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| STUDY protocol |
| ASAPP: Efficacy of Azithromycin for Short cervix and Amniotic fluid sludge for the Prevention of Preterm birth, a pilot randomised controlled trial |
| Version 1.2 dated 08 April 2024 |
| **Authors: Prof Joanne Said, Prof Michelle Giles, Dr Penelope Sheehan,**  **Dr Su Lynn Khong and Dr Arun Sett**  **Sponsor: The University of Melbourne** |
| **Statement of Compliance**  This study will be conducted in compliance with all stipulation of this protocol, the conditions of the ethics committee approval, the NHMRC National Statement on ethical Conduct in Human Research (2007) and the Note for Guidance on Good Clinical Practice (CPMP/ICH-135/95). |

Summary of Amendments

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| **Date** | **Protocol Version** | **Amendment Type** | **Summary of Changes Made** |
| 28 November 2023 | 1.1 | Response to HREC Queries | *Title page*  Prof Michelle Giles, Dr Penelope Sheehan, Dr Su Lynn Khong and Dr Arun Sett added to the list of authors. |
| *Section 7.1 Recruitment Procedure*  Eastern Health added to the list of recruitment clinics. |
| *Section 9.1 Sample Size Estimation and Justification*  Minor revisions made to improve clarity of sample size justification. |
| 08 April 2024 | 1.2 | Response to ANZCTR Queries | *Title page*  The title has been amended to reflect the suggested title.  ASAPP Pilor Trial: Efficacy of Azithromycin for Short cervix and Amniotic fluid sludge for the Prevention of Preterm birth. |

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# Study Synopsis

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| Title | ASAPP: Efficacy of **A**zithromycin for **S**hort cervix and **A**mniotic fluid sludge for the **P**revention of **P**reterm birth, a pilot randomised controlled trial |
| Short Title | ASAPP |
| Design | Multi-centre pilot randomised controlled trial (RCT) to assess the efficacy of oral azithromycin in eliminating amniotic fluid sludge (AFS) as a surrogate outcome for preterm birth |
| Study Centres | Joan Kirner Women’s & Children’s at Sunshine Hospital, St Albans, Victoria  Monash Medical Centre, Clayton, Victoria  The Royal Women’s Hospital, Parkville, Victoria |
| Study Question | In asymptomatic pregnant women with cervical length ≤25.0mm *and* AFS identified between 13+0-24+0 weeks’ gestation, does treatment with oral azithromycin result in eradication of amniotic fluid sludge two weeks after randomisation? |
| Study Objectives | 1. To determine whether azithromycin treatment in asymptomatic women at high risk for preterm birth is efficacious in eliminating AFS. 2. To determine whether azithromycin treatment prevents further shortening of cervical length. 3. To determine whether azithromycin treatment alters the vaginal flora / microbiome 4. To determine the feasibility of undertaking a larger multicentre trial to determine whether azithromycin therapy is effective in preventing neonatal morbidity associated with preterm birth. Feasibility will be determined by:    * 1. Participant acceptance of the study (recruitment rate)      2. Clinician acceptance of the study (clinicians willing to offer the trial and randomise participants) |
| Primary Objectives | The primary objective for this pilot study is to determine whether azithromycin treatment in asymptomatic women at high risk for preterm birth (with a cervical length below 25.0 mm and presence of AFS) is efficacious in eliminating AFS. The primary outcome for this pilot trial is the presence of AFS two weeks post-randomisation (primary endpoint). |
| Secondary Objectives | * To contribute to current knowledge about the relationship between cervical insufficiency, AFS and preterm birth * To assess the feasibility for a larger definitive RCT powered to assess the impact of oral azithromycin treatment on preterm birth (and its associated morbidity and mortality) as its primary outcome * To report on core pregnancy and neonatal outcomes as described in the CROWN initiative, specifically the Core outcomes set for Primary Prevention of Preterm Birth (COPOP).   **Secondary outcomes specific to ASAPP:**   * Change in cervical length two weeks after randomisation and at 26+0 weeks’ gestation * HVS microscopy, culture and sensitivity (MCS) and vaginal microbiome two weeks after randomisation and at 26+0 weeks’ gestation, relative to baseline   **The following Secondary outcomes derived from CROWN and COPOP will also be reported:**  For the mother:   * Maternal mortality * Maternal infection or inflammation (clinical or histological chorioamnionitis) * Preterm pre-labour rupture of membranes (PPROM) * Prelabour rupture of membranes (PROM) * Maternal adverse effects from azithromycin   For the infant:   * Gestational age at birth * Birthweight * Perinatal mortality * Early neurodevelopmental morbidity (Intraventricular Haemorrhage) * Gastrointestinal morbidity (Necrotising Enterocolitis) * Infection (suspected or confirmed neonatal sepsis) * Respiratory morbidity (Respiratory distress syndrome, Bronchopulmonary dysplasia/Chronic lung disease, or requirement for respiratory support) |
| Inclusion Criteria | * Asymptomatic (absence of symptoms suggestive of labour at the time of randomisation) * 13+0- 24+0 weeks’ gestation * Cervical length ≤25.0mm * Sonographic appearance of AFS |
| Exclusion Criteria | * Multiple gestation * Major or lethal fetal congenital abnormality * Placenta praevia * Vasa praevia * PPROM * Clinical signs suggestive of chorioamnionitis * Indicated preterm delivery at time of randomization * Known allergy to azithromycin * Use of medications likely to prolong QT interval * History of cardiac disease (congenital long QT syndrome, Torsades de Pointes, arrhythmia, uncompensated heart failure) * Known hepatic impairment |
| Number of Planned Subjects | 84 |
| Investigational product | Oral azithromycin tablets, 250mg. Patients randomised to receive the investigational product will take two 250mg tablets on day one followed by a 250mg tablet daily on days two-five. |
| Safety considerations | Azithromycin is a category B1 drug shown to be safe for use in pregnant and breastfeeding women. Azithromycin has not been associated with increased rates of fetal malformations above the baseline risk of 1-3%. Serious but very rare adverse effects include fatal arrhythmias such as QT prolongation and Torsades de Pointes. To minimise this risk, any women with a history of Congenital long QT syndrome, Torsades de Pointes, arrhythmia, prolonged QT interval on baseline ECG or uncompensated heart failure will be excluded from this study. There are currently no other safety concerns with respect to this drug. |
| Statistical Methods | We will undertake descriptive statistical analysis reporting the proportion of women screened, eligible, approached, consented and refused. All outcomes will be compared between intervention and control groups using an intention-to-treat analysis. The proportion of women with sonographic appearances of AFS in both the treatment and control groups will be compared at 2 weeks following randomisation (primary endpoint) and at 26+0 weeks’ gestation (secondary endpoint) using the Chi squared statistic. The mean cervical length in both the treatment and control groups will be compared at 2 weeks following randomisation and at 26+0 weeks’ gestation using the Mann-Whitney U test (assuming the cervical lengths will not be normally distributed). All other secondary outcomes will be compared using either the Chi squared statistic (for categorical data), the t-test (for normally distributed continuous data) or the Mann-Whitney U test (for non-normally distributed continuous data). Where appropriate, logistic regression will be used to estimate the odds ratio (OR) and 95% confidence interval (CI) for the corresponding population OR, stratified by cervical length, gestation at recruitment and study site. Linear regression will be used for non-categorical outcomes. |

