**CUT umbilical cord milking to prevent Encephalopathy in infants with prenatal drug exposure – the CUTE Project**

CUTE Project

**Sponsor**

The Royal Hospital for Women, Randwick

**PROJECT TEAM ROLES AND RESPONSIBILITIES**

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Name: Professor Ju-Lee Oei

Position: Senior Neonatologist

Institution: Royal Hospital for Women, Randwick NSW

Responsibilities: Principal Investigator and Clinical Lead SESLHD

Principal Investigator - South Western Syndey Local Health District:

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Position: Clinical Academic, Obstetrics - Gynaecology

Institution: Liverpool Hospital, Liverpool NSW

Responsibilities: Clinical Co-Lead SWSLHD

Associate Investigator:

Name: Dr Jon Hyett

Position: Head of Maternal and Foetal Medicine

Institution: South Western Sydney Local Health District

Responsibilities: Clinical Co-Lead SWSLHD

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Position: Conjoint Senior Principal Scientist

Institution: Neuroscience Research Australia (NeuRA), Randwick NSW

Responsibilities: MRI specialist

Associate Investigator:

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Position: Senior Lecturer

Institution: University of New South Wales (UNSW) School of Psychology, Randwick NSW

Responsibilities: Biological sample processing and analytics

Associate Investigator:

Name: A/Prof Keith Chee Ooi

Position: Associate Professor of Medicine and Consultant Paediatric Gastroenterologist at the Sydney Children’s Hospital (Randwick)

Institution: University of New South Wales (UNSW) School of Psychology, Randwick NSW

Responsibilities: Stool sample processing and bioinformatics

Associate Investigator:

Name: Patricia Symons

Position: Pathologist at the Department of Anatomical Pathology, Liverpool Hospital

Institution: Liverpool Hospital, Liverpool NSW

Responsibilities: Placental sample processing and analysis

Associate Investigator:

Name: A/Prof Atul Malhotra

Position: Neonatologist at Monash Children’s Hospital and Monash University, Melbourne

Institution: Monash University

Responsibilities: Extensive pre-clinical and clinical expertise on the impact of cord milking on cell fractions of cord blood

**Additional collaborators and contributing services**

NSW Health Pathology Services

- Royal Hospital for Women, Randwick: SEALS Pathology

- Fairfield, Bankstown-Lidcombe and Liverpool Hospitals: South West Pathology Service (SSWPS)

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**NOTE**: SWSLHD recommends getting a Peer Review prior to submitting the ethics application and disclosing any conflicts of interests in the protocol. Please see the [*Peer Review Guide and the Disclosure of Interests and Management of Conflicts of Interest Guide, which support the implementation of the Australian Code for the Responsible Conduct of Research (the Code).*](https://www.nhmrc.gov.au/about-us/publications/australian-code-responsible-conduct-research-2018?utm_source=Release+of+Peer+Review+Guide+and+Disclosure+of+Interests+and+Management+of+Conflicts&utm_campaign=9a5a43c870-EMAIL_CAMPAIGN_2019_08_28_03_25&utm_medium=email&utm_term=0_e66256a329-9a5a43c870-64999547https://www.nhmrc.gov.au/about-us/publications/australian-code-responsible-conduct-research-2018?utm_source=Release+of+Peer+Review+Guide+and+Disclosure+of+Interests+and+Management+of+Conflicts&utm_campaign=9a5a43c870-EMAIL_CAMPAIGN_2019_08_28_03_25&utm_medium=email&utm_term=0_e66256a329-9a5a43c870-64999547)

**1. SUMMARY**

|  |  |
| --- | --- |
| **Study Title** | CUT umbilical cord milking to prevent Encephalopathy in infants with prenatal drug exposure – the CUTE project |
|  |  |
| **Aims/Objectives** | **Primary** - To determine whether prenatal opioid exposure increases inflammation in neonates**Secondary** – To determine whether umbilical cord milking (UCM) reduces inflammation in infants with prenatal opioid exposure, and whether it improves their postnatal developmental outcomes |
| **Study design** | 1. Healthy control (standard Cord Clamping, SCC)2. Opioid-exposed (standard Cord Clamping, SCC)3. Opioid-exposed (Umbilical Cord Milking, UCM) |
| **Planned sample size** | 48 mother-infant dyads, 3 groups x 16 participants |
| **Inclusion criteria** | **Maternal** – 18-35 years, healthy weight, singleton pregnancy, antenatal informed consent, willingness to participate/comply, absence of pregnancy complications, self-report and urine toxicology report confirmation of opiate use during pregnancy (opioid-exposed group only)**Infant** – Full term (>39-41 weeks), uncomplicated delivery |
| **Study procedures** | 1. Participant identification2. Screening, consent and enrolment3. Maternal urine, blood and stool collection (antenatal)4. (for opioid-exposed group only) assignment to SCC or UCM groups.5. Cord blood and placenta samples collection at birth6. Neonatal urine, meconium and neonatal saliva collection within first 24 hours. 7. Neonatal blood collection (during heel prick testing) and stool collection 2-3 days after birth. 8. (*For opioid-exposed group only*) MRI scan within 2 weeks of birth. 9. Neurodevelopmental and behavioural testing and infant stool collection at 3 months.  |
| **Analysis considerations** | **Sample size calculation** Group sizes are based of similar studies used in the past to permit justification for a full RCT.**Analysis plan** Planned comparisons will be made between data obtained from controls versus opioid exposed (groups 1 vs 2/3) to detect the impact of prenatal opioid exposure on the infant across all measures. A second comparison between opioid SCC and opioid UCM will permit assessment of the impact of UCM on this group. Regression analysis will then permit detection of trends and relationships between all measures gathered to assess the overall impact of prenatal opioids and UCM treatment. A UCM control is not included due to likely ceiling effects in healthy controls and challenges with recruiting otherwise healthy infants into a treatment condition.  |
| **Study duration** | 3 years.  |

**2. BACKGROUND AND RATIONALE**

*Context:* This project is a collaboration across a highly skilled and experienced team of specialists from neonatology, paediatrics, gastroenterology (including microbiome) and neuroscience with all the necessary expertise, laboratory space and connections to make this project a success.

Further personnel supporting the project are a part-time Research Assistant (Melissa Bebbington; 0.6 FTE) who is responsible for project management, assistance with MRI and all administrative duties; a full-time PhD student (Rinjani Soengkoeng, supervised by CI Clemens) who is responsible for sample transport, storage and processing. A neonatal registrar at the Royal Hospital for Women (Kok Joo Chan, supervised by Prof JuLee Oei) will assist with the MRI studies. A microbiologist (Dr Josie van Dorst, post-doctoral fellow to A/Prof Keith Ooi) will assist with analysis and interpretation of stool samples. We will also shortly be employing a casual Midwife Researcher at each site (we have identified and contacted candidates, this will be confirmed following ethics approval) who will be already embedded within each hospital and will be responsible for sample collection at antenatal appointments, during birth and across the postnatal period.

The project is funded by the Ross Trust ($100K) across 2023. We are currently seeking renewal across 2024 (a further $100K). Sample analysis is further supported by internal funding from CIs Clemens and Ooi. Biospecimens are stored free of charge in the School of Psychology at UNSW. Current funding from the Ross Trust is sufficient for conducting MRIs in the pilot study. Overall, the pilot study is relatively low-cost due to the non-invasive nature of the experimental design. If successful it will form the basis of larger grant submissions (NHMRC, March of Dimes) to enable a fully randomised clinical trial.

Although the project outlined below includes an intervention (umbilical cord milking), we have not considered it to be a clinical trial as the intervention is a variant on normal birth procedures, does not include administration of any external substance, and relies on the transfer of endogenous substances typically transferred during vaginal births and compression of the placenta. Indeed, anecdotal evidence from our colleagues indicates that this procedure is performed in hospitals across Australia, and published studies indicate it is a routine procedure in many countries (Aydogan et al., 2021; Liyanage et al., 2020).

*Background:*

Over the last 20 years the global use of opioid drugs has dramatically increased (Degenhardt et al., 2019; Karanges et al., 2018). In 2012, 15 times more opioids were dispensed in Australia than in 1991, while numbers of opioid-related hospitalizations more than doubled between 1998 and 2009 (Blanch et al., 2014). Worryingly, women of reproductive age are one of the most at-risk populations for developing an opioid addiction (Varney et al., 2022). Opioid use in the antenatal period is particularly dangerous as both licit and illicit opioids readily cross the placenta, enter the foetal bloodstream and impact the brain across critical periods of development.

Once circulating, opioids impact growth and development across a range of domains. At birth, prenatal opioid exposure leads to poorer obstetrical outcomes including life-threatening withdrawal symptoms in >55-94% of newborns (Maguire et al., 2016; Wachman et al., 2018). In the longer term, prenatal opioid use is linked to visual system dysfunction, cognitive impairment and delayed motor development (Lee et al., 2020; Maeda et al., 2014; McGlone et al., 2014; Merhar et al., 2019). Children and adults with a history of prenatal opioid exposure are at greater risk of falling behind academically, being hospitalized due to misadventure, developing a mental health disorder, and committing suicide (Oei et al., 2017; Oh et al., 2020; Sherman et al., 2019). Unfortunately, there are currently no early life interventions available for opioid-exposed infants to prevent, buffer or reverse these long-term consequences.

