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Microbiome Understanding in Maternity Study (MUMS), an Australian prospective longitudinal cohort study of maternal and infant microbiota: study protocol

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ABSTRACT

Introduction Pregnancy induces significant physiological and cardiometabolic changes, and is associated with alterations in the maternal microbiota. Increasing rates of prepregnancy obesity, metabolic abnormalities and reduced physical activity, all impact negatively on the microbiota causing an imbalance between the commensal microorganisms (termed dysbiosis), which may drive complications, such as gestational diabetes or hypertensive disorders. Considerable work is needed to define the inter-relationships between the microbiome, nutrition, physical activity and pregnancy outcomes. The role of the microbiota during pregnancy remains unclear. The aim of the study is to define microbiota signatures longitudinally throughout pregnancy and the first year post birth, and to identify key clinical and environmental variables that shape the female microbiota profile during and following pregnancy.

Methods and analysis The Microbiome Understanding in Maternity Study (MUMS) is an Australian prospective longitudinal cohort study involving 100 mother–infant pairs. Women are enrolled in their first trimester and followed longitudinally. Assessment occurs at <13+0, 20+0–24+6 and 32+0–36+6 weeks gestation, birth and 6 weeks, 6 months and 12 months postpartum. At each assessment, self-collected oral, vaginal and faecal samples are collected with an additional postpartum skin swab and breastmilk sample. Each infant will have oral, faecal and skin swab samples collected. Measurements include anthropometrics, body composition, blood pressure, serum hormonal and metabolic parameters and vaginal pH. Dietary intake, physical activity and psychological state will be assessed using validated self-report questionnaires, and pregnancy and infant outcomes recorded. Parametric and non-parametric hypothesis tests will be used to test the association between high-risk and low-risk pregnancies and their outcomes.

Ethics and dissemination The study received the following approval: South Eastern Sydney Local Health District Research Ethics Committee (17/293 (HREC/17/POWH/605). Results will be made available to the participants of MUMS, their families and the funding bodies; in the form of a summary document. Results for the greater maternity care community and other researchers will be disseminated through conferences, local, national and international presentations and peer-reviewed publications.

Trial registration number ACTRN12618000471280 (prospectively registered).

Strengths and limitations of this study

- ► Microbiome Understanding in Maternity Study (MUMS) will provide the first Australian study to longitudinally define the stool, oral, vaginal and skin microbiota throughout pregnancy and postpartum.
- ► Our longitudinal study design will allow us to look at changes over time in the same patient, defining the temporal sequence of changes and providing a stronger evidence for causality.
- ► Detailed maternal characteristics including anthropometric measures, nutritional intake, physical activity and serum biological markers will enable the identification of confounding factors that influence the microbiota.
- ► The mother–infant pair design and collection of birth, feeding method and infant developmental details will highlight factors influencing the developing infant microbiota.
- ► The primary limitation of this study is loss to follow-up and missing data points that would challenge internal validity of reported results from MUMS.

INTRODUCTION

Pregnancy is a period of complex, simultaneous physiological changes that are essential to facilitate the development of a healthy baby, including hormonal, metabolic, immunomodulatory and cardiovascular adaptations. Unfortunately, pathophysiological pregnancy

states are also becoming more common. Globally, 10% of women suffer from hypertension in pregnancy,[1] and one in seven pregnancies are affected by some form of glucose intolerance.[2] Increasingly, mothers are older and more overweight prior to pregnancy, with disorders such as diabetes and hypertension, both pre-existing and developing during pregnancy, leading to poorer maternal and fetal outcomes.[3]

The human microbiota, the population of microbes occupying various body sites, is thought to have an impact on human health through effects on metabolism, immunity and hormones.[4] Defining the optimal or healthy composition of microbes within the human body is a currently unresolved question. This was the aim of the Human Microbiome Project (HMP) launched in 2007.[5] Since then a rapid expansion in human microbiota studies in various geographic populations and disease states has occurred.[6–8] There is now increasing evidence that a change in microbiota signatures, often referred to as dysbiosis, is associated with many disease states.[9 10] However, understanding the interplay between the host, their environment and their microbiota is the key to understanding the degree to which the microbiota has a causal effect on disease process or in this study, pregnancy complications and infant microbiota composition.