# Glossary of Abbreviations & Terms

|  |  |
| --- | --- |
| **ABBREVIATION** | **DESCRIPTION** |
| AE | Adverse event |
| AFS | Amniotic fluid sludge |
| AUC | Area under the curve |
| BPD | Broncho-pulmonary dysplasia |
| CI | Confidence interval |
| CLD | Chronic lung disease |
| CROWN | Core Outcomes in Women’s and Newborn health |
| COPOP | Core Outcomes set for Primary Prevention of Preterm Birth |
| CS | Caesarean section |
| GCP | Good Clinical Practice |
| GMP | Good Manufacturing Practice |
| HVS | High vaginal swab |
| MCS | Microscopy, culture and sensitivity |
| MIC | Mean inhibitory concentration |
| NHMRC | National Health and Medical Research Council |
| NICE | National Institute for Health and Care Excellence |
| OR | Odds ratio |
| PPROM | Preterm premature rupture of membranes |
| PROM | Premature rupture of membranes |
| RANZCOG | Royal Australian and New Zealand College of Obstetrics and Gynaecologists |
| RCT | Randomised controlled trial |
| RDS | Respiratory distress syndrome |
| SAE | Serious adverse event |
| SUSAR | Suspected Unexpected Serious Adverse Reaction |
| TGA | Therapeutic Goods Administration |
| WHO | World Health Organisation |

# Study Sites

### Study Location/s

|  |  |  |  |
| --- | --- | --- | --- |
| **Site** | **Address** | **Contact Person** | **Contact Details** |
| Joan Kirner Women’s & Children’s Sunshine Hospital, Western Health | 176 Furlong Rd, St Albans VIC 3021 | Prof Joanne Said | 0411460940  jsaid@unimelb.edu.au |
| Dr Su Lynn Khong | sulynn.khong@wh.org.au |
| Monash Health | 246 Clayton Rd, Clayton VIC 3168 | Prof Michelle Giles | m.giles@alfred.org.au |
| Eastern Hospital | 8 Arnold Street, Box Hill VIC 3128 | Dr Penelope Sheehan | 0413338795  [penelope.sheehan@easternhealth.org.au](mailto:penelope.sheehan@easternhealth.org.au) |
| Royal Women’s Hospital | 20 Flemington Rd,  Parkville 3052 | Dr Penelope Sheehan | 0413338795  penelope.sheehan@monash.edu |

# Introduction/Background Information

### Lay Summary

Babies born preterm are more likely to be unwell when they are born. They are also more likely to experience disability and other health problems when they grow up. There are a number of risk factors which make it more likely that a baby will be born preterm. These risk factors include previous preterm birth, socio-economic disadvantage, and short cervix length. Several research projects suggest that amniotic fluid sludge (AFS), a finding seen on ultrasound, increases the risk of preterm birth. AFS is material found inside the amniotic sac thought to be associated with infection and inflammation. Having both a short cervix *and* AFS increases the risk that a baby will be born early.

The purpose of this study is to determine whether azithromycin is an effective treatment for AFS. In some previous studies, the use of azithromycin has appeared to prolong pregnancy in women with AFS. However, in other studies the benefit of giving azithromycin is less clear. There are currently no studies that show how effective azithromycin is for treating and eliminating AFS specifically. We hope that the results from this study will assist the development of a bigger trial which will directly assess the impact of azithromycin in reducing the risk of preterm birth and the associated morbidity and mortality.

### Introduction

Preterm birth represents the single greatest cause of death and disability in children up to five years in the developed world, disproportionately affecting Indigenous Australians and disadvantaged communities. Leading risk factors for spontaneous preterm birth include previous preterm birth, cervical insufficiency, and low socio-economic status. A growing body of research suggests that amniotic fluid sludge (AFS), a finding seen on ultrasound, is associated with increased risk of preterm birth, particularly when other risk factors are present. AFS is thought to be associated with infective and inflammatory processes within the amniotic sac, thus empiric treatment with antibiotics has become commonplace despite a lack of definitive evidence in the form of a randomised controlled trial to support this.

A number of observational and historical studies suggest that azithromycin given to women with AFS may reduce the risk of preterm delivery and prolong gestation. However, other studies claim that the use of azithromycin in this population has become commonplace without sufficient evidence of efficacy. Furthermore, there are currently no randomised controlled trials that evaluate the efficacy of oral azithromycin in eliminating AFS specifically as a primary outcome.

We propose a multi-centre, placebo-controlled double-blinded trial to assess the efficacy of oral azithromycin in eliminating AFS. Women at high risk for preterm birth will be recruited from specialist antenatal preterm birth clinics at three major maternity hospitals in Victoria. Eligible women will be randomised to receive either five days of oral azithromycin, or five days of placebo. The primary outcome of this study will be presence of AFS after treatment. Secondary outcomes will include reporting on a range of maternal and neonatal outcomes.