Animal and human research has revealed that the widespread developmental harms associated with prenatal opioid exposure may be unified by a single causal system – inflammation. Opioids interact with the maternal and foetal immune system and can promote inflammatory responses in the body and brain of infants (Ahluwalia et al., 2000; Chan et al., 2016; Gessi et al., 2016; Jantzie et al., 2020; Pascual et al., 2017). This is hypothesised to result in disrupted cell signalling and functioning, apoptosis, and changes in tissue structure, which may result in reduced cortical volume and white matter aberrations in the brain (Becher et al., 2017; Marsland et al., 2015; Wang et al., 2012). Animal models of prenatal opioid exposure have repeatedly demonstrated the link between prenatal opioid-associated inflammation is associated with neurological, behavioural, and cognitive changes that persist into adulthood, closely mimicking the developmental trajectory observed in opioid-exposed children (Jantzie et al., 2020; Yuan et al., (2014). Evidence of inflammation in human infants with prenatal opioid exposure is currently lacking due to an absence of research in this area.

Inflammation presents a promising target for clinical intervention in infants affected by prenatal opioid exposure. The inflammatory response is characterized by increased expression of specific protein factors – cytokines, chemokines, signalling molecules, and receptors – by immune cells (Becher et al., 2017). This up-regulation can be detected across a range of biological sample types, including blood and saliva, via both transcriptomic and proteomic analyses (Mally et al., 2014; Romano-Keeler et al., 2014). Additionally, inflammation in the neonate can be validated through histological examination of placental tissue (Goldstein et al., 2020). Thus, assessing biomarkers of inflammation in opioid-exposed infants and mothers could allow early detection of pathophysiological states associated with negative long-term outcomes. To investigate this possibility, the CUTE project will compare inflammation in opioid-exposed and healthy mother-infant dyads by analysing protein, mRNA, and anatomical changes in plasma, saliva, and placental samples at birth. These samples will be gathered exclusively as part of standard practices (e.g. heel prick for infant blood) or using non-invasive techniques (e.g. saliva collection).

Early life inflammation can have a significant impact on the development of key biological systems, such as the central nervous and digestive systems. MRI studies of neuroinflammatory diseases have shown that peripheral inflammation correlates with neurological and structural changes in the brain (Marsland et al., 2015). Opioid-exposure has been linked to poor white matter development (Yen et al., 2022), with animal studies suggesting that this may be due to abnormal differentiation of precursor cells into myelin (Hahn et al., 2010), a process that is critically dependent on normal patterns of opioid receptor activation (Eschenroeder et al., 2010). By conducting MRIs and volumetric analysis on a subset of opioid-exposed infants, the CUTE project will assess whether drug exposure and systemic indicators of inflammation (i.e., blood at birth) can predict inflammation-related morphological changes in the brain.

Regulation of inflammatory processes is increasingly linked to gut health, where an imbalance of bacteria in the gut can increase the production of toxic metabolites (de Vos et al., 2022). The impact of this in the context of opioid exposure is particularly significant, as opioid exposure increases the permeability of both the gut-blood barrier (Thomas et al., 2022) and the blood-brain barrier (Seleman et al., 2014) such that toxic metabolites more readily pass into the blood, crossing into the brain to initiate a neuroinflammatory response (Jalodia et al., 2022). Poor gut health is common in adult opioid users (Acharya et al., 2017; Gicquelais et al., 2020), and recent work in the lab of CI Clemens indicates gut dysbiosis in rat pups born prenatally exposed to opioids. Thus, infants prenatally exposed to opioids may not only experience inflammation due to chronic opioid exposure, but their capacity to buffer against additional sources of inflammation is likely impaired, further contributing to altered immune functioning and poor physical and mental health outcomes (Shobeiri et al., 2022; Yang et al., 2016). To determine if gut health is impacted by prenatal drug exposure and inflammatory state, gut microbiome diversity will be analysed in stool samples collected from the mother, stool samples from all infants after birth and up to 3 months later.

The second primary objective of this study is to promote transfer of placental and umbilical blood transfusion into the infant via umbilical cord milking (UCM) at birth as a potential buffer against the acute impact of prenatal opioid exposure. UCM involves cutting and clamping the umbilical cord further away from the newborn than in standard practice, leaving a long section (20-30 cm) of cord attached to the infant. The umbilical cord blood within this section is then gently pushed into the newborn’s body. UCM is a standard practice in many countries (e.g., Japan), and anecdotal evidence indicates it is performed in Australia (*pers. comm.*). UCM supports haemodynamic stability and steady red blood cell volumes as the infant transitions from having the majority of its cardiac output being directed towards the placenta, to being directed towards the lungs (Koo et al., 2023). Randomised controlled trials have demonstrated that UCM improves APGAR scores and cerebral fractional oxygen extraction and reduces the need for cardiorespiratory support and the risk of hypoxic-ischemic encephalopathy in term and late preterm infants, indicating UCM promotes effective blood flow and tissue perfusion (Katheria et al., 2023; Okulu et al., 2022). Placental transfusion also increases ferritin levels, which has been linked to improved white matter maturity of the brain 4 months later (Mercer et al., 2018). Cord blood also contains high levels of stem cells, anti-inflammatory cytokines, and growth factors with regenerative properties (Katheria et al., 2020; Romanov et al., 2019). These cord blood components immune-regulating properties (Henning et al., 2011; Serrenho et al., 2021), and could ameliorate the early inflammation that appears to characterise prenatal opioid exposure (Jantzie et al., 2020; Yen et al., 2022). Furthermore, a meta-analysis by Balasubramanian et al. (2020) found that infants who were assigned to UCM at birth had higher cognitive and language scores on the Bayley Scales of Infant Development at two years old, compared against those who received an alternate form of placental transfusion. The potential for improved brain functioning in UCM-receiving infants is particularly relevant to clinical management of opioid-exposed infants, as this population is highly vulnerable to impaired cognitive development and academic delays throughout childhood (Andersen et al., 2020; Oei et al., 2017).

UCM can be performed as quickly as immediate cord clamping should any birth-related complications arise (Katheria et al., 2023). UCM can safely be performed with vaginal birth but is particularly effective with caesarean births where anaesthetics may limit placental transfusion (Consonni et al., 2020). Across the numerous studies available on UCM in full-term infants, no harm associated has been reported (Gomersall et al., 2021; Jeevan et al., 2022; Koo et al., 2023). Considering that UCM boosts many of the same factors impaired in opioid-exposed infants, the CUTE project will perform a pilot study to test whether this non-invasive, rapid and free variation of normal birth procedures could be used to improve outcomes for opioid-exposed infants.

**3. STUDY AIMS/OBJECTIVES**

***Aim:*** The overall aim of the CUTE project is to improve outcomes for infants born with prenatal opioid exposure

***Primary Objective:*** The first objective of this project is to gather biological samples (blood, saliva, urine, faeces) and evaluate brain and behavioural health (MRI, socioemotional and movement assessment) from opioid exposed infants and their mothers to understand factors that contribute to the poor long-term outcomes of infants, and with a view to developing biomarkers that predict level of risk in opioid-exposed babies, allowing better targeting of treatment.

*Hypothesis:* Compared to non-drug exposed controls, we hypothesise prenatal opioid exposure will be associated with systemic inflammation evident as increased proinflammatory markers in mother and infant blood and saliva. We hypothesise that both mother and infant with show evidence of gut dysbiosis, evident as reduced gut bacterial diversity and altered ratio of ‘good’ to ‘bad’ bacteria. We hypothesise that infants with prenatal opioid exposure will have reduced brain volume and white matter density, and poorer cognitive, emotion and motor development at 3 months of age. Finally, we hypothesise that these measures will be inter-related, where infants with greater evidence of inflammation will have poorer outcomes on the MRI and 3-month follow-up.

***Secondary Objective:*** The second objective is to pilot Umbilical Cord Milking (UCM) as a means of improving the physiological and developmental outcomes of full-term infants with prenatal opioid exposure. An extension of delayed cord clamping, UCM increases the transfer of vital stem cells, anti-inflammatory cytokines and elements such as iron into the newborn infant to boost growth and development (Bora et al., 2023; Daskalakis et al., 2018; Katheria et al., 2020).

*Hypothesis:* We hypothesise that UCM will reduce evidence of inflammation in the days following birth and that this will be related to improved outcomes at 3-month follow-up.

**4. PARTICIPATING SITES**

The following sites have been selected due to the presence of in-hospital Chemical Use in Pregnancy Services (CUPS; SESLHD), Substance Use in Pregnancy and Parenting Services (SUPPS; SWSLHD) and higher reported incidence of opioid use in the SWSLHD areas by service staff.

Royal Hospital for Women (South Eastern Sydney Local Health District)

Liverpool Hospital (South Western Sydney Local Health District)

Fairfield Hospital (South Western Sydney Local Health District)

Bankstown-Lidcombe Hospital (South Western Sydney Local Health District)

NSW Health Pathology – East (SEALS; South Eastern Sydney Local Health District)

NSW Health Pathology – South West Sydney (SSWPS; South Western Sydney Local Health District)

**5. STUDY DESIGN**

**5.1 Study Type**

This is a pilot study to examine the feasibility of a larger scale study. This feasibility arises through three considerations: 1) logistical considerations when working with a vulnerable population; 2) estimates of variance to inform power calculations around feasibility for a larger RCT; and 3) identification of predictive factors to carry forward into a larger study i.e., whether all measures differ between groups, or that some may not be required moving forward.