Given the mother's or host's major physiological changes during pregnancy, it would be expected that significant adaptations will occur in maternal microbiota signatures within the body, including within faecal, oral and vaginal sites. Koren *et al*'s work suggested that the gut microbiota in the first trimester of pregnancy resembles that of the non-pregnant state, and the changes from the first to the third trimester appear to be marked.[11] These changes are predominantly characterised by increased abundance of Actinobacteria and Proteobacteria phylum members, and an increase in lactic acid producing bacteria, as well as a reduction in α-diversity.[11] The other significant change that has been identified is a reduction in *Faecalibacterium prausnitzii*, a butyrate-producing bacteria with anti-inflammatory properties. The proinflammatory state of the third trimester is reflected in increased microbial beta (ß)-diversity, weight gain, insulin insensitivity and raised faecal cytokine levels.[11] In people with metabolic syndrome, *Faecalibacterium prausnitzii* is similarly depleted.[12] More recent studies have also shown that diet[13] and overweight or obese body mass index status[14] also impact the gut microbial composition during pregnancy. Jost *et al*[15] demonstrated that the maternal gut microbiota remained stable from the last trimester of pregnancy through to 1 month postpartum, despite concurrent findings of altered metabolic activity and low-grade inflammation. This, once again, highlights the importance of larger longitudinal studies to elicit the timing of gut microbiota changes during pregnancy.

As with the gut, it has been widely shown that the vaginal microbiota undergoes changes during pregnancy,[16 17] including a significant reduction in bacterial species richness and enrichment of *Lactobacilli*.[16] A more recent study did not identify significant vaginal microbial changes during pregnancy,[18] although significant alterations in the postpartum microbial composition were found. Changes in the oral microbiome during pregnancy have also been demonstrated, with increased total viable microbial counts in all three trimesters of pregnancy when compared with the non-pregnant state.[19] Although the mechanisms that underlie these changing oral microbiota in pregnancy are unclear, associations have been made between pregnancy complications, particularly preterm birth, and periodontal disease and oral microbial dysbiosis.[20–22] Similarly, pregnancy complications have been associated with dysbiosis of the gut microbiota, as seen in newly diagnosed pre-eclamptic patients.[23]

In addition to the study of the maternal microbiota, the study of mother–infant pairs is pivotal in the understanding of the impact of the maternal microbiota on infant microbiota colonisation and development. Mueller *et al*[24] highlighted the ongoing need for this area of research. Necessary treatments and interventions in pregnancy such as antenatal antibiotic use and caesarean delivery impact on the maternal microbiota and appear to perturb the development of the healthy infant microbiome, while vaginal delivery and breastfeeding appear to be paramount to newborn development and long-term metabolic and immune function.[24] The impact of pregnancy on the baseline maternal microbial population has not been studied longitudinally in low-risk and high-risk pregnant women in the same geographical location. Additionally, detailed data that examine the inter-relationships between maternal history, physical examination, maternal nutrition, exercise, mental state, serological markers of cardiometabolic health, pregnancy and postpartum microbiome and pregnancy complications and outcome are lacking. The primary objective of the Microbiome Understanding in Maternity Study (MUMS) cohort is to analyse multisite microbial changes that occur over the course of a woman's pregnancy, how these change postpartum for the woman, how similar or dissimilar the neonatal and maternal microbial signatures are from birth to 1 year of life and the relationships between the maternal and infant microbiome and clinical, physical and nutrition/physical activity factors.

The specific aims of this cohort study are:

1. To establish what constitutes normal microbiota from early pregnancy to postpartum in the gut, mouth and vagina in Australian women.
2. To examine how the pregnancy microbiota differs in women with pregnancy complications; in particular, women with excessive gestational weight gain, hypertension and gestational diabetes.
3. Identify key/critical variables that shape the pregnancy and postpartum microbiota profiles.
4. To establish an infant population baseline of gut, oral and skin microbiota over the first 12 months of life and examine how neonatal/infant microbiota relate to: mode of birth; maternal microbiota; breastmilk

composition and the infant's health in the first 12 months of life.

METHODS AND ANALYSIS

Study design and setting

MUMS is a longitudinal prospective observational cohort study that includes mother–infant pairs followed from the first trimester of pregnancy through to 1 year postpartum in an Australian population. The cohort is recruited from women booking in for pregnancy care at St George Hospital in metropolitan Sydney, Australia. This hospital services a sociodemographically diverse population, with approximately 40% of antenatal patients born overseas.

Participant characteristics

Inclusion criteria are pregnant women (18 years or over) booking in for pregnancy care at the study hospital with a singleton pregnancy, under 13 weeks and 0 days gestation at the time of enrolment, and who have sufficient understanding of written and spoken English to complete the study questionnaires and procedures. Women are excluded if they do not meet the inclusion criteria, are pregnant with twins or higher order multiples, planning a home birth or if they are suffering from a major active mental illness or disability that precluded them giving informed consent. Exclusions after enrolment are pregnancies complicated by late miscarriage, stillbirth or fetal anomalies incompatible with life. Written informed consent is obtained from all participants, for each woman and their infant.