It is anticipated that the results of this pilot study will inform the development of a larger trial which will be adequately powered to determine whether treatment with oral azithromycin reduces the risk of preterm birth and the associated perinatal morbidity and mortality in women with AFS.

### Background information

Preterm birth, defined as birth prior to 37 weeks completed gestation, has an overall incidence of 8.7% in Australia [1]. In babies born to Indigenous mothers, the incidence of preterm birth is as high as 14% [1]. Globally, this represents the single greatest cause of morbidity and mortality in children up to five years in the high income countries [2]. In the short-term, prematurity is associated with higher rates of neonatal respiratory illness, necrotising enterocolitis, sepsis, neurological conditions, feeding difficulties and problems with vision and hearing [3]. In the medium-term, babies born preterm are more likely to experience developmental delay compared to term-born babies, particularly in the language domains [4]. Later in life, prematurity has been associated with increased risk factors for cardiometabolic disease including hypertension, lower lean body mass and impaired glucose regulation [5].

While approximately 30% of preterm births are iatrogenic due to a maternal or fetal indication(s) such as preeclampsia or fetal growth restriction, the vast majority occur spontaneously and may be associated with underlying risk factors such as previous preterm birth, sonographic short cervix or low socioeconomic status [6]. Studies show that short cervical length is associated with preterm birth, with a length below 25mm commonly used as a cut-off [7]. The shorter the cervix, the greater the risk of preterm birth [7]. Cervical shortening is linked with multiple aetiologies such as prior excisional cervical surgery [8], the declining actions of progesterone [9], intra-amniotic infection [10] and inflammatory processes [11]. Current treatments aimed at preventing preterm birth and prolonging pregnancy include progesterone therapy and cervical cerclage. RANZCOG guidelines recommend vaginal progesterone be offered to asymptomatic women with a short cervix below 25mm [12]. Progesterone should also be considered for women with a singleton pregnancy and a history of previous spontaneous preterm birth [12].

The presence of free-floating hyperechogenic material within the amniotic cavity in close proximity to the uterine cervix has been coined amniotic fluid “sludge” (AFS) [13]. Multiple studies have shown this sonographic finding to be an independent risk factor for spontaneous preterm birth [14-17]. Furthermore, AFS has also been shown to be associated with histological chorioamnionitis [17, 18], short cervix, obesity, previous preterm delivery, second trimester vaginal bleeding and cervical cerclage [14]. The prevalence of AFS amongst women with risk factors for preterm birth varies between studies. In a case series reported by Fuchs et al, prevalence of AFS was 7.4% [14], however this was a heterogenous cohort as it included women with threatened preterm labour as well as asymptomatic woman at high risk for preterm birth [14]. A retrospective study by Espinoza et al reported the prevalence of AFS in their population to be 22.6%, which included women in preterm labour with intact membranes [18]. Kusanovic et al reported a prevalence of AFS of 23.5% in asymptomatic women at high risk for preterm birth [19].

The composition of AFS has been investigated in several studies. Romero et al analysed the nature of AFS by collecting amniotic fluid in the region of the sludge under sonographic guidance for microscopic examination [10]. They reported that the amniotic fluid white cell count was markedly elevated and bacterial culture was positive for *Streptococcus mutans*, *Mycoplasma hominis* and *Aspergillus flavus.* Espinoza et al also analysed microorganisms isolated from the amniotic fluid of patients with AFS and reported positive growth of *Ureaplasma urealyticum* [18]. Romero et al suggest AFS is an indicator of microbial invasion and inflammation of the amniotic cavity. In a further study the same researchers used scanning electron microscopy to further characterise the appearance of AFS and found the bacteria to be arranged in a biofilm formation [20].

Other studies have investigated the relationship between cervical shortening and intra-amniotic infection. In a retrospective cohort study, Hassan et al concluded that 9% of women with sonographic short cervix had microbial invasion of the amniotic cavity with the most frequent organism cultured being *Ureaplasma urealyticum* [21]. Romero et al also reported a relationship between microbial invasion of the amniotic cavity and cervical dilation [10], supporting the theory that intraamniotic infection may be a *cause of* cervical insufficiency or may occur *secondarily* due to exposure of membranes to genital tract flora. The combination of AFS and cervical length of 25mm or less was shown to be more sensitive in predicting preterm birth than either of these measures alone in high-risk women [22].

Azithromycin is a second-generation macrolide with broad-spectrum activity imparted by its bacteriostatic, immunomodulatory and anti-inflammatory profile [23]. Macrolides are clinically active against Gram-positive cocci, Gram-negative cocci and Gram-negative bacilli with azithromycin having exhibited superior activity against Gram-negative pathogens in particular [24]. Intracellular pathogens such as *Chlamydia* species, *Mycoplasma* species and *Ureaplasma* species show variable susceptibility [24]. Azithromycin accumulates more effectively in cells than other macrolides, is derived in high concentrations at sites of infection and has an extended plasma half-life, thus allowing effective single dosing for acute bacterial infections [23]. Azithromycin is a pregnancy category B1 drug considered safe to use in pregnant and breastfeeding women [25].

