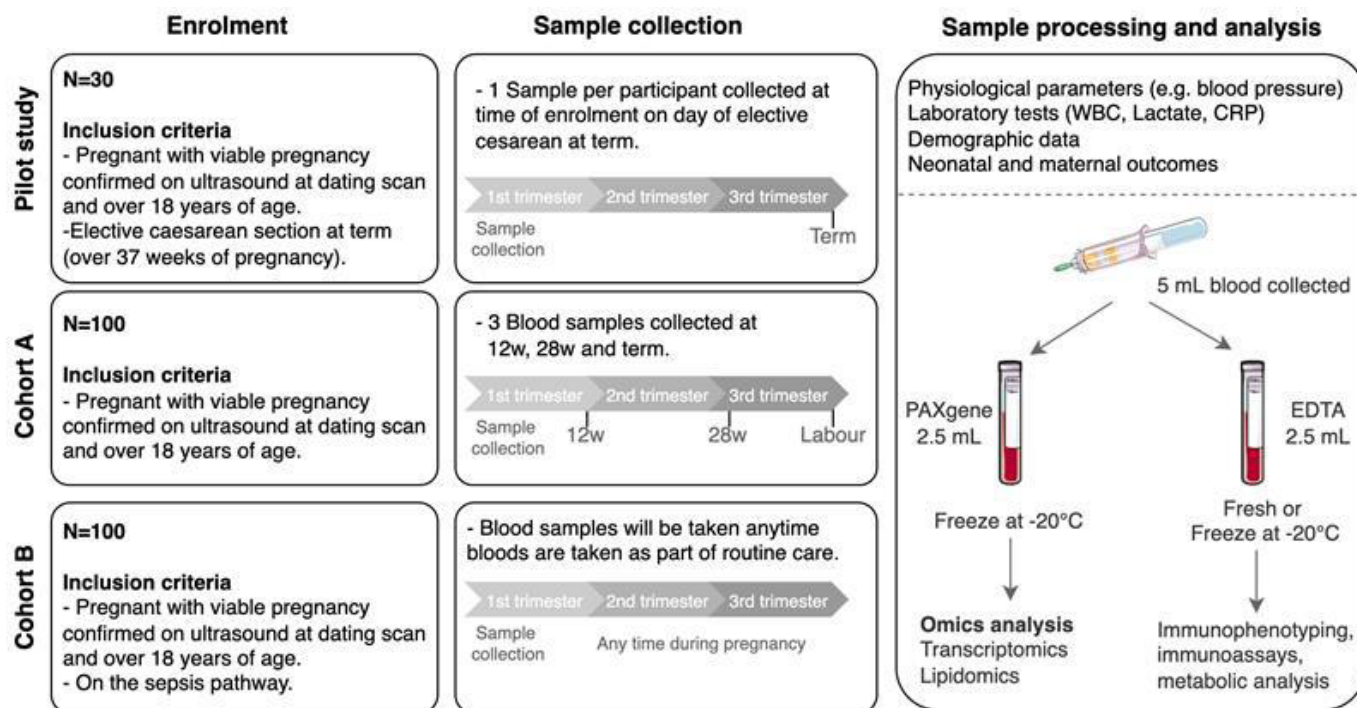
Figure 1 Study design. CRP, C reactive protein; WBC, white blood cell count.

suspected sepsis in pregnancy and labour. Suspected sepsis is defined by fulfilling either one high risk or two medium risk criteria (online supplemental appendix 1), which places a patient on the local sepsis pathway (online supplemental appendix 2). Deferred consent for study participation will be sought once the woman is well enough to do so, as per ethical permissions. Whenever a patient has blood samples taken (as part of their clinical care), a study blood sample will also be taken and routine physiological parameters recorded (shown in online supplemental appendix 3). A minimum of two research samples will be taken (at presentation and 24 hours after sepsis episode). Due to the complex nature of sepsis, more samples may be taken if clinically indicated, so the number of samples will vary based on the woman's clinical condition.