**5.2 Expected Study Duration**

The study is expected to start in November 2023 and finish in December 2025, pending ethics approval. The period of recruitment is expected to extend from November 2023-December 2024, with the remaining 12 months for sample analysis.

**5.3 Data Source and Population**

We aim to recruit 48 mother-infant dyads: 32 with histories of primary opioid use during pregnancy, and 16 controls without histories of substance use during pregnancy. Participants will be recruited across all study sites as they are identified by midwifery staff. Due to the unpredictable nature with which women with a history of opioid use present at the hospital, it is likely that there will be uneven numbers of opioid-dependent women recruited across study sites. To counteract this, study groups will be matched for study site wherever possible (see below).

The study groups are:

1) Controls/no UCM (n=16): Pregnant women with no history of substance use across pregnancy. No UCM.

2) Opioid-exposed/no-UCM (n=16): Pregnant women with a history of substance use across pregnancy. No UCM.

3) Opioid-exposed/**UCM** (n=16): Pregnant women with a history of substance use across pregnancy. UCM at birth.

There is no control group receiving UCM in this design for two reasons: 1) obstetric and midwife staff conclude it would be difficult to recruit otherwise healthy pregnant women to receive UCM; 2) there would be a ceiling effect in these infants where UCM would not noticeably improve outcomes. Nonetheless, the control group is essential to provide a baseline reading across all measures while controlling for all external factors (e.g., socio-economic status) and to indicate a direction of change for the measures to be gathered i.e. whether infant inflammatory cytokines are higher or lower compared to controls, and whether the UCM normalises these values.

For considerations of sample size and study design we consulted a biostatistician through Stats Central at UNSW. A typical power analysis approach does not apply here as this is a pilot study and we do not have an accurate estimate of the standard deviation of the majority of our outcome measures in the opioid-dependent dyads (hence the requirement for the pilot study). For a main trial of 90% power and two-sided 5% significance, pilot trial samples per treatment arm of 15 would offer a detection of a small effect size (Whitehead et al., 2016). This is consistent with other pilot studies in this area that detected significant group differences with similar group sizes, including resting state MRI of infants (n=10 exposed, 12 controls; Radhakrishnan et al., 2021), MRI of unborn children (n=12 opioid exposed, 16 controls; Radhakrishnan et al., 2022), saliva for detection of reward and inflammatory genes (25 opioid exposed, 25 controls; Yen et al., 2022).

We have employed a minimisation randomisation approach to our study design (Jin et al., 2021). As it is unknown how many opioid-exposed women will be recruited from each study site, it is important that study groups are balanced across study sites using a dynamic or adaptive randomisation approach that permits continuous adjustment for imbalance between groups, whilst taking into account the total number of participants recruited. This approach preserves the group allocation ratio at every allocation. Thus, for every opioid-exposed pregnant woman recruited to a specific site, the next opioid-exposed recruit to the site will be allocated to the opposite group (i.e., UCM vs non-UCM), and a control will be recruited. As there will be many more controls than opioid-exposed, recruitment will primarily focus on opioid-exposed women, with controls matched as best possible. Factors for matching will be site, mother age, sex, vaginal versus caesarean birth, other medical conditions including complications with pregnancy (e.g., gestational diabetes, pre-eclampsia) or chromosomal disorders.

**5.4 Recruitment and Screening**

Participants will be recruited from mothers who are admitted to each study site for an antenatal appointment. As part of standard admission procedures, detailed drug and alcohol histories are taken by midwives. This information will be used to identify potential participants.

***Recruitment of Substance-dependent Participants***

Women who self-report substance use during pregnancy are internally referred to either the Chemical Use in Pregnancy Service (CUPS) for SESLHD or the Substance Use in Parenting and pregnancy Service (SUPPS) for SWSLHD, which provide specialised ante- and post-natal support for infants and families dealing with substance use. The treating clinician at the service will determine if the woman is a suitable candidate for the research project, i.e., she does not meet any of the exclusion criteria outlined in Section 5.6 ‘Exclusion Criteria’. After sufficient rapport is established, the clinician will briefly introduce the study to the mother. As contact with CUPS or SUPPS typically occurs between 15-19 weeks gestation (Oei et al., 2009), there will be sufficient time for rapport to be built.

At this point, the recruitment process will differ between the SESLHD and the SWSLHD sites, based on feedback provided by CUPS and SUPPS clinicians from these districts – see the following table:

|  |  |  |
| --- | --- | --- |
| Local Health District | SESLHD | SWSLHD |
| Applicable sites | The Royal Hospital for Women | Fairfield Hospital, Liverpool Hospital, Bankstown-Lidcombe Hospital  |
| Recruitment & Informed Consent Process | 1. The CUPS clinician will request that the individual’s contact details can be passed on to the research team.2. The CUPS clinician will contact a Research Assistant and inform them of the potential participant and provide the person’s contact details.3. The Research Assistant will call the participant, confirm they have time to complete the informed consent process, and provide a digital copy of the following documents via email or SMS:* Participant Information and Consent Form (PICF) with attached Sample Collection Appendix and Withdrawal Form.
* A Visual Aid

The Research Assistant will discuss the study and answer any questions the individual has.4. The individual may sign the PICF during the call, or after a certain period of time at their request. | 1. The research team will provide the SUPPS clinic with paper copies of the PICF with attached Sample Collection Appendix and Withdrawal Form, and Visual Aids (together, the Informed Consent Package).2. The SUPPS clinician will request that a Research Assistant can call the individual during their next appointment with SUPPS to further discuss the study.3. The SUPPS clinician will contact a Research Assistant and inform them of the potential participant and their next appointment time.4. During the individual’s next appointment, the Research Assistant will call the individual to introduce the study and obtain informed consent, using the Informed Consent Packages previously provided.5. The individual may sign the PICF during the call, or after a certain period of time at their request. |
| Screening | Women who do not consent to all applicable procedures included within the study – collection of neonatal, maternal and placental tissue, collection of medical and neurodevelopmental data, infant MRI scan, and urine/meconium toxicology analysis – will be screened out. | Women who do not consent to all applicable procedures included within the study – collection of neonatal, maternal and placental tissue, collection of medical and neurodevelopmental data, infant MRI scan, and urine/meconium toxicology analysis – will be screened out. |

While the majority of mothers with substance use issues are Australian-born and able to understand conversational English (Zhao et al., 2017), the research project may enrol mothers from Culturally and Linguistically Diverse Backgrounds, especially within the South Western Sydney catchment. If the mother is deemed suitable by the SUPPS clinician for the study, NSW Health interpreters will be engaged to explain the study to the mother. This is standard procedure for health care for all patients in NSW.

If legal guardianship is removed from the biological parent the Department of Communities and Justice (DCJ) in NSW will be approached for consent. The case worker for the child will be approached to review the study and present the case to DCJ for participation of the child. This pathway of enrolling infants has been used successfully in other studies (Yuan et al., 2014).

***Recruitment of Control Participants***

Control mothers will be identified by treating clinicians at hospital antenatal care clinics using the exclusion criteria outlined in Section 5.6 ‘Exclusion Criteria’. The clinician will briefly introduce the study to the mother and request that their contact details be provided to the research team. A Research Assistant will contact the mother, confirm they have time to complete the informed consent process, and introduce the study more thoroughly. They will also provide digital copies of the Informed Consent Package. The Research Assistant will discuss any questions the mother has. The individual may sign the PICF during the call, or after a certain period of time at their request.

Women who do not consent to all applicable procedures included within the study – collection of neonatal, maternal and placental tissue, collection of medical and neurodevelopmental data, and urine/meconium toxicology analysis – will be screened out.

**5.5 Inclusion Criteria**

|  |  |  |
| --- | --- | --- |
|  | Opioid-exposed | Control |
| Maternal Inclusion Criteria | * 18-35 yrs, healthy weight (BMI 25-29.9; Australian Institute of Health and Welfare, 2023)
* Singleton pregnancy
* Willingness and ability to give written informed consent prior to birth
* Willingness to participate in and comply with the study
* Use of opiates (heroin, methadone, buprenorphine, codeine, oxycodone, fentanyl, tramadol, pethidine) during pregnancy as confirmed via self-report and/or maternal and neonatal urine toxicology
 | * 18-35 yrs, healthy weight (BMI 25-29.9; AIHW, 2023)
* Singleton pregnancy
* Willingness and ability to give written informed consent prior to birth
* Willingness to participate in and comply with the study
* No prior history of opioid use disorder.
 |
| Infant Inclusion Criteria | * Born at >39-41 weeks of gestation (or, if gestation unknown, an estimated birthweight of ≥ 2.5 kg)
 | * Born at >39-41 weeks of gestation (or, if gestation unknown, an estimated birthweight of ≥ 2.5 kg)
 |

**5.6 Exclusion Criteria**

|  |  |  |
| --- | --- | --- |
|  | Opioid-exposed | Control |
| Maternal Exclusion Criteria | * HIV positive status, diabetes
* complications with pregnancy (e.g. gestational diabetes, pre-eclampsia, chorioamnionitis)
* chromosomal, inflammatory or autoimmune disorders with or without medication
* use of any anti-inflammatory or steroid medications
* Consent unable to be obtained before birth (e.g., insufficient time, precipitous labour, cognitive impairment, inebriation)
* Patients with a history of a psychological illness or condition such as to interfere with the patient's ability to understand the requirements of the study.
 | * HIV positive status, diabetes
* complications with pregnancy (e.g. gestational diabetes, pre-eclampsia, chorioamnionitis)
* chromosomal, inflammatory or autoimmune disorders with or without medication
* use of any anti-inflammatory or steroid medications
* Consent unable to be obtained before birth (e.g., insufficient time, precipitous labour, cognitive impairment, inebriation)
* Patients with a history of a psychological illness or condition such as to interfere with the patient's ability to understand the requirements of the study.
* Drug use (any) during pregnancy as confirmed via maternal and/or neonatal urine toxicology
 |
| Infant Exclusion Criteria | * Non-vigorous status at birth
* Major congenital or chromosomal anomalies
* Auto-immune or infectious diseases
* Admission to NICU or requiring ventilatory support
 | * Non-vigorous status at birth
* Major congenital or chromosomal anomalies
* Auto-immune or infectious diseases
* Admission to NICU or requiring ventilatory support
 |

**5.7 Consent Process**

Informed consent will be ensuring the participants are well informed on all aspects of

the purpose of the study, the procedures involved, the potential risks and benefits, and the rights and responsibilities of participants to mothers prior to the birth of their baby.