The mean in vitro inhibitory concentration (MIC50) of azithromycin against *Ureaplasma spp*. ranges from 250ng/ml to 1000ng/ml [26]. In a study by Sutton et al, a 500mg intra-venous dose of azithromycin was given as an infusion 15, 30 or 60 minutes prior to planned caesarean section (CS) [26]. Maternal serum plasma samples were repeatedly drawn up to eight hours post-infusion, and all tissue samples were collected intraoperatively. This resulted in a plasma area under the curve (AUC) of 6030ng x hr/ml, with a median myometrial concentration of 402 ng/g, thus achieving the MIC50 required for prophylaxis against *Ureaplasma spp* in these two compartments [26]. However, amniotic fluid only reached a peak concentration of 33ng/ml, well below the MIC50 [26].In a similar study reported by Ramsey et al, 1 gram of oral azithromycin was given 6, 12, 24, 72 or 168 hours before elective CS with samples collected intraoperatively [27]. Ramsey et al recorded mean drug concentrations of 1792ng/ml in myometrial tissue (at 12 hours), 1041ng/ml in adipose tissue (at 6 hours) and 2130ng/ml in placental tissue (at 6 hours), thus exceeding the MIC50 in all three compartments and achieving higher concentrations than Sutton et al despite using an oral dose [27]. Importantly, these concentrations remained above the MIC50 for up to 72 hours after administration. They also observed a peak amniotic fluid concentration of 151ng/ml at 6 hours with an estimated half-life of 30 hours, which although below the MIC50 is still considerably higher than that reported by Sutton et al. [27]. It is important however to consider the greater time between drug administration and tissue sampling in the Ramsey study compared to Sutton et al, which may explain the higher tissue concentrations achieved. Finally, Fisher et al studied women who had been treated with oral azithromycin in pregnancy with 500mg-1g on day one followed by 250mg/day for another four days [28]. Although only plasma concentrations of azithromycin were measured, in non-African American pregnant females the plasma AUC for azithromycin was 21,000 ng x hr/ml [28]. Unfortunately, Ramseyet al did not report plasma AUC measurements, however the far higher AUC measurement reported by Fisher et al compared to that reported by Sutton et almay suggest slower oral clearance of azithromycin leading to greater systemic and amniotic fluid exposure. This may also be attributed to multiple dosing as opposed to a single IV dose.

Fuchs et al [14] reported a case series of women with AFS who all were treated with azithromycin and compared these to a historical control comprising those with AFS who were untreated. They concluded that azithromycin appeared to reduce the risk of preterm delivery before 34 weeks in the presence of AFS, however **they identified the need for a randomised placebo-controlled trial of antibiotic administration in the presence of AFS**. In an observational study, Hatanaka et al concluded that antibiotic treatment with macrolides in high-risk pregnant women with AFS can be effective in reducing the frequency of spontaneous preterm birth and can increase birthweight [29]. Another study argues that the empiric use of azithromycin in women at risk of preterm birth has become routine without sufficient evidence of efficacy [30]. In this retrospective observational cohort study, it was reported that women treated with azithromycin had greater risk factors for preterm birth however when adjusted for confounders there was no clear benefit that azithromycin prevented preterm birth [30]. A retrospective cohort study by Cuff et al is the only study that reports on the efficacy of azithromycin for resolution of AFS [31]. Although these authors concluded that the rate of resolution of AFS in treated and untreated women was not significantly different, this was not the primary outcome of the study and unfortunately assessment for resolution of AFS was not performed in all participants [31].

We conducted a retrospective study across three tertiary centres in Melbourne (Monash Hospital for Women, The Royal Women’s Hospital and Joan Kirner Women’s and Children’s Hospital) and demonstrated that there was no difference in the primary outcome of preterm birth prior to 37 weeks’ gestation amongst women with a short cervical length with or without AFS (≤15mm between 13 and 24 week’s gestation) who were treated with azithromycin compared to those who were not (Adjusted Hazard Ratio 1.36 (1.04 – 1.77), p = 0.299, n = 374, 129 treated with azithromycin, 245 not treated with azithromycin [32]. However, as this was an unblinded, retrospective study, it is plausible that those treated with azithromycin represented a much higher risk cohort compared with those who were not treated with azithromycin. In particular, those treated with azithromycin were more than four times more likely to have ultrasound appearances of AFS suggesting a much greater risk of preterm birth (79.8% AFS in those treated with azithromycin, 18.0% AFS in those who were not treated with azithromycin, p<0.001) [32].

Despite this conflicting evidence and the lack of randomised controlled trials demonstrating efficacy, Azithromycin is sometimes used in this cohort of women. Current guidelines, including those published by the World Health Organisation (WHO) [33], The Royal Australian and New Zealand College of Obstetrics and Gynaecologists (RANZCOG) [34] and National Institute for Health and Care Excellence (NICE) [35], do not provide any specific guidance regarding this therapy which is increasingly being offered in an ad hoc manner. Given the potential for this treatment to reduce preterm birth, and the associated morbidity and mortality, **there is an urgent need for a randomised controlled trial to determine the efficacy of this treatment.** While a definitive, appropriately powered trial to demonstrate the efficacy of azithromycin in prolonging gestation, reducing the rate of preterm birth, and preventing the neonatal morbidity associated with preterm birth is urgently needed, a pilot study to confirm the efficacy of azithromycin in eliminating AFS (an established risk factor for preterm birth) is essential.

# Study Objectives

### Hypothesis

In asymptomatic pregnant women with cervical length ≤25.0mm *and* AFS identified between 13+0-24+0 weeks’ gestation, treatment with azithromycin will result in eradication of amniotic fluid sludge two weeks after randomisation.

### Study Aims

The aim of this project is to determine whether azithromycin treatment in asymptomatic women at high risk for preterm birth is efficacious in eliminating AFS. We also aim to report on cervical length and high vaginal swab MCS and vaginal microbiome two weeks post-randomization and at 26+0 weeks’ gestation. We hope this will contribute to current knowledge surrounding the relationship between short cervix, AFS and preterm birth. We also seek to report on core maternal and neonatal outcomes as defined by the Core Outcomes in Women’s and Newborn health (CROWN) initiative [36], specifically the core outcomes set for Primary Prevention of Preterm Birth (COPOP) [37]. Finally, we will assess the feasibility of undertaking a larger multicentre trial to determine whether azithromycin is effective in preventing neonatal morbidity associated with preterm birth.

### Outcome Measures

The primary outcome for this pilot study is the presence of AFS two weeks post-randomisation (primary endpoint). Secondary outcomes will include change in cervical length, plus High vaginal swab MCS and vaginal microbiome two weeks post-randomisation and at 26+0 weeks’ gestation.

Secondary outcomes will also include pregnancy and neonatal outcomes as described in the CROWN initiative, specifically the Core Outcomes Set for Primary Prevention of Preterm Birth (COPOP). The CROWN initiative provides recommendations for reporting in women’s health research to address the widespread variation in reporting of outcomes which makes comparison between and combination of results across studies difficult [36]. Furthermore, these neonatal outcomes reflect the major complications associated with prematurity. Maternal and neonatal outcomes will be reported up to the point of discharge of mother and baby from hospital.