Data collection timeline

Data timepoints and the samples and data to be collected at each timepoint are summarised in figure 1. Participants are provided verbal, written and pictorial instructions for each sample collection.

Data collection

Clinical data

Maternal demographic and pregnancy information is recorded at each visit, and includes age (years), parity, prior history, current medications, exposure to antibiotics and development of pregnancy complications (such as gestational diabetes mellitus or hypertensive disorder of pregnancy).

Infant clinical data recorded includes anthropometrics (weight, length and head circumference), feeding method, exposure to medications, antibiotics, supplements and growth and development questionnaires.

Pregnancy outcome data are obtained from eMaternity (electronic maternity record), including complications (gestational diabetes mellitus and hypertensive disorder of pregnancy), length of labour, exposure to antibiotics, labour augmentation requirements and mode of birth.

Physical measures

Maternal physical measures include weight (kg), waist and hip circumference (cm), body impedance analysis (fat mass and fat free mass) and blood pressure (mm Hg).

Infant physical measures include Apgar Score, weight (kg), length (cm) and head circumference (cm).

Questionnaires

Maternal dietary intake is assessed using the validated Australian Eating Survey, an online food frequency questionnaire for an Australian population.[25]

Maternal physical activity is assessed using the International Physical Activity Questionnaire).[26]

Maternal mental health screen is assessed using the validated Edinburgh Depression Scale.[27]

Infant breastfeeding status and dietary intake is assessed using 'The study Infant Feeding Questionnaire' adapted from the Growing Healthy Trial.[28]

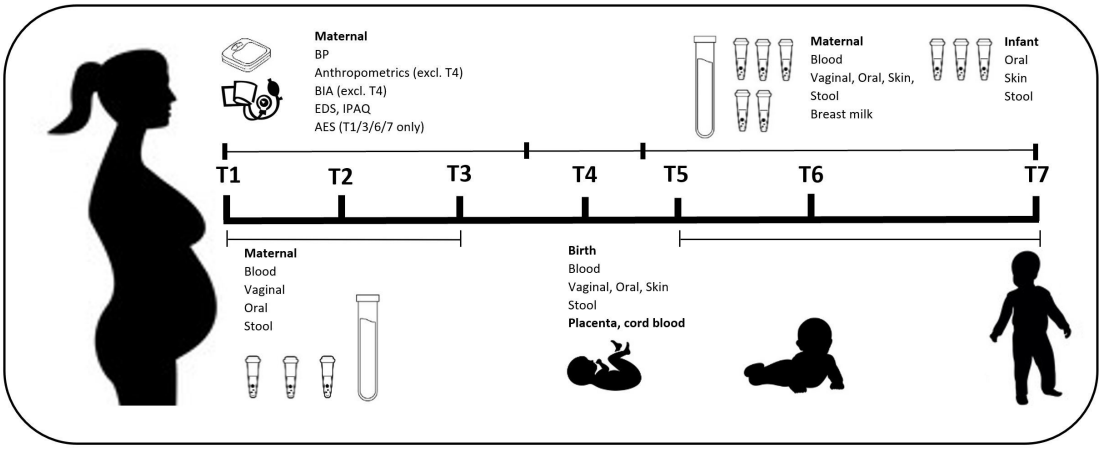

Figure 1 Microbiome Understanding in Maternity Study timeline of visits and sample collection. Maternal visits=T1:<13+0 weeks gestation, T2: 20–24 weeks gestation and T3: 30–36 weeks gestation. Maternal and infant visits=T4: birth, T5: 6 weeks postpartum, T6: 6 months postpartum and T7: 12 months postpartum. AES, Australian Eating Survey; BIA, body impedance analysis; BP, blood pressure; EDS Edinburgh Depression Scale; IPAQ, International Physical Activity Questionnaire.

Biological samples

Blood: Both plasma and serum venous maternal blood samples will be collected at each time point. Cord blood was also collected from cordocentesis following birth.

Breastmilk: Breastmilk samples will be collected from lactating postpartum participants from the 6-week visit onwards and stored at −80 °C.

Samples for microbiome analysis

At the seven designated time points, non-invasive faecal, oral and vaginal samples are self-collected by participants, and blood samples will be collected during their clinic visit. At the time of delivery, cord blood and placental samples will be collected and stored at −80 °C. In addition to the maternal samples collected during pregnancy, once birth occurs, additional skin (areola and cubital fossae) swabs and breastmilk samples will be collected. Faecal, oral and skin swab samples will be collected at four time points from the infant.