Mothers will be informed about the limits of their consent, and that they can withdraw their consent (for their own participation and that of their infant) at any point during the study without penalty. It will be explained that participating or not participating in the study will have no impact on the healthcare they and their baby receive at any of the sites involved.

The Research Assistant will ensure that eligible mothers adequately understand the study, its requirements, and the possible implications by:

* Providing all information in lay language.
* Providing enough time for all information to be explained and for mothers to ask questions as needed.
* Providing each mother with an informed consent package consisting of the Participant Information Sheet (PIS) with attached Sample Collection Appendix and Withdrawal Forms, and a visual aid to illustrate study components while the Research Assistant discusses it (see ‘CUTE Project Visual Information Sheet’).
* Providing each mother with their contact details should they have further questions.
* Clearly outlining what will happen to the data obtained and how they are able to access the conclusions from the study.

Participants will be provided with this information in written format PIS, which is written in lay language with minimal scientific jargon (part of the Informed Consent Package). Once these processes have occurred, the participant will be asked to fill out the Participant Consent Form attached to the PIS, either digitally or on paper. A copy of the signed Consent Form will be given to the participant for their personal record; the original will be kept by the CI as documentation of participant consent.

All efforts will be taken to minimise risk of coercion or pressure to ensure consent is voluntary. To ensure that coercion of potential participants is minimised, the research team consulted with a representative from the NSW Users and Aids Association (NUAA) with lived experience of opioid dependence. Based on this consultation, the following actions will be put in place:

* The Research Assistant will have been familiarised with the vulnerabilities of substance-dependent mothers through extensive consultation with the CUPS/SUPPS clinicians at all four research sites.
* The Research Assistant will not be a treating clinician, and therefore will have no ongoing role in patient care if the pregnant mother declines to take part in the study.
* A Peer Contact with lived experience of substance dependence during pregnancy will be onboarded as part of the research team, to mitigate the potential power imbalance of treating clinicians being Principal Investigators. Their contact details will be provided to participants during the informed consent process; participants can discuss their involvement with the project and any concerns they have with the Peer Contact, who will act as a source of support and guidance that is independent of the NSW healthcare system. The research team is currently liaising with the Queensland Injectors Voice for Advocacy and Action, based on the recommendations of the CEO of NUAA, to identify a suitable Peer Contact.

**5.8 Study Procedures**

***Overview of experimental plan***



***Investigation Plan***

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Study Procedures | Enrolment Visit | Birth | Birth-24 hrs | Postnatal Day 2-3 | 40 weeks term/1 week old | 3-months |
| Informed Consent |  |  |  |  |  |  |
| Inclusion/Exclusion Criteria |  |  |  |  |  |  |
| Maternal urine, blood, and stool collection |  |  |  |  |  |  |
| Treatment |  |  |  |  |  |  |
| Cord blood and placenta collection |  |  |  |  |  |  |
| Neonatal meconium and saliva collection |  |  |  |  |  |  |
| Birth data collection |  |  |  |  |  |  |
| Neonatal blood collection  |  |  |  |  |  |  |
| Neonatal stool collection |  |  |  |  |  |  |
| MRI |  |  |  |  |  |  |
| Neurodevelopmental & Socio-emotional assessment |  |  |  |  |  |  |
| Infant stool sample collection |  |  |  |  |  |  |
| Study payment |  |  |  |  |  |  |
| Adverse Event & Serious Adverse Event Assessment |  |  |  |  |  |  |

The study procedure is largely equivalent across all sites, although some differences exist between SESLHD and SWSLHD. Any such differences are clearly indicated in the text below.

***Study Procedures***

Participant Enrolment

*Opioid exposed:* Potential participants will be identified by research midwives upon referral to the CUPS (SESLHD) or SUPPS (SWSLHD) units. Research midwives are provided with a tailored information sheet outlining inclusion and exclusion criteria to assist with this process. Once a potential participant is identified, details will be passed to the research assistant to make contact via the phone or in person, depending on the circumstances of the individual and the frequency of hospital visits.

*Controls:* Potential participants are identified by midwives at their first antenatal visit to the hospital. Once identified, details will be passed to the research team and assessed against the inclusion criteria relative to the minimisation randomisation approach to ensure balance across study groups.

After providing informed consent, mothers that meet the inclusion criteria and none of the exclusion criteria will be enrolled into the study. As part of this they will receive a maternal enrolment number which will be documented in their medical record and used for all associated study documents.

Upon delivery, neonates that meet the infant inclusion criteria and none of the exclusion criteria will also receive an infant enrolment number. This will also be documented in the baby’s medical record and used on their study documents.

Summary of Biospecimen Collection and Storage

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **BIOSPECIMEN**  | **SAMPLE COLLECTOR** | **SHORT-TERM STORAGE**  | **TRANSPORTER** | **LONG-TERM STORAGE**  |
| Cord blood  | Attending midwife  | -20 freezer, pathology dept  | Research assistant  | -80°C freezer, UNSW Psychology, Mathews Building, Level 6, Room F23  |
| Infant blood   | Attending midwife  | -20 freezer, pathology dept  | Research assistant  | -80°C freezer, UNSW Psychology, Mathews Building, Level 6, Room F23  |
| Maternal blood  | NSW Health Pathology Phlebotomists at St George and Liverpool sites | -20 freezer, pathology dept  | Research assistant  | -80°C freezer, UNSW Psychology, Mathews Building, Level 6, Room F23  |
| Saliva (infant)  | Attending midwife  | 4°C fridge, maternity ward   | Research assistant  | -80°C freezer, UNSW Psychology, Mathews Building, Level 6, Room F23  |
| Urine  | Participant (parent/carer) at the hospital  | Tox. screen immediately after collection  | Pathologist, SEALS/ SSWPS  | Disposed after toxicology screening  |
| Stool (maternal) | Participant (parent/carer) at home  | Participant’s freezer  | Research assistant  | -80°C freezer, UNSW Psychology, Mathews Building, Level 6, Room F23 |
| Stool (infant) | Attending midwife | -20 freezer, onsite | Research assistant | -80°C freezer, UNSW Psychology, Mathews Building, Level 6, Room F23 |
| Meconium  | Attending midwife  | 4°C fridge, maternity ward  | Pathologist, SEALS/ SSWPS  | -20 freezer, pathology dept > disposed after tox screen |
| Placenta  | Attending midwife  | 4°C fridge, maternity ward  | Research assistant  | -80°C freezer, UNSW Psychology, Mathews Building, Level 6, Room F23  |

Toxicology Screening and Inclusion Criteria

Testing for drug use in pregnant women can be challenging and influenced by a range of factors including willingness to disclose, time since last use and frequency of use. For this reason we will assess drug use as an inclusion criteria through multiple non-invasive and routine routes: self-report, mother urine, infant urine, infant meconium; with the assumption that not all participants will be comfortable with all measures.

* Self-reporting of drug use will be reported at the first visit and initiates transfer to the CUPS and SUPPS unit.
* Maternal urine will be obtained as part of routine procedures during the earliest possible antenatal visit. This will be obtained by a Research Midwife and transferred to SEALS Pathology (SESLHD) or South West Pathology Service (SSWPS) for SWSLHD for screening of opioids, cannabinoids, amphetamines, cocaine, benzodiazepines.
* Infant urine will be collected at first possible void (within 24 hours of birth and prior to start of pharmacotherapy) by the Research Midwife and transferred to pathology for toxicological screening. Urine is a reliable indicator of recent exposure.
* A sample of infant meconium will be collected by the Research Midwife and transferred for pathological analysis. Meconium can be a more reliable indicator of sustained use.

Blood collection

Blood will be collected from mother and infant as part of routine care and to test for inflammation. It will not require any additional visits or blood draws.