In order to assess the feasibility of undertaking a larger multicentre trial, we will collect data regarding feasibility measures such as the proportion of eligible women who consent, patient satisfaction with trial participation and the time taken to complete trial related assessments and data collection based on our experience with undertaking pilot trials [38].

**Table 1: Primary and secondary outcomes for this pilot study with further details and justifications.**

|  |  |
| --- | --- |
| **PRIMARY OUTCOMES** | |
| **Outcome** | **Details / justification** |
| Presence of AFS two weeks after randomisation | The primary outcome has been selected based on the association of AFS with intra-amniotic infection and preterm birth. Presence or absence of AFS after azithromycin treatment provides the most mechanistic measure of the efficacy of this antibiotic.  Two weeks post-randomisation was chosen pragmatically as the time of primary outcome measurement for several reasons outlined below:   * To allow sufficient time for azithromycin to resolve AFS such that the primary outcome can be measured categorically (ie AFS is either “present” or “absent”) * To reflect the usual timing of hospital visits typical for women with risk factors for preterm birth |
| **SECONDARY OUTCOMES** | |
| **Outcome** | **Details/justification** |
| Change in cervical length two weeks after randomisation and at 26+0 weeks’ gestation | This secondary outcome has been selected as despite cervical length being associated with AFS, the mechanism for this is less clear. The two time points of 2 weeks post-randomisation *and* 26+0 weeks accounts for different gestational ages at the time of recruitment and treatment. |
| High vaginal swab (HVS): microscopy, culture and sensitivity and maternal vaginal microbiome | This outcome has been included based on the association between AFS, short cervical length and microbial invasion of the amniotic cavity. In addition, Australian government guidelines recommend that testing for urogenital infection be offered to women who have risk factors for preterm birth (50). |
| Maternal mortality | Although maternal mortality is a rare outcome and we do not specifically anticipate any maternal deaths during this pilot trial, we will nevertheless report maternal deaths occurring between time of randomisation and discharge from hospital following delivery. This is one of the key recommendations outlined in the CROWN initiative to ensure we can harmonise the reporting of outcomes across any future trials in this area. |
| Maternal infection or inflammation | This outcome will be assessed based on either a clinical or histological diagnosis of chorioamnionitis. Included based on recommendations outlined in the CROWN initiative. |
| PPROM | Defined as spontaneous premature rupture of membranes before onset of labour prior to 37 weeks. Included based on recommendations outlined in the CROWN initiative. |
| PROM | Defined as prelabour rupture of membranes at term. Included based on recommendations outlined in the CROWN initiative. |
| Maternal adverse effects | Based on a questionnaire conducted two weeks after randomisation which will collect data on a range of side effects. Included based on recommendations outlined in the CROWN initiative. |
| Gestational age at birth | Was not chosen as the primary outcome as it was felt the current evidence does not yet support a large definitive trial to assess preterm birth as a primary outcome in the absence of a smaller, more feasible pilot study. Included based on recommendations outlined in the CROWN initiative. |
| Birthweight | Included based on recommendations outlined in the CROWN initiative. |
| Perinatal mortality | Including stillbirth and neonatal death. Included based on recommendations outlined in the CROWN initiative. |
| Early neurodevelopmental morbidity | This will be diagnosed based on the presence of Intraventricular Haemorrhage seen on cranial ultrasound or MRI scan if performed in the neonate up until the time of discharge. Included based on recommendations outlined in the CROWN initiative. |
| Gastrointestinal morbidity | Measured as Necrotising Enterocolitis. Included based on recommendations outlined in the CROWN initiative. |
| Infection | Measured as suspected or confirmed neonatal sepsis according to the definitions described by the Australian and New Zealand Neonatal Network [39], requiring treatment with intravenous antibiotics. Included based on recommendations outlined in the CROWN initiative. |
| Respiratory morbidity | Measured as diagnosis of respiratory distress syndrome (RDS), Bronchopulmonary Dysplasia (BPD)/Chronic lung disease (CLD) or requirement for respiratory support. Included based on recommendations outlined in the CROWN initiative. |
| ***Table 1:*** *Primary and secondary outcomes for this pilot study appear in the left-hand column. Information in the right-hand column provides additional details or justifications for each specific outcome.* | |

# Study Design

### Study Type & Design & Schedule

**Type of study:** The ASAPP trial is a triple-blind, randomised, placebo-controlled trial across three major maternity service providers in Victoria. This study will adopt a parallel group design with an allocation ratio of 1:1.

**Trial entry:** Women will be identified and counselled by trained research midwives. Written consent will be obtained. In accordance with the CONSORT statement for pilot and feasibility trials (46) we will collect data regarding the number of eligible participants, the number approached, and the number of participants who decline to take part in the trial.

**Randomisation and Treatment allocation:** Eligible, consenting women will be randomised using a computer-generated random number sequence. The randomisation schedule, using a 1:1 randomisation and variable block size will be prepared in advance. Randomisation will be stratified by cervical length (≤10mm OR 10.1-25.0mm), gestational age (<20+0 weeks OR ≥20+0 weeks), and clinical site.

Assignment is to either the ‘azithromycin group’ or the ‘placebo group’. A study number will be allocated to the participant that corresponds to the relevant trial treatment pack, each of which look identical and contain five tablets, either:

* 6x 250mg azithromycin tablets (for the treatment group)
* 6x placebo tablets (for the placebo group)

The azithromycin tablets and placebo tablets will be supplied by PCI Pharma Services. All drugs will be prescribed by a medical practitioner as “Azithromycin 250mg x6 doses” or “Placebo tablets x6 doses” and dispensed by hospital pharmacy staff. Two doses should be taken on day 1, and then 1 dose daily on days 2 to 5. Tablets should be taken on an empty stomach at least 30 minutes prior to food to maximise absorption.

**Treatment schedules:** Women in both the interventional and control groups will be given their treatment packs on the day of randomisation and instructed to commence the five-day treatment period the same day. Researchers, participants, and staff will be blinded to the treatment allocation.