The University Hospital of Wales is a large tertiary hospital with approximately 6000 deliveries a year. Cohort A will run a recruitment period of 6 months, with another 6-month follow-up until the completion of pregnancy. Recruitment of participants for cohort B will also take place over this 1-year period. We anticipate that some candidates from cohort A (about 8%, based on local audit results) will be placed on the sepsis pathway and will therefore have additional study blood tests.

Clinical data and analysis

Detailed clinical data will be collected from participant notes. All trial data will be collected using bespoke clinical report forms (online supplemental appendix 3) and will be collated and anonymised into an electronic database. Data will be processed in line with the University and Health Board Data Management standard operating procedures using validated data management systems to ensure consistency, viability and quality of data. It will be stored in line with the Data Protection Act (2018).

Detailed clinical data will include the following:
► Demographic data (gestation, age, mode of delivery and sex).
► Medical history, including comorbidities (eg, diabetes).
► Past and current obstetric data (including chorioamnionitis, antenatal corticosteroids, intrapartum antibiotics and type of delivery).
► Infection history during current and previous pregnancies.
► Drug history and immunisation status.
► Sepsis pathway triggers.
► Haematological markers of infection at presentation (CRP, WBC, neutrophil count and lactate).
► Vital physiological monitoring data.
► Microbiological and histological results.
► Antimicrobials.
► Pregnancy outcome, including death, intensive care admission, requirement for neonatal antibiotics and neonatal sepsis observations.
► Maternal outcome including high-dependency or intensive care admission and death.
► Final diagnosis (in cases with suspected sepsis).
  – Sepsis confirmed—culture positive: the clinical, microbiological and histological data of patients recruited into cohort B and/or cohort A with a sepsis episode will be reviewed by at least two clinicians. Sepsis will be confirmed if culture-positive microbiological samples (confirmed as contributing to
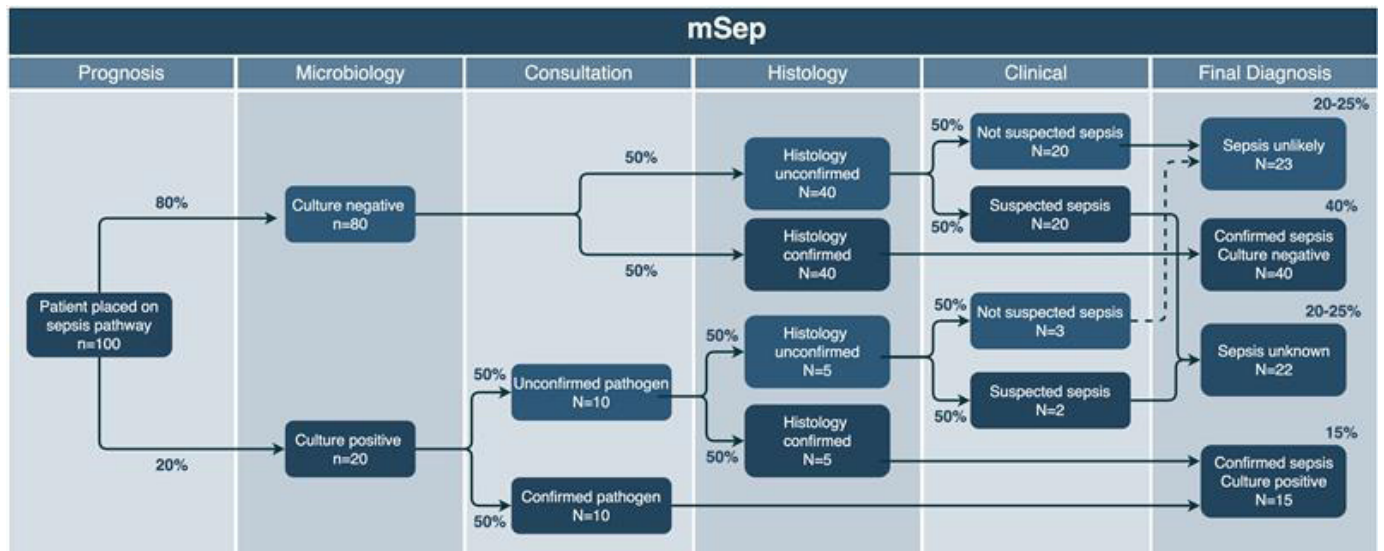
Figure 2 Stratification logic diagram showing the decision pathway and the estimated percentages and the number of patients expected in the four groups of the final diagnosis based on local audit data.