* For the mother, this will occur during the 24–28-week glucose tolerance testing visit to the hospital where blood sampling occurs as part of standard procedures. An additional <5 mL of blood will be withdrawn at the same time by NSW Health Pathology Phlebotomists at St George and Liverpool sites
* For the infant, this will occur at two time points: 1) collection of cord blood and 2) heel prick blood. For cord blood, once the cord is clamped and cut, <5 mL blood will be milked from the cord into a collection tube. At 2-3 days postnatal, the heel prick occurs as part of routine care and testing for metabolic disease. An additional 0.1 mL will be collected via capillary sampling. Blood will be collected by the attending midwife in both instances.

Blood samples will be retained on ice and transferred to the NSW Health Pathology Service (SEALS or SSWPS) where they are centrifuged to separate red blood cells and plasma. Plasma will be frozen and retained for analysis of pro- and anti-inflammatory cytokines as indicators of inflammation using equipment at NeuRA in collaboration with Dr Adam Walker.

Stool samples

Maternal and infant stool samples will be collected to assess gut health and microbiome. At the first antenatal visit, mothers will be given a stool sample collection kit for use at home. This stool sample will then be collected at the 24–28-week glucose tolerance test as an indicator of gut health in the mother. Infant stool samples will be collected at 2-3 days after birth and at a 3-month follow-up. Parents will be provided with an infant nappy liner for the clean collection of samples. Stool samples will then be collected from the parent by the research assistant and undergo sequencing analysis at the Ramaciotti Centre for Genomics and in collaboration with A/Prof Keith Ooi and Dr Josie van Dorst from the Sydney Childrens Hospital.

Umbilical Cord Milking

Opioid using mothers will be assigned to receive umbilical cord milking or standard cord clamping in the 2 weeks prior to birth according to the randomisation approach outlined in Section 5.3. ‘Data Source and Population’. The Research Midwife will be responsible for ensuring that the correct cord clamping technique is employed through notation on hospital records. An information sheet and video will be provided to all mid-wives involved. Note that this is a variation of normal cord clamping procedures, and does not disrupt the birth process.

Infants randomized to the UCM condition will have their umbilical cord clamped 20-30 cm from their body within 30-60 seconds of birth. The cord will then be cut, leaving the long section attached to the infant. This section of the cord will be squeezed slowly (‘milked’) towards the infant once or twice to push the blood within inside the infant’s body. The cord is then clamped closer to the infant and care proceeds as usual. Once each cord is milked, it will be measured (cut end to infant umbilicus).

Placental Pathology

Inflammation associated with early development can often be evident as changes in the placenta. Upon request, the placenta will be processed for histological examination by NSW Health Pathology laboratories at SESLHD and SWSLHD. This examination will provide information on gross anatomical changes, e.g., abnormal placental weight or presence of abscesses, as well as cellular changes in placental functioning association with intra-uterine inflammation, e.g., acute villitis, as indicated by infiltration of neutrophils into intervillous space.

To further assess inflammatory changes in the placenta, three small (1-cm diameter) cotyledon biopsies will be collected from each placenta by the attending midwife. This will occur within 2-8 hours of delivery, prior to requesting histological exam. Biopsies will be placed in RNA*later* and stored at 4°C on-site until a research team member collects them. Analysis of molecular markers of inflammation will occur using multiplex immunoassay (in collaboration with Dr Adam Walker at NeuRA) and PCR (performed at the UNSW School of Psychology by CI Clemens).

Saliva Collection

Saliva samples have previously been used to evaluate a range of inflammatory genes in opioid exposure (Yen et al., 2022). Saliva offers the assessment of a wide array of protein-coding and non-coding genes associated with the regulation of inflammatory processes. Within 24 hours of birth, two saliva samples will be collected from all infants. To prevent sample contamination, saliva will be collected when over an hour has elapsed since the infant’s last feeding. In the opioid-exposed infants, collection will also occur before the commencement of any withdrawal treatment (e.g., morphine hydrochloride), as this may influence their inflammatory state. Treatment latency in the majority of opioid-exposed infants lies within 48-72 hours postpartum (Gaalema et al., 2013; Kaltenbach & Jones, 2016). Samples will be stored on-site until a research team member collects them. Analysis of inflammatory genes will occur using PCR (performed at the UNSW School of Psychology by CI Clemens.

Magnetic Resonance Imaging (MRI)

Within the first 2 weeks of age, opioid-exposed infants will undergo an MRI. Where possible, this will happen prior to the infant leaving the hospital. This is particularly the case for infants receiving pharmacotherapy who may have an extended hospital stay. All participating sites have a clinical MRI capable of scanning infants to the parameters required for the study.

Under the supervision of the Research Midwife and Research Assistant, the infant will be transferred to the scanning facility and feeding will occur immediately prior to the scan to facilitate sleeping. The infant will be wrapped in a swaddling blanket and then placed inside a MedVac Vacuum Splint immobilization bag. Paediatric ear muffs will be used to reduce the experience of machine-related noise. Prof Rae and Prof Oei have successfully employed this approach in the past (Yuan et al., 2014).

The MRI scanning, analysis and reporting will be on site at each hospital, with Professor Caroline Rae providing technical oversight and analysis. A 3T MRI scanner will be used, following a protocol with robust sequences and short scan times that is well tolerated by infants (Yuan et al., 2014). The scan protocol is designed to allow us to measure brain structure and pathology, including inflammation, microbleeds and baseline tissue conductivity. Scans will include T1 and T2 weighted images, a fast diffusion scan for fibre connectivity and estimation of water diffusivity, a susceptibility weighted image (for microbleeds), a tissue conductivity image (balanced fast field echo) and a quantitative magnetization transfer image. The total scan time, including scout images and setup will not exceed 45 min. Excessive infant movement may necessitate a second scan. A total time for the scan of 90 min will be reserved to allow time for settling or resettling of the infant.

Developmental Assessments

Developmental and health data for all opioid-exposed infants will be obtained. Infants will be assessed at 3-4 months during the fidgety General Movements Assessment; socioemotional development will be assessed via maternal self-report questionnaire:

1. *General Movements Assessment* (GMA)

The GMA is a measure of neuromotor development and provides a sensitive and specific prediction of risk of future developmental and neurological issues, including cerebral palsy. It consists of a 3-minute video at 40-44 weeks (writhing phase) and then another one at 3-4 months (fidgety phase). This can be sent to blinded assessors for evaluation.

1. *Infant Behaviour Questionnaire Revised – Very Short Form* (IBQR-VSF; (Putnam et al., 2014).

The IBQR-VSF measures infant temperament across three domains – Negative Affect, Surgency (extraversion), Effortful Control. It is suitable for 3- to 12-month-olds and takes approximately 15 minutes to complete. Parents will be asked to complete the IBQR-VSF either during their child’s 3-month check-up or via an SMS link.

Adverse BSID or GMA results will be reported to the clinical team in charge of the patient for subsequent management.

Participant Payment

After completion of the 3-month follow-up visit, mothers will be offered a $50 supermarket voucher (not cash) for participating in the study. This will include control mothers. The voucher will be texted/emailed to the mother, unless the child is in foster care, after the 3-month follow-up visit.

Birth Data Collection

A detailed neonatal chart review will be conducted for all infants, including:

* Sex,
* Gestational age,
* Length, weight, and head circumference at birth,
* 1- and 5-minute APGAR scores,
* Duration of hospital stay,
* If relevant, neonatal withdrawal scores and dose, duration and type of withdrawal medication required.

Maternal data from admission and ongoing medication notes as part of standard care will be collected, including:

* Maternal drug use history,
* Maternal psychosocial screening scores.

Specific Biomarkers of Interest

|  |  |
| --- | --- |
| BIOSPECIMEN | BIOMARKERS |
| Blood | FGF basic, Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF.Ref: Bio-Rad Laboratories. *Bio-Plex Pro Human Cytokine 27-plex Assay* *#M500KCAF0Y* |
| Saliva | DRD2, IL1β, IL6, IL10, TNFα, CXCL1, CCL2.Ref: Yen, E., Madan, N., Tarui, T., Kaneko-Tarui, T., Breeze, J. L., Davis, J. M., & Maron, J. L. (2023). Sex-specific inflammatory and white matter effects of prenatal opioid exposure: a pilot study. *Pediatric research, 93*(3), 604–611. https://doi.org/10.1038/s41390-022-02357-5 |
| Stool | Analysed for microbial composition, alpha and beta diversity and gut dysbiosis to give us an indication of gut health.16s rRNA sequencing used to identify and bacterial diversity. v3-v4 amplicon library prep to be used. |
| Placenta | TLR4, NLRP3, IL-1β, TNF-α, SERT, TPH1, IDO.Ref: Ruyak, S. L., Noor, S., DiDomenico, J., Sun, M. S., Fernandez Oropeza, A. K., Rodriguez, D. E., Marquez, L. E., Milligan, E. D., & Bakhireva, L. N. (2022). Effects of prenatal opioid and alcohol exposures on immune and serotonin factors in human placenta. *Experimental Neurology*, *353,* 114057. https://doi.org/10.1016/j.expneurol.2022.114057 |

***Data Analysis Plan***

A Biostatistician from Stats Central at UNSW was consulted for the experimental design and analysis of this project. The primary group comparisons to answer the two primary objectives of the study will be planned orthogonal contrasts to test for the impact of prenatal opioid exposure: [control] vs. [opioid-exposed UCM and opioid-exposed no UCM] and then to test for the impact of UCM: [opioid-exposed UCM] vs. [opioid-exposed no-UCM].