**Standard Care:** Care for both groups of women will otherwise be according to standard practice. Management of women with cervical insufficiency can include vaginal progesterone or cervical cerclage. Both treatments are effective as demonstrated by randomised controlled trials and systematic reviews [40, 41], hence it would be unethical to prevent women from accessing these treatments. As the decision regarding these treatments varies among clinicians and can differ according to gestation and cervical length, we have not mandated strict protocols with respect to these management options. We anticipate that randomisation will achieve an adequate balance of these additional treatments.

**Concomitant Medications / Treatments:** All concomitant medications and treatments received by the mother at any time during the pregnancy will be recorded. There are no restrictions regarding concomitant medications except those known to prolong QT interval (which constitute an exclusion criteria).

**Compliance:** Compliance with the allocated treatment schedule will be recorded, however as analysis will be by intention to treat, non-compliance with the allocated treatment schedule (including failure to complete the five-day course) will not be regarded as an indication for exclusion or withdrawal from the trial. Women will be asked to return their used blister sheet at their two-week follow-up visit as a measure of compliance.

**Data Collection:**

Cervical length measurement*:* Cervical length will be measured at baseline, two weeks post-randomisation and at 26+0 weeks’ gestation following a standardized protocol as outlined by the RANZCOG guideline *Measurement of cervical length for prediction of preterm birth [42].* Cervical length will be reported as a continuous variable expressed in millimetres.

AFS measurement:Assessment for AFS will take place at baseline, two-weeks post-randomisation and at 26+0 weeks’ gestation. AFS will be assessed using the transvaginal sonographic approach following the same technique as above. AFS will be defined as “*presence of dense aggregates of particulate matter in the proximity of the internal cervical os”* [18]. Assessment of AFS will be binary (either “present” or “absent”).

Both measurement of cervical length and assessment for AFS will be undertaken by an experienced study clinician who is blinded to the treatment allocation. Validation of the sonographic appearances at all three study visits will be undertaken by a minimum of three study investigators who will review still images and stored cineloops to verify the cervical length and presence or absence of AFS blinded to the timepoint (before randomisation, at two weeks after randomisation and at 26+0 weeks’ gestation) and the treatment allocation.

High vaginal swabs for MCS and vaginal microbiome: A HVS will be performed at baseline, two-weeks post-randomisation at and 26+0 weeks’ gestation. Two samples will be taken, one swab for MCS and another swab for analysis of maternal vaginal microbiome.

Adverse effects from azithromycin: Women will complete a brief questionnaire during their two-week follow-up appointment which will evaluate any adverse effects experienced during the 5-day treatment period. Participants will record each symptom as either ‘present or absent’ during the treatment period, and any symptoms present will be ranked as ‘mild’, ‘moderate’, or ‘severe’. The following symptoms will be included in the questionnaire: nausea, vomiting, abdominal cramping/ pain, diarrhoea, dizziness, headache, heart palpitations, per-vaginal discharge, change in fetal movements. There will also be a free-text option where participants may record any additional symptoms. These symptoms are included as they are the most common symptoms associated with oral azithromycin or are common symptoms reported in pregnancy.

Maternal and neonatal CROWN outcomes: Data will be sourced with consent from the medical records of both mother and infant up until the point of hospital discharge. All study data will be stored centrally on the secure REDCap server and access to the data will be accessible only to study investigators. Participants may request access to their trial data. Data monitoring will involve independent review of 10% of participants to ensure accuracy with source data.

**Unblinding:** In the unlikely event that study participants experience a Suspected Unexpected Serious Adverse Reaction (SUSAR), the participant will be unblinded and treatment discontinued. For our study we identify a SUSAR as any of the following: anaphylaxis, severe allergic reaction, development of QT prolongation or Torsades de Pointes.

**Table 2: Proposed schedule of enrolment, interventions, key visits, and data collection.\***

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | TIMEPOINT | | Initial Visit/s | | At home | | | | Visit 2 | Visit 3 | Discharge data collection |
|  |  | **Gestation range** | 13+0 – 24+0 weeks’ gestation | | *after initial visit* | | | | | 26+0 weeks’ gestation | *after birth* |
|  |  | **Study days** | *as per gestation range* | D1 | D2 | D3 | D4 | D5 | D14±3 | *as per gestation timepoint ±7 Days* | *data entry within 4 weeks of discharge* |
| PROCEDURE | Informed Consent | | x |  |  |  |  |  |  |  |  |
| Demographic Information | | x |  |  |  |  |  |  |  |  |
| Randomisation | | x | |  |  |  |  |  |  |  |
| Dosing: Azithromycin or placebo | |  | x | x | x | x | x |  |  |  |
| Transvaginal cervical length and assessment of AFS | | x |  |  |  |  |  | x | x |  |
| HVS for MCS and vaginal microbiome | | x |  |  |  |  |  | x | x |  |
| Symptoms questionnaire | |  |  |  |  |  |  | x |  |  |
| Collection of birth and neonatal data | |  |  |  |  |  |  |  |  | x |
| Collection of maternal and neonatal outcome data | |  |  |  |  |  |  |  |  | x |

*\*Procedures are outlined in the left-hand column and relevant timepoints in the shaded horizontal rows. Procedures occurring at a certain timepoint are denoted by an X.*

# Study Population

### Recruitment Procedure

Eligible women will be recruited from the *Cervical Surveillance Clinic* at Joan Kirner Women’s and Children’s at Sunshine Hospital, the *Amethyst Clinic* at Monash Health, the *Preterm Labour Clinic* at the Royal Women’s Hospital, and corresponding clinics at Eastern Health. These are specialist obstetric services with a specific focus on preterm birth prevention. Women are typically referred to these clinics with a history of preterm birth or mid trimester pregnancy loss, deep or repeated cervical excisional procedures, congenital uterine anomalies or the finding of an incidental short cervix on a routine ultrasound scan [42]. These women undergo serial assessment of cervical length and may be offered cervical cerclage or vaginal progesterone.