sepsis episode by a consultant microbiologist) support the clinical diagnosis of sepsis.

– Sepsis confirmed—culture negative: the clinical and histological data of patients recruited into cohort B and/or cohort A with a sepsis episode will be reviewed by at least two clinicians. Sepsis and the underlying cause will be confirmed if any of the following are met:
  – Positive placental histology.
  – Clinical course suggestive of sepsis with prescription of over 24-hour intravenous antibiotics and then converted to an oral course.
– Sepsis unknown: if the clinical course was unclear and intravenous antibiotics were given for less than 24 hours and an oral course prescribed, or intravenous antibiotics were given for over 24 hours and no oral course prescription.
– Sepsis unlikely: if another clear clinical diagnosis was identified or none of the previous criteria was met.

Histological analysis will be considered as evidence of infection; however, to align with studies of neonatal sepsis,[35] [36] microbiologically confirmed sepsis (culture positive) cases will be used to assess Sep3. Therefore, the stratification of cohort B patients will follow the logic outlined in figure 2, with four groups for final diagnosis: sepsis unlikely, sepsis unknown, sepsis confirmed—culture positive and sepsis confirmed—culture negative.

Sample size

Power analysis for this study has been restricted to testing Sep3 for maternal sepsis; other preliminary findings will be used to calculate power for future studies.

Cohort A

The main goal of cohort A is to be able to follow a pregnancy journey that then provides a reference interval for the Sep3 biomarkers in this population. In addition, women who have an episode of suspected sepsis will also be opportunistically captured, with approximately 8% of women anticipated to have suspected sepsis during labour (based on local audit data). The dropout rate for participants recruited into cohort A is estimated to be 25% due to potential missed blood samples (since the timing of labour is unpredictable and appointments may be missed/redirected to other healthcare settings). Microarray studies of whole blood have shown that 30–50 women are sufficient to detect differential gene expression.[37] However, RNA sequencing data yield greater complexity (and associated heterogeneity), with the capability for a more comprehensive expression analysis. We therefore aim to enrol 100 women into cohort A.

Cohort B

Power calculations on Sep3 indicate that for 95% power to detect differences observed between sepsis and control (alpha 0.01, minimum effect size 1.74), 14 recruits are required.[35] We anticipate that most women placed on the sepsis pathway will not have microbiological culture-positive confirmed sepsis (figure 2), and so to reach at least 14 patients we aim to recruit 100 women.

Sample processing

Blood samples will be taken during routine venepuncture and handled as per local standards and protocols. Storage will be appropriate to the specific tests and will include refrigeration and freezing. The sample storage and handling process will be validated during the pilot testing. Once signed consent is obtained from the patient, the sample will be analysed by the study team. If consent is declined, then the sample of blood will be discarded.

The 5 mL blood samples in both cohort A and cohort B will be subdivided equally into two collection tubes. The first tube is a PAX gene (Qiagen) vacutainer tube for

extraction and stable storage of RNA at −20°C. This tube will be used primarily for both RNA sequencing analysis (to validate the biomarker network) and lipidomic analysis. The second tube will contain EDTA and will either be frozen at −20°C or opportunistically taken fresh for more indepth analysis (within 24 hours). In the case of the latter, peripheral blood mononuclear cells (frozen in 10% dimethyl sulfoxide) and plasma will be collected for further immunoassays.