We will also account for interdependencies in data (e.g., body weight and length are likely to be correlated) by careful inclusion of covariates and interpretation of data while taking this into account. We will investigate the use of principal component analysis and partial least squares discriminate analysis to summarise groups of related variables for the purpose of modelling. Another strength of our experimental design is the repeated measures across multiple variables. For example, we will be able to integrate time into our analysis to assess changes in inflammatory markers and microbiome to build up a complete picture of inflammatory state.

For a summary of the data analysis plan, see the below table:

|  |  |  |
| --- | --- | --- |
| Objective | To understand factors that contribute to the poor long-term outcomes of infants with prenatal opioid exposure and so identify early biomarkers of risk. | To pilot UCM as a means of improving the physiological and developmental outcomes of infants with prenatal opioid exposure. |
| Independent Variable | Prenatal exposure status: - Group 1 = Control infants - Group 2 = All opioid-exposed infants (UCM and no-UCM) | Cord management at birth: - Group 1 = Opioid-exposed infants that received standard cord clamping - Group 2 = Opioid-exposed infants that received UCM |
| Dependent Variables |  - Frequency of abnormalities in placental histology. - Mean levels of molecular markers of inflammation in cord and neonatal blood, neonatal saliva, and placental samples. - Mean levels of inflammatory gut microbiota in neonatal and 3-month stool samples. - Frequency of abnormal scores on neuro-behavioural assessment (GMA) at 3-months. - Mean scores on socioemotional development assessment (IBQ-R-VSF) at 3-months. - Rate of physical growth, as measured by anthropometric data. | - Mean levels of molecular markers of inflammation in cord and neonatal blood, neonatal saliva, and placental samples. - Mean levels of inflammatory gut microbiota in neonatal and 3-month stool samples. - Mean brain volume and frequency of abnormalities in white and grey matter structure.- Frequency of abnormal scores on neuro-behavioural assessment (GMA) at 3-months. - Mean scores on socioemotional development assessment (IBQ-R-VSF) at 3-months. - Rate of physical growth, as measured by anthropometric data. |
| Covariates |  - Maternal age, mental health status, and polysubstance use. - Birth weight, length, and head circumference. |  - Maternal age, mental health status, and polysubstance use. - Birth weight, length, and head circumference. |
| Analysis type |  -Orthogonal contrasts -Principal component analysis -Partial least squares discriminate analysis |  -Orthogonal contrasts -Principal component analysis -Partial least squares discriminate analysis |

One of the main benefits of this pilot study is the ability to obtain reliable estimates of variance to carry forward into a power analysis for the design of a fully powered RCT. Estimates of variance and confidence intervals will be calculated for each measure, and we will perform a factor analysis to determine which variables are most predictive of study group. Factors that do not vary across each group will not be carried forward into the RCT.

Data from all relevant participants will be used in the following analyses, including data from participants that have withdrawn from study, up until the point at which they withdrew.

**5.9 Randomisation (if applicable)**

The randomisation procedure is outlined in Section 5.6 ‘ and repeated here. It will be performed within 2 weeks of the due date:

We have employed a minimisation randomisation approach to our study design (Jin et al., 2021). As it is unknown how many opioid-exposed women will be recruited from each study site, it is important that study groups are balanced across study sites using a dynamic or adaptive randomisation approach that permits continuous adjustment for imbalance between groups, whilst taking into account the total number of participants recruited. This approach preserves the group allocation ratio at every allocation. Thus, for every opioid-exposed pregnant woman recruited to a specific site, the next opioid-exposed recruit to the site will be allocated to the opposite group (i.e., UCM vs non-UCM), and a control will be recruited. As there will be many more controls than opioid-exposed, recruitment will primarily focus on opioid-exposed women, with controls matched as best possible. Factors for matching will be site, mother age, sex, history of drug and alcohol use, vaginal versus caesarean birth and ethnicity.

**6. TISSUE COLLECTION**

Sample collection is a mandatory requirement for this study. However, all sample collection is either non-invasive (e.g., stool sample) or is part of routine care and procedure (e.g., infant heel prick). All samples will be deidentified at collection. All analysis will be performed by on-site NSW Health Pathology Services or by UNSW researchers associated with this study.

* Sample collection will be performed by Research Midwives, Research Assistants or at home by parents (e.g., stool sample) as appropriate.
* All samples transferred to NSW Health Pathology Services will be disposed of according to NSW Health Guidelines once analysis is complete. All samples analysed at UNSW facilities will be retained in a secure facility for replicate testing for up to 21 years.
* All samples will either be tested on-site by NSW Health Pathology Services (urine, placenta) or deidentified and transferred to a locked -80°C freezer in a secure PC2 laboratory in the School of Psychology at UNSW (blood, stool, saliva). For analysis, samples may then be transferred to NeuRA (plasma for cytokine assays) or the Sydney Children’s Hospital (DNA extraction from stool samples).
* All samples will be used for the intended research purposes as outlined in this application; they will not be banked for future use not otherwise described in this application.

**6.1 Sample-specific Details of Collection**

***Urine: Toxicology analysis***

Mothers will be asked to collect a spot urine void as part of routine antenatal care using sterile urine cups. Infant urine will be collected at first void (within 24 hours of birth) using newborn urine-bags. All urine samples will be taken for routine toxicological testing of illicit substances by NSW Health Pathology Services. After analysis, samples will be disposed of according to NSW laboratory guidelines. Researchers will have access to the analysis output only.

***Meconium: Toxicology analysis***

Meconium will be collected within the first 24 hours after birth. Bio-liners will be inserted into the infant’s nappy to collect passed meconium. This will be transferred by the Research Midwife to sterile containers with a spatula and then refrigerated. The collection process will be repeated until approximately 10g of meconium is collected. Within 72 hours of collection, the samples from each infant will be pooled to form a single sample. The sample will then be transferred to NSW Health Pathology Services and dried to produce ~1g of meconium. This sample will be stored in de-identified collection tubes, then frozen at -20 ⁰C for toxicology analysis by NSW Health Pathology Services. After analysis, the samples will be disposed of according to NSW laboratory guidelines.

***Blood: Inflammatory Markers***

All blood samples will be collected for analysis of inflammatory cytokines. Maternal blood (<5 mL) will be collected via venepuncture as part of standard antenatal care at the time of Glucose Testing performed at the hospital and at 24-28 weeks gestation. Cord blood (0.5-1 mL) will be collected at the time of birth by the Research Midwife. Infant blood (0.1 mL) will be collected by sterile capillary tube as part of the standard heel prick procedure at 2-3 days. Samples will be placed on ice and transferred to on-site NSW Health Pathology Services for immediate centrifuging to separate red blood cells and plasma. Plasma will be removed and stored at -20 ⁰C before collection by the research assistant. From there it will be transferred on dry ice to the UNSW School of Psychology in Randwick and stored in -80 ⁰C freezers until analysis. Samples will be tested using multiplex cytokine assays (for details, see document titled ‘Protein Targets of Cytokine Assay’). Any excess plasma will be retained for duplicate testing if required for up to 21 years.

***Placenta:* *Inflammation***

After birth the placenta will be weighed. While on-site, three samples (approximately 1 cm3) of placental villous tissue will be collected from the maternal face of the placenta. The biopsies will be rinsed in ice-cold 0.9% saline, then placed into cryovials containing RNA*later*. They will be temporarily stored at 4 ⁰C on-site prior to collection by a research assistant. From there they will be transferred on dry ice to the UNSW School of Psychology in Randwick and stored in -80 ⁰C freezers until analysis via multiplex immunoassay and PCR. Any excess tissue will be retained for duplicate testing if required for up to 21 years.

The remaining tissue will undergo histopathological examination by NSW Health; afterwards, it will be disposed of according to NSW laboratory guidelines. The report of this examination will be requested.

***Saliva: Inflammation***

Saliva will be collected form infants within 24 hours of birth to compliment blood analysis and provide additional indicators of inflammatory biomarkers in addition to other molecular changes not detectable in the blood (Prasad et al., 2016; Yen et al., 2022). To prevent sample contamination, saliva will be collected when over an hour has elapsed since the infant’s last feeding. In the opioid-exposed infants, collection will also occur before the commencement of any withdrawal treatment (e.g., morphine hydrochloride), as this may influence their inflammatory state. Treatment latency in the majority of opioid-exposed infants lies within 48-72 hours postpartum (Gaalema et al., 2013; Kaltenbach & Jones, 2016). Saliva samples will be collected by gently suctioning the oropharynx of the infant for 20-30 seconds using a 1 mL syringe (plunger and endcap removed) attached to low wall suction (≤ 10 mm Hg). Due to the low levels of saliva production in neonates, it is predicted that 10-100 µL of saliva will be collected per sample; this volume has been successfully used for transcriptomic analysis in previous research (Dietz et al., 2012; Yen et al., 2022). The saliva will be immediately transferred into collection tubes containing RNAProtect Saliva Reagent (Qiagen), vortexed, and stored at 4 ⁰C on-site prior to collection by a research assistant. From there they will be transported to a locked -80°C freezer in a secure PC2 laboratory in the School of Psychology at UNSW.

In the laboratory of CI Clemens, the samples will be centrifuged to separate cellular and cell-free components. Salivary supernatant will be removed and kept at 4 ⁰C for a minimum of 48 hours (no longer than 28 days). RNA will be extracted, and qRT-PCR performed for expression of genes relevant to inflammation. Extracted RNA will be stored in de-identified vials in a locked -80 ⁰C fridge pending analyses for 21 years.