### Inclusion Criteria

**Table 3: Inclusion criteria for this pilot study with further details and justifications.\***

|  |  |
| --- | --- |
| **INCLUSION CRITERIA** | |
| **Criterion** | **Details and justification** |
| Asymptomatic | Defined as absence of symptoms suggestive of labour at randomisation (including vaginal bleeding, loss of mucus plug, rupture of membranes, uterine contractions). Women with overt symptoms of premature labour are unlikely to benefit from this treatment and will more likely require intensive antibiotic treatment. |
| 13+0-24+0 weeks’ gestation | Range of gestational ages at which high-risk women are typically seen in Cervical Surveillance Clinic/Amethyst Clinic/Preterm Labour Clinic. Prevention of early preterm birth < 28 weeks is likely to have the greatest beneficial effect. |
| Cervical length ≤25.0mm | 25 millimetres or less is a commonly used cut-off for prediction of preterm delivery [7]. The mean cervical length for women with AFS has been quantified in several studies. In a Fuchs et al study, mean cervical length was 22.9mm in patients with AFS [14] and in another paper by Ventura et al, mean cervical length was 20.5mm [43]. |
| Sonographic appearance of AFS | Defined as the presence of dense aggregates of particulate matter in the proximity of the internal cervical os [18] as seen on transvaginal ultrasound. Sonographic appearance of AFS will be determined by the sonologist performing the ultrasound scan. As this is a subjective diagnosis, images demonstrating AFS for all participants will be curated and reviewed by the study investigators blinded to the timing (pre or post intervention) and treatment allocation. |

*\*Inclusion criteria for this pilot study appear in the left-hand column. Information in the right-hand column provides additional details or justifications for each specific inclusion criteria.*

### Exclusion Criteria

**Table 4: Exclusion criteria for this pilot study with further details and justifications.\***

|  |  |
| --- | --- |
| **EXCLUSION CRITERIA** | |
| **Criterion** | **Details and justification** |
| Multiple gestation | Excluded as multiple gestation carries a substantial risk of preterm delivery, accounting for 15-20% of all preterm births [44]. Uterine overdistention is thought to be the underlying mechanism [45] as opposed to intra-amniotic infection or inflammation. |
| Major or lethal fetal congenital abnormality | Including chromosomal, central nervous system and cardiac abnormalities. These conditions will be excluded as they may confound secondary newborn outcomes. |
| Placenta praevia, vasa praevia | Excluded as placenta praevia is associated with a very high risk of indicated preterm delivery in the presence of vaginal bleeding [46]. |
| PPROM | Defined as spontaneous premature rupture of membranes before the onset of labour, prior to 37 weeks. Excluded as antibiotic prophylaxis in this setting has been well established [47]. |
| Clinical signs suggestive of chorioamnionitis | Defined as temperature > 37.8 degrees C, uterine tenderness, tachycardia. Excluded as women with clinical evidence of chorioamnionitis should be treated with antibiotics and delivery expedited. |
| Indicated preterm delivery at time of randomization | Examples include preeclampsia, eclampsia, fetal growth restriction. Excluded as this will prevent primary outcome data from being collected 2 weeks after randomisation. |
| Known allergy to Azithromycin | Excluded to minimise the risk of maternal allergic reaction. |
| Use of medications likely to prolong QT interval | Serious adverse effects of Azithromycin include QT prolongation and torsades de pointes [48]. This is based on a retrospective cohort study which showed a small absolute increase in cardiovascular deaths most pronounced in patients with high baseline risk of cardiovascular disease [48]. Excluded for maternal safety reasons. |
| History of cardiac disease | Including congenital long QT syndrome, Torsades de Pointes, arrhythmia, and uncompensated heart failure. Excluded due to risk of QT prolongation and torsades de pointes as outlined above. |
| Known hepatic impairment | Azithromycin is hepatically cleared so hepatic impairment may alter azithromycin bioavailability. |

*\*Exclusion criteria for this pilot study appear in the left-hand column. Information in the right-hand column provides additional details or justifications for each specific inclusion or exclusion criteria.*

### Consent

Women will be identified and counselled by trained research midwives under the supervision of the research team. A patient information and consent form will be provided. Written consent will be obtained.

# Participant Safety and Withdrawal

### Risk Management and Safety

Australian guidelines identify azithromycin as a category B1 drug, which is safe to use in pregnant and breastfeeding women [25]. Azithromycin is generally well-tolerated with only a mild side effect profile, occurring in 1-5% of patients; these side effects typically include gastro-intestinal upset, headache and dizziness [25]. Serious but uncommon adverse effects include fatal arrhythmias such as QT prolongation and Torsades de pointes, which were reported in a retrospective cohort study that suggested an increase in cardiovascular deaths and deaths from any cause in people treated with a 5-day course of azithromycin [48]. This observed increase was said to be small, and most pronounced in those with a higher baseline risk [48]. To minimise this risk, any women with a history of Congenital long QT syndrome, Torsades de Pointes, arrhythmia, prolonged QT interval on baseline ECG or uncompensated heart failure will be excluded from this study. Azithromycin has not been associated with increased rates of fetal malformations above the baseline risk of 1-3% [49]. All participants will be provided with verbal and written information regarding the risks and benefits of receiving azithromycin and written informed consent will be documented.

Should participants experience psychological distress as a result of participation in this research, arrangements will be made for counselling or other appropriate support. Any counselling or support will be provided by qualified staff that are not members of the research project team. This counselling will be provided free of charge.

### Handling of Withdrawals

Participants who choose to withdraw from the study will be invited to continue to provide data for the study including birth, neonatal and maternal outcome data. Should they decline to contribute any data to the study, their data will be deleted from the database, and they will be regarded as a complete withdrawal from the study. Withdrawals will be specifically recorded and reported.

### Replacements

Replacements will be allowed in this study only in the event of withdrawals to ensure that the target sample size is achieved.

# Statistical Methods

### Sample Size Estimation & Justification

We estimate a minimum treatment effect rate (eradication of sludge) of 37.5% with oral azithromycin. This is based on an absolute estimate of a spontaneous eradication rate of 20% in the absence of treatment and an eradication rate of 50% with azithromycin. This effect rate has been guided by clinical consensus, as currently no trials assess the efficacy of oral azithromycin in resolving AFS. There is however some evidence to suggest that in some cases AFS may regress spontaneously [31]. A sample size of 84 participants (42 in each arm, allowing for 10% drop-out) will have 80% power to detect a relative difference in the rate of eradication of AFS of 37.5% with two-sided alpha of 0.05 (residual AFS in the placebo group after intervention of 80%, residual AFS in the azithromycin group after intervention of 50% - that is a 30% absolute reduction in the presence of AFS). This sample size will also provide over 80% statistical power to detect a 20% difference in the mean cervical length two weeks following treatment. This sample size was calculated using ClinCalc sample size calculator.