Faecal samples are collected using sterile ColOff catchment bags with samples placed in Stratec PSP Spin Stool DNA Plus Kit. Oral, vaginal and skin microbiome samples will be collected using Copan eNat sterile swabs and guanidine thiocyanate-based DNA stabilising medium. Once the samples are returned to the University of New South Wales Microbiome Research Centre (UNSW MRC) located at St George Hospital, the samples will be aliquoted and stored at −80 °C.

Primary outcome and covariate assessment

The primary outcome will be quantification and characterisation of the change in the microbial community composition and function of the mothers' gut microbiota from trimester one to trimester three of pregnancy.

The secondary outcomes are:

1. Identification of the maternal microbial community, diversity and function during pregnancy (gut, vaginal and oral) and for 1 year postpartum (gut, vaginal, oral, skin and breastmilk) for the entire cohort. Subgroup analysis; comparing low-risk and high-risk pregnancies and pregnancies with and without maternal complications of Gestational Diabetes Mellitus (GDM), Hypertensive Disorders of Pregnancy (HDP) and gestational weight gain.
2. Investigate the mother–infant microbial transmission and its relationship, for the first year of the infants' life.

Potential confounding factors that will be assessed as covariates when modelling associations between maternal and infant microbiota include:

- Pathological pregnancy outcome including HDP and GDM.
- Weight in the first trimester and total weight gain and body fat in pregnancy.
- Mode of birth.
- Maternal and infant diet, and maternal physical activity.
- Antibiotic exposure during birth.
- Breastmilk composition.

Other covariates included in the study are: maternal age, medications, recent illness, antenatal antibiotic or corticosteroid exposure, prior history and family history of diabetes and hypertension.

Microbiota DNA extraction, quantification and sequencing

DNA extraction from the faecal samples is to be obtained by the use of the commercial kit, the PSP Spin Stool Kit (Stratec, USA), with an enzymatic and bead beating step to enhance DNA recovery and concentration. DNA extraction from the oral, vaginal and skin samples are to be obtained using the commercial kit, QIAamp DNA Mini Kit (Qiagen, USA) following previously published methodology.[29] DNA concentration is measured using the Qubit 2.0 Fluorometer (Life Technology, USA). Bacterial quantitative PCR analysis of samples will be undertaken to confirm the presence of bacterial DNA, prior to sequence analysis. PCR primers (926F[30] and 1062R[31]), targeting total bacteria, will be performed using Quantstudio (Thermo Fisher Scientific) using SYBR Green chemistry (Roche). To rule out possible reagent and collection kit contamination, sample collection buffers and double distilled water will be included for DNA extraction, Qubit, qPCR and sequencing. Shotgun metagenomic libraries will be generated with the Illumina Nextera DNA Flex, sequenced on the NovaSeq 6000 Sequencing platform at the UNSW Ramaciotti Centre for Genomics.

Metabolomic analysis

From serum

Serum samples will be analysed for untargeted, then targeted metabolic profiling. This will be performed using liquid chromatography–mass spectrometry. The data acquired in the untargeted scan will be processed and analysed using established protocols.[32]

From stool

The PSP Spin Stool Kit allows targeted short chain fatty acid analysis; once the stool is homogenised and aliquoted and prepared, ultrahigh performance liquid chromatography is performed using established protocols.[33]

Human milk oligosaccharide (HMO) analysis

HMO identification in milk samples are carried out by the use of the high-performance anion exchange chromatography with pulsed amperometric detection analysis via Dionex BioLC system on the whey milk following centrifugation and removal of fat.

Maternal and cord blood profiling analysis

Immune parameters and cytokine profiles from maternal and cord blood serum and plasma samples will be analysed. Samples will be assayed for C-reactive protein, adipokines (adiponectin and leptin) and cytokines (IL-6, IL-10, IL-17 and TNF-α), all measured by ELISA. Angiogenic factors (sFlt-1, sENg and PIGF) will be assayed by immunoassay (Roche).