***Stool samples: Microbiome and gut health***

Stool samples will be collected to provide an indication of gut health. This will be once from mothers and twice from infants. At the earliest antenatal visit, mothers will be provided with a stool collection kit (containing instructions, gloves, a specimen jar, and a specimen bag), a freezer pack, and a small Styrofoam esky. Three days prior to the glucose tolerance test, mothers will receive a SMS reminder to freeze the freezer pack and collect their stool, transferring a small sample of the collected faeces without urine into the specimen jar, and placing this jar together with the cold freezer pack inside the specimen bag, and into a freezer. On the day of the glucose tolerance test, they will be requested to place the specimen bag and ice pack into the esky and bring with them to the hospital. The research assistant will collect the sample and transfer to UNSW where the stool samples will be transferred into 2 mL de-identified collection tubes and stored in a locked -80 ⁰C fridge.

Stool samples will be collected from each infant at two time points. 2-3 days post-birth, and at the 3-month follow-up. For the on-site sampling at 2-3 days, Research Midwives will place a Bio-liner into the infant’s nappy to collect passed faeces. A sample of the faeces (approximately 2-3g) will be transferred to a sterile, de-identified specimen container using the scoop in the lid. At 3-month follow-up, a sample of stool (approximately 2-3g) will be collected from infant’s nappies where possible. Infants with dry nappes will have a Bio-liner placed into the infant’s nappy to collect faeces passed during the visit and stool will be collected at the end. Samples will be transferred into sterile, de-identified specimen containers. Samples from all time points will be kept at -20 ⁰C until collected by the research assistant for transfer to a restricted-access PC2 laboratory on UNSW Randwick campus where they will be stored in a locked -80 ⁰C fridge.

The stool samples from both time points will be sent to the Ramaciotti Centre for Genomics for DNA sequencing. Remaining stool will be stored for 21 years in a locked -80 ⁰C fridge within a restricted-access PC2 laboratory.

**7. ETHICAL CONSIDERATIONS**

**7.1 Study Procedure Benefits**

This research has multiple benefits for the infant, the family and the research area.

The primary benefit is to the infant born with prenatal opioid exposure. As outlined in the background, long-term outcomes for infants with prenatal opioid exposure are poor across physical development, cognition and mental health. There are currently no treatments to prevent, mitigate or reverse the impact of this insult. Here we offer the first possibility of a non-invasive, rapid and free adaptation of normal cord clamping procedures that could reduce the acute and long-term impact of prenatal opioid exposure. This could include reduced hospital stays, reduced dependence on medication and reduced requirement for health support across development and improved mental health and educational outcomes. This not only benefits the infant, but also the family and carers of the infant. If successful, this approach could be rapidly adapted to a full RCT and then implemented as standard hospital procedure for infants identified with prenatal opioid exposure.

The benefits also extend beyond the immediate impact on the opioid-dependent infants in the UCM cohort in this this study. This project will for the first time provide a complete assessment of inflammatory markers in the mother and infant across multiple tissue types and link these to brain and behavioural development. This combination of data has not previously been gathered for prenatal opioid exposure and will provide support for the theory that inflammatory processes are critical to the poor outcomes observed in infants with prenatal opioid exposure. This will provide direct evidence of inflammation in opioid exposed infants and will inform screening of treatment options in animal studies performed by CI Clemens.

The data gathered will also feed directly into international study efforts in the area of prenatal opioid exposure. For example, the MRI will use parameters consistent with the HEALthy Brain and Child Development Study (HBCD) guidelines, a National Institutes of Health (US)-led initiative to understand brain development following perinatal exposure to opioids (Jordan et al., 2020). CI Rae will co-ordinate this effort to ensure consistency of sampling and manage transfer of deidentified data.

This pilot data will be critical to securing further funding (NHMRC, March of Dimes) with a view to conducting a full RCT with a much larger cohort and longer-term follow-up to comprehensively assess the viability of UCM in improving outcomes for prenatally exposed infants.

Finally, this study will offer a proof of concept for the study of prenatal drug exposure in NSW hospitals. As such, it will provide a template to extend this approach to the study of other drugs of abuse, in particular to assess the impact of prenatal methamphetamine on the brain and behaviour of infants. Much less is known about the long-term impact of prenatal methamphetamine on infant outcomes, which is of particular concern considering that methamphetamine is the most consumed illicit drug in Australia (Australian Criminal Intelligence Commission, 2021). If successful, this pilot study will facilitate research with such a vulnerable and frequently overlooked population.

**7.2 Study Procedure Risks**

The majority of the procedures outlined in this project are either non-invasive or part of standard antenatal or postnatal care. The UCM is a variant of normal cord-clamping procedures. The majority of research associated with UCM has identified

very few risks for term and late preterm infants.

There is some preliminary evidence from a single study that umbilical cord milking may increase the risk of severe intraventricular haemorrhage (IVH) in very preterm infants, i.e., those born at 23-27 weeks old (Katheria et al., 2019). However, studies of full-term or late preterm infants have not found that UCM increases the risk of haemorrhage; additionally, the population assessed by Katheria et al. (2019) was unique as prematurity of this extent is associated with an immature blood-brain barrier and fragile germinal matrix, which places infants at high-risk of haemorrhage. A network meta-analysis by Jasani et al. (2021) on studies of cord management strategies in preterm infants born at less than 37 weeks reported that UCM had no effect on rates of severe IVH, and actually reduced incidence of any grade of IVH when compared to immediate cord clamping. A recent large randomised controlled trial across three countries did not find any difference in risk of IVH between non-vigorous term infants assigned to UCM or immediate cord clamping. To completely mitigate the risk of IVH in the study, ***only infants that are born full-term, i.e., 39-41 weeks old, will be enrolled***. If mothers who have consented to their infants’ participation antenatally deliver preterm, i.e., at less than 35 weeks old, the infant will be excluded from the study.

Blood collection from infants and adults is part of standard procedures. In very rare cases, improper capillary sampling in neonates may cause complications such as scarring, nerve damage, haematoma, necrosis, and osteomyelitis; and improper venepuncture in adults may be cause arteriospasm, haematoma, nerve damage, or fainting ("WHO Guidelines on Drawing Blood: Best Practices in Phlebotomy," 2010). To minimize the risk of these outcomes, capillary sampling and venepuncture will follow established clinical guidelines, e.g., The Royal Hospital for Women Heel Prick for Blood Sampling Local Operating Procedure. Capillary sampling and venepuncture is a common procedure in the sites included within this protocol, and clinical staff are familiar with the appropriate methods and risk management.

There is no evidence of long-term risks associated with MRI scans. Metal objects within or on the subject of an MRI scan may move or heat up in response to the magnetic field generated by the machine; however, it is not expected that the infants who will undergo the MRI scan as part of this study will have metal implants, jewellery, or other attached metal objects. If the infant is unsettled at the time of scanning, the MRI will be rescheduled.

The analysis of gene and protein expression in tissue samples via RT-qPCR and multiplex immunoassay will not be performed in a way that could provide genetic information on any molecular targets other than those specified by the protocol. These analyses will also be unable to provide diagnostic information, information about the infant’s future health risks or their risk of having children with a genetic disorder, or information that may be relevant to the health of family members who are not a part of the project.

The analysis of microorganism diversity within meconium and stool samples via microbiome analysis will not be performed in a way that could provide human genetic information. This analysis cannot provide diagnostic information, information about the infant’s future health risks or their risk of having children with a genetic disorder, or information that may be relevant to the health of family members who are not a part of the project.

Toxicology analysis may reveal that a opioid-identified or control participant has used illegal substances. However, care of mothers with substance use issues in Australia is based on a harm minimisation policy; thus, healthcare providers and researchers are not required to report maternal use of illicit substances to legal authorities. If maternal substance use is associated with a separate child protection issue, healthcare workers may need to disclose the activity as part of their requirements as mandatory reporters. If there are no other child protection issues, illicit substance use in mothers does not need to be disclosed. As part of the informed consent process, it will be ensured that participants are fully aware of these possibilities prior to obtaining informed consent. Only the treating clinicians will have access to these results together with identifying information, to preserve participant privacy and confidentiality.

All efforts will be taken to minimise risk of coercion or pressure to ensure consent it voluntary. For this reason, when possible, consent will be obtained by the research assistant as they will have no ongoing role in patient care if the pregnant mother declines to take part in the study.

Overall, there are two risks that have been identified as potentially occurring as a result of study procedures – IVH after UCM, and complications as a result of blood collection. The former risk has only been reported in extremely preterm infants and will be completely mitigated by entirely excluding this population from the study. Blood collection complications are very rare as the collection procedures – capillary sampling and venepuncture – are standard procedures across all hospitals in NSW and are always performed by trained staff. All other study procedures are entirely non-invasive and have no associated risks.

On the other hand, the study could produce significant benefits to the community by building the foundations for the development of the very first treatment option for infants with prenatal opioid exposure, who are currently at significantly higher risk of poor health and developmental outcomes. The data will be critical in the development of a future randomised controlled clinical trial to evaluate UCM in this population more broadly, which could also allow for research into UCM as an intervention for other, less-studied groups of substance-exposed infants (e.g., those with prenatal methamphetamine exposure). Another benefit of the study is the potential to identify biomarkers of risk that will allow clinicians to flag particularly vulnerable opioid-exposed infants early in life and thereby enable more efficient and effective clinical management. Therefore, the considerable potential benefits of the study outweigh the two possible risks that have been identified.