### Statistical Methods To Be Undertaken

All outcomes will be compared between the intervention and control groups using an intention-to-treat analysis, in line with recommendations by the CONSORT Group [50]. The proportion of women with sonographic appearances of AFS in both the treatment and control groups will be compared at 2 weeks following randomisation (primary endpoint) and at 26+0 weeks’ gestation (secondary endpoint) using the Chi squared statistic, as this represents categorical data. The mean cervical length in both the treatment and control groups will be compared at 2 weeks following randomisation and at 26+0 weeks’ gestation using the Mann-Whitney U test (assuming the cervical lengths will not be normally distributed), as this represents metric data.

All other secondary outcomes will be compared using either the Chi squared statistic (for categorical data), the t-test (for normally distributed continuous data) or the Mann-Whitney U test (for non-normally distributed continuous data). Where appropriate, logistic regression will be used to estimate the odds ratio (OR) and 95% confidence interval (CI) for the corresponding population OR, stratified by cervical length, gestation at recruitment and study site. Linear regression will be used for non-categorical outcomes.

We will undertake descriptive statistical analysis reporting the proportion of women screened, eligible, approached, consented, and refused.

# Supply and Accountability of Investigational Product

Stocks of investigational product will be purchased directly from PCI Pharma Services at a minimum order quantity for Azithromycin and Placebo. Sufficient stocks will be held to ensure no disruption in the trial supply.

### Drug Accountability

The Pharmacy Department at each clinical site will maintain a record of drugs dispensed for each participant and subsequent returns. The Pharmacy will also maintain a record of receipt and destruction for all study treatments.

# Data Security & Handling

### Details of where records will be kept & How long will they be stored

All hard copy data will be stored in locked filing cabinets located at the recruiting sites. All electronic data will be securely stored in a password protected REDCap database on a secure password protected server. All data will be retained for 25 years consistent with requirements for interventional studies in pregnancy. After 25 years all paper data will be destroyed by secure shredding. All electronic data will be deleted.

### Confidentiality and Security

All participants will be allocated an individual study number. This study number will allow re-identification of patient research data. The code linking the participant identifying data with the study number will be securely stored separately to the main database. All hard copy data will be stored at the recruiting sites. All electronic data will be securely stored in a password protected REDCap database on a secure password protected server.

# Safety Reporting

### Definitions

**ADVERSE EVENT (AE)** is any untoward medical occurrence in a participant administered an investigational product (or placebo) and which does not necessarily have a causal relationship with this treatment. 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 (see below).

Adverse events include the following:

* All suspected adverse drug reactions
* All reactions from drug or device – overdose, abuse, withdrawal, sensitivity, toxicity or failure of expected pharmacological action (if appropriate)
* Apparently unrelated illnesses, including the worsening (severity, frequency) of pre-existing illnesses
* Injury or accidents
* Abnormalities in physiological testing or physical examination that require clinical intervention or further investigation (beyond ordering a repeat examination)
* Laboratory abnormalities that require clinical intervention or further investigation (beyond ordering a laboratory test).
* Any untoward event that occurs after the protocol-specified reporting period which the Investigator believes may be related to the drug or device.

AEs are not required to be reported unless they meet SUSAR and/or Outcome criteria.

**SERIOUS ADVERSE EVENT (SAE)** is 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:**

(i) 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.

(ii) Important medical events which may not be immediately life-threatening or result in death or hospitalization 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.

(iii) Note that admission to hospital for birth is not regarded as an SAE, however unplanned admission to hospital prior to birth, or readmission following discharge from hospital after birth will be regarded as an SAE.

**A SUSPECTED UNEXPECTED SERIOUS ADVERSE REACTION (SUSAR)** is an SAE that:

* is related to the drug and is unexpected (i.e., not listed in the investigator brochure or approved Product Information; or
* is not listed at the specificity or severity that has been observed; or
* is not consistent with the risk information described in the Patient Information and Informed Consent Form or elsewhere in the protocol. (FDA, Safety Reporting Requirements for INDs and BA/BE Studies, draft guidance, September 2010).

An event is causally related if there is a reasonable possibility that the drug caused the AE, i.e., there is evidence to suggest a causal relationship between the drug and the event (FDA, Safety Reporting Requirements for INDs and BA/BE Studies, draft guidance, September 2010).

### Reporting of SUSARs

All SAE’s and SUSARs will be reported to the Trial Safety Monitoring Committee within 3 days of becoming aware of them by using the trial SAE / SUSAR form. The Trial Safety Monitoring Committee will commence investigation of the SAE / SUSAR within 7 days and provide a report to the HREC and Trial Steering Group within 21 days.

The following information will be recorded for each SAE / SUSAR and reported to the Safety Monitoring Committee**:**

* Event description
* Primary and secondary diagnoses of event (If death/hospitalisation)
* Severity
* Attribution to study intervention
* Action taken with study intervention, including rechallenge, if applicable
* Outcome of SUSAR including end date if recovered.

# Trial Committees

### Trial Steering Group

The ASAPP Study Steering Group will be chaired by A/Prof Joanne Said and will include Dr Penelope Sheehan, Prof Michelle Giles, and Dr Su Lynn Khong. Additional members will be coopted as necessary.

### Safety monitoring Committee

A Safety Monitoring Committee comprising an obstetrician, a neonatologist and an epidemiologist at minimum with established terms of reference will review all Serious Adverse Events and Suspected Unexpected Serious Adverse Reactions. The Safety Monitoring Committee will report to the Trial Steering Group.

# Publication Policy

The Trial Steering Group will appoint a Writing Committee to draft manuscript(s) based on the study data. If the study fails to achieve the expected recruitment target the Trial Steering Group may elect not to publish the data.

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