Power calculation and sample size

The aim of MUMS is to establish whether there are microbiota differences of sufficient magnitude between low-risk and high-risk pregnancies to justify further larger scale studies, including interventional trials. The sample size for MUMS is based on detecting a sizeable difference between groups in microbial β-diversity (evolutionary distance between species). Most available data in and outside pregnancy relate to gut microbiota (as opposed to oral or vaginal), and its possible links to cardiovascular and metabolic complications, thus differences in gut microbial β-diversity have been chosen for the basis of the sample size calculation. To demonstrate a moderate effect size of 0.3 on gut microbiota β-diversity of being in high-risk vs low-risk group by the time of third trimester sample, a total sample size of 82 (41 in high-risk and low-risk groups) is required with a power of 0.8 and alpha (α) of 0.05.[34] Given that the study of the microbiome in pregnancy is largely an unresolved subject, the decision to use a faecal microbial β-diversity metric for the power calculation is considered suitable for this exploratory clinical microbiome study in pregnancy.[35]

High-risk women included in recruitment were women with one or more of the following: a Body Mass Index (BMI)>30, previous GDM or hypertensive disorder of pregnancy.

In order to recruit and retain 82 women to the time of birth, initial recruitment of 100 women, expecting a 15% loss to follow-up over the course of pregnancy, was done.

Analytical methods

Metagenomic reads will undergo preprocessing prior to compositional and functional assignment. In brief, PCR duplicates will be removed from shotgun metagenomic reads that were using BBmap/clumpify.sh (Bushnell, BBMap). Low-quality metagenomic reads will be removed using fastp (V.0.19.5).[36] Sequence reads will be mapped against the human genome (GRCm38.p6) using minimap2 (V.2.16),[37] and human host sequence will be removed. Taxonomic compositional profiling will be performed using KrakenUniq (V.0.5.8).[38] The HMP Unified Metabolic Analysis Network (HUMAnN2 V.2.8.1) pipeline will be used for functional profiling of processed metagenomic reads. α-diversity metrics will be calculated from the resulting datasets using the otusummary[39] package within R (V.3.6.1).

Statistical analysis and data integration

A novel feature of this project is to describe the microbiome in this pregnant population. Descriptive statistics (mean and SD, median IQR, number and percentage) will be used to summarise the microbiota data according to distribution, and data visualisation tools will be used to graphically depict the data. Statistical analysis of the resulting microbial taxonomic, functional and diversity datasets will be conducted for the primary research aims using R (V.3.6.1).

Data management, ethical procedure and confidentiality

Procedures are taken to ensure confidentiality of the women participating in this study. Written and informed consent occurs at the first visit. No identifying information is recorded in the metadata exports, and all participants are assigned a unique anonymised identification code. Personal information will not be made public at any point. The participating women may withdraw consent for participating in the research at any timepoint.

Patient and public involvement statement

Three community representatives were on the St George Hospital Obstetric Medicine Research Committee from the time of the MUMS inception. Regular feedback about the structure of the proposed MUMS protocol was discussed in the monthly meetings, in order to ensure: acceptability of the measures to be taken, the usability of the surveys and samples to be collected and time commitment requirements. Once the results of the study are generated and analysed, the community committee members will be part of the discussion and decision-making regarding result dissemination to the participants, community and the wider research community.

DISCUSSION

The MUMS cohort will provide an in-depth examination of the microbiome throughout pregnancy and its evolution for the first year of life. While the study of the microbiome is evolving and becoming increasingly sophisticated in the laboratory, the clinical and public health importance worldwide is yet to be well defined.

This unique study will allow a comprehensive and longitudinal insight into multisite maternal and infant microbiome data using shotgun metagenomic analysis, and be the first to correlate these results with rigorous metadata collected by a single research team. Every woman participating in MUMS will be recruited from one location, St George Hospital, Sydney, Australia. The cohort will be physically and ethnically diverse, adding to the generalisability and reproducibility of the results.

We will examine whether changes in the composition of the maternal microbiome are associated with the development of abnormal pregnancy physiology and disease processes to a degree that is clinically meaningful and potentially suitable for future intervention trials.

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Contributors Study concept and design: AH, DS, EME-O, GLH, GD, MEC, GM, AJO'S and KH. Drafting of the manuscript: DS, EM, AH, KH and EME-O. Recruitment of the participants, collection of samples and record keeping: DS, Michelle Bai, AJO'S and LMR. Sample management, processing and analysis: GLH, DS, EM and KH. All authors read and approved the final manuscript.

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Disclaimer SSMRF and RANZCOG have neither direct role in the design of this study nor the data collection, analysis or interpretation of the data or manuscript writing.

Competing interests None declared.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

Patient consent for publication Not required.

Ethics approval Ethical approval was granted by the South East Sydney Local Health District Human Research Ethics Committee (SESLHD HREC); registration number HREC/17/POWH/605 on the 9 January 2018. The study was prospectively registered with the Australian and New Zealand Clinical Trials Registry Number: ACTRN12618000471280. Written informed consent is obtained from all participants prior to enrolment in the study.

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