**7.3 Confidentiality and Privacy**

Once enrolled in the study, mothers and babies will each be assigned a Participant Number. This number will be linked to clinical characteristics (e.g., experimental group, treatment condition, birthweight, polysubstance exposure, etc.) in a document separate to the one containing identifying participant details. Each blood and tissue sample collected from each participant will be labelled with the Participant Number. To maintain participants’ confidentiality and anonymity, identifying participant details will only be accessible to members of the study team who need to know this information, such as their treating clinician or the Research Assistant who will go through the informed consent procedure with the participant. All other team members will only be able to access the document linking Participant Numbers with clinical characteristics.

The results of this pilot study will be used to determine the feasibility of a randomised controlled trial designed to examine the impact of CUT umbilical cord milking in infants with prenatal opioid exposure.

**7.4 Data Storage and Record Retention**

Physical study records (e.g., signed Consent Forms) will be kept in locked cabinets stored at the Royal Hospital for Women, Liverpool Hospital, Fairfield Hospital, and Bankstown-Lidcombe Hospital. Electronic data will be recorded on an electronic Case Report Form (e-CRF) hosted by REDCap and stored on secure institutional network drives at SWSLHD, SESLHD, and the University of New South Wales that have password-controlled access and backup protocols to prevent data loss.

Source documents pertaining to the study will be maintained by the investigational sites. Source documents may include a subject's medical records, hospital charts, clinic charts, the investigator's subject study files, as well as the results of diagnostic tests such as pathology reports. The investigator's copy of the e-CRF serves as part of the investigator's record of a subject's study-related data.

* The following information will be entered into the subject's medical record:
* Subject’s name, contact information and protocol identification.
* The date that the subject entered the study, and subject number.
* A statement about whether informed consent was obtained (including the date).
* Relevant medical history
* Dates of all subject visits and results of key study parameters.
* Occurrence and status of any adverse events.
* The date the subject exited the study, and a notation as to whether the subject completed the study or reason for discontinuation.

Study documents will be securely archived in a mixture of paper and electronic formats. Paper records will be processed to be stored in the Governance Records Repository managed by State Records NSW for long-term storage. All records will have standard barcodes affixed to them. Electronic records will be maintained by the Royal Hospital for Women on existing Network servers. An Archive Log will be compiled, encompassing the location and nature of protocol-specific archived records, the date of archiving, and the minimum period they are to be retained. A copy of the Archive Log will be securely retained by CI Oei. As study documents will pertain to obstetric/maternal healthcare, they will be retained for a minimum of 50 years after the date of the birthing episode, then destroyed, as required by the State Records NSW Health Services, Public: Patient/Client records (GDA17).

# SAFETY REPORTING

***Adverse Event Reporting***

An adverse event (AE) in the context of this project is defined as any untoward medical occurrence in a mother or infant which does not necessarily have a causal relationship with the study treatment (UCM). An AE can therefore be any unfavourable or unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal investigational product, whether or not considered related to the medicinal product.

AEs are not required to be reported unless they meet criteria for a Serious Event, or there is a reasonable possibility that the intervention caused the AE, i.e., there is evidence to suggest a causal relationship between the treatment and the event.

***Serious Adverse Event Reporting***

A serious adverse event (SAE) in the context of this project is defined as any untoward medical occurrence that:

* results in death,
* is life-threatening (i.e., the subject is at risk of death at the time of the event),
* requires inpatient hospitalisation or prolongation of existing hospitalisation,
* results in persistent or significant disability or incapacity,
* is a congenital anomaly/birth defect,
* other important medical events which, in the opinion of the investigator, are likely to become serious if untreated, or as defined in the protocol.

*Notes*:

1. The term “life-threatening” in the definition of “serious” refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.
2. Important medical events which may not be immediately life-threatening or result in death or hospitalisation but which may jeopardize the patient or may require intervention to prevent one of the listed outcomes in the definition above should also be considered serious.

The investigator or delegate at each participating institution is responsible for reporting, within 1 working day of becoming aware of the event, all SAEs occurring up to 30 days after the study intervention to their HREC. All SAEs will be recorded in the participant’s medical record and entered into the study’s e-CRF.

Details of the SUSARs will be reviewed by the independent Data and Safety Monitoring Board.

# Data Safety and Monitoring Board

The Data Safety and Monitoring Board (DSMB) will be formed and begin to review safety after 25-50% of participants have been recruited. The DSMB will report directly to the HREC that provides approval for the trial. The DSMB is accountable through the HREC to the National Health and Medical Research Council. The DSMB will be independent of the study and blinded to treatment arm, unless review of non-blinded data is required.

The members of the DSMB will oversee:

1) conduct of the study and,

2) any sudden unexpected serious events that are related to the study intervention (cord milking).

The DSMB will be activated at 25%, 50% and 75% recruitment. The DSMB will also take into account any emerging/new information regarding the intervention that could alter the study objectives, priorities and methodologies and advice the researchers accordingly. Stopping rules will be based on a difference of >2 SD of the chosen outcome between the control and intervention group.

All data will be reviewed by the DSMB which will meet monthly. It will provide independent assessment of patient safety and study progress and make recommendations about the continuation of the study. If the DSMB feel there may be ethical problems to continue the trial, or if the study team wishes a formal interim analysis to be carried out which has not been foreseen in the Protocol or pose a question of principle (for example modification of an endpoint), then a written request should be made to the HREC.

# Early Termination

The possible circumstances for early termination of the study include:

* Failure to recruit participants within 18 months of study inception.
* Recognition of substantial research evidence indicating that UCM is contraindicated for opioid-exposed infants.
* Incidence of health issues in UCM-receiving infants that are directly related to UCM.
* Incidence of multiple adverse events not necessarily related to UCM, that prevent continuation of data collection.

The reasons for discontinuing the study intervention will be documented in the participant's medical record and eCRF. Early termination of the study will be managed by the independent DSMB.

# BLINDING AND UNBLINDING

Blood, saliva, and placental samples will be labelled with the assigned Participant Number prior to being sent to CI Clemens for molecular analysis; stool samples will be labelled with Participant Numbers and then sent to the Ramaciotti Centre for Genomics. Upon completion of molecular analyses, the results for all sample types will be matched to the relevant clinical characteristics by the research assistant for subsequent statistical analyses. Infant MRI results, identified by Participant Numbers only, will be sent to Professor Caroline Rae for analysis and reporting.

As there is no suitable placebo treatment that mimics UCM, participating mothers and associated clinicians will be cognisant of what intervention the infant is receiving. The current protocol in NSW for managing perinatal care of mother-infant dyads with chemical exposure history also requires that clinicians are aware of mothers’ substance use history. As a result, collection of medical and developmental data (growth, GMA results, BSID scores, etc.) will not occur in a blinded fashion.

The assessor scoring the GMA videos will be provided with the infant’s date of birth, their gestational age, and the date of the video only. The scores will then be matched to relevant clinical characteristics.

**12. CONFLICT OF INTEREST**

The investigators declare that there are no conflicts of interest.

**13. FUNDING**

The research is to be funded by a grant of $93,119 from the Ross Trust Foundation. The investigator and sponsor of the project do not have any obligations to the Ross Trust Foundation.

The biospecimens are stored for 21 years as per NHMRC requirements for a child-related study. These are stored free of charge in the School of Psychology at UNSW. Current funding from the Ross Trust is sufficient for conducting MRIs in the pilot study.

All non-Standard of Care blood draws, biochemical tests, assays, biospecimen archiving and clinical imaging related to the study will be paid for via. the grant provided by the Ross Trust Foundation.

**14. RESEARCH OUTCOMES**

At the conclusion of the study, a summary of findings in lay language will be prepared and provided to the participants via email or post. Individual data will not be available. Participants will also be invited to attend a presentation of findings delivered at each hospital site. These plans are communicated to participants in the Participant Information Sheet. Research data will be archived for a maximum of 2 years and thereafter will be deleted from local servers.

As data is obtained, it will be presented at local and international symposia and conferences by students and staff involved in the project. This may include the Developing Brain’ Clinical Research Colloquium and Perinatal Society of Australia and New Zealand. It is anticipated that the data gathered in this project will contribute to multiple high impact publications.

The data generated from this project will allow for the following research outcomes:

* Accurate estimates of variance to generate a power analysis to determine the feasibility of a full RCT. The data will also inform the continuing inclusion and exclusion of dependent variables into the RCT.
* The development of funding applications to continue this research program (e.g., NHMRC, NIH, March of Dimes, Thresher). Given the number of dependent variables being assessed, we are confident that the data will be sufficient to justify further funding.
* The extension of the study protocol to studies of other drugs of abuse. It is unlikely that the mothers are single-drug users, therefore information about polydrug use may be informative to predict long term outcomes. For this reason, the data plan and variables assessed are highly relevant to investigating the impact of a range of other drugs of abuse used across the prenatal period. In Australia, this is particularly relevant to methamphetamine, which is the country’s most consumed drug (ACIC, 2021). If successful, this project will offer a template to assessing prenatal drug exposure more broadly.

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