Statistical analysis plan

Each physiological parameter (routinely collected) and laboratory test (including the transcriptomic, immunological and metabolic plans in the next paragraph) will be tested individually and in combination to determine their accuracy in predicting our cases of confirmed sepsis (eg, positive predictive values, relative risk). In collaboration with nSep,[36] we will test whether maternal parameters or limited fetal parameters, such as concerns regarding fetal heart rate on cardiotocography, can predict neonatal sepsis.

Transcriptomic data processing and analysis plan

The main aim of the transcriptomic analysis will be to validate the ability of Sep3 to accurately predict bacterial maternal sepsis. The secondary aim will be to define changes in systemic immune health throughout the pregnancy journey.

RNA will be extracted from the PAX gene/blood solution and globin mRNA will be depleted before RNA samples are analysed by single-cell RNA sequencing. Paired-end reads from Illumina sequencing were trimmed with Trim Galore[38] and assessed for quality using FastQC,[39] using default parameters. Reads will be mapped to the reference genome (downloaded from the Ensembl FTP site) using STAR[40] and counts assigned to transcripts using feature-Counts[41] with the (GRCh38.96) Ensembl gene build GTF. Differential gene expression analyses will use the DESeq2 package[42] applying Benjamini-Hochberg correction for multiple hypothesis testing. Further data processing and analysis will be performed with R Language and Environment for Statistical Computing,[43] followed by quality control and data processing using Bioconductor packages.[44] Additionally, InnateDB[45–47] and STRING[48] will be used to examine the biological network relationships and the association with known pathways (REACTOME[49 50]) among differentially expressed genes. For classification a variety of machine learning and statistical pathway biology approaches, for example as described in Smith et al,[35] will be used. Pathway analyses will be carried out stepwise using a pathway biology approach, becoming more focused.

Immunological phenotyping and metabolic analysis

Immunological and metabolic assays are designed to discover new pathophysiological drivers and to test novel biomarkers that can accurately predict maternal and feto-maternal sepsis. Collectively, these investigations will

define changes in systemic immune health throughout the pregnancy journey. As with Sep3, these parameters can be compared with current clinical parameters for diagnosing maternal sepsis.

For lipidomic analysis, lipids will be extracted from PAX gene/blood solution via methanol precipitation and analysed by targeted reverse-phase lipidomic methods on high-pressure liquid chromatography/tandem mass spectrometers. Fresh EDTA samples will provide an opportunity for indepth immunophenotyping by multiparameter flow cytometry (eg, CD3, CD4, CD8, CD14, CD15, CD16, HLADR, CD123, CD10) along with functional analysis, such as reactive oxygen species production and metabolic profiling (by flow cytometry; eg, mitochondrial membrane potential). The Attune NXT analyser (ThermoFisher) will be used for data acquisition and analysed by FlowJo (BD Life Sciences). Plasma will be used for further immunoassays, including cytokine quantifications by ELISA (eg, interleukin (IL) 6 and IL-10) and investigation of other immune-signalling molecules. Frozen EDTA samples can be used for other immunoassays such as complement quantification by ELISA (eg, C3a, C5a) and additional targeted lipidomic assays (medium chain fatty acids).

Patient and public involvement

Patients and advisory groups were directly involved in the planning and preparatory stages of this research study and study results will be disseminated through sepsis advisory groups.

ETHICS, CONSENT AND DISSEMINATION

Ethical approval has been obtained from the Wales Research Ethics Committee 2 (SPON1752-19, 30 October 2019). Participants in cohort A and the pilot study will be given detailed written information regarding the study and time to review this prior to obtaining written consent. Participants in cohort B will be consented by a health professional who has been trained in gaining study-specific consent once the woman is well enough to do so. Samples will be securely disposed of according to local protocol if consent is not obtained or withheld.

Results will be disseminated through national and international presentations at scientific and clinical meetings and publications in peer-reviewed journals. The study has been registered at ClinicalTrials.gov (trial registration number: NCT05023954).

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