Official Title: Clinical Study of STI Screening to Prevent Adverse Birth and New-born Outcomes (Philani Ndiphile)

NCT Number: NCT04446611

Unique Protocol ID: R01AI149339

Document Date: 19 August, 2024

- PROTOCOL

R01 STI Screening and Microbiome Study:
Clinical Study of STI Screening to Prevent Adverse Birth and New-born Outcomes

Version 1.3

19 August 2024

Funding:

U.S. National Institutes of Health (NIH)

Swiss National Science Foundation (SNSF) (Aim 3)

Contents

STUDY TEAM

Desmond Tutu Health Foundation, South Africa & Perelman School of Medicine, University of Pennsylvania
Andrew Medina-Marino, PhD (co-Primary Investigator)

David Geffen School of Medicine, The University of California, Los Angeles (UCLA)
Jeffrey Klausner, MD (co-Primary Investigator)

Foundation for Professional Development, Research Unit, East London, South Africa
Remco Peters, MD, PhD (co-Investigator)

School of Public Health and Family Medicine, University of Cape Town
Susan Cleary, PhD (co-Investigator)

University of Alabama at Birmingham
School of Medicine
Christina Muzny, MD (co-Investigator)
Ashutosh Tamhane, PhD (Epidemiologist)
School of Public Health
David Redden, PhD (co-Investigator)
Department of Biostatistics
Dustin Long, PhD (Biostatistician)

School of Medicine, Louisiana State University
Christopher Taylor, PhD (co-Investigator)

South African National Health Laboratory Service
Koleka Mlisana, MBChB, MMedPath

Foundation for Professional Development, East London, South Africa
Freedom Mukomana, MEng (Data Manager)
Mandisa Mdingi, MSc (Research Manager)
Ranjana Gigi, MSc, (PhD student)

Institute of Social and Preventive Medicine, University of Bern, Switzerland
Nicola Low, MD (Co-Investigator: Primary Investigator for Aim 3)

Clinical Study of STI Screening to Prevent Adverse Birth and New-born Outcomes

SUMMARY

Purpose:	Prevalence of STIs is high among pregnant women in South Africa and most infections remain untreated. Untreated infections impact on pregnancy and birth outcomes. Good diagnostic and point-of-care (POC) tests are available, such as the GeneXpert platform. The health impact, cost-effectiveness and approaches to optimization of STI diagnostic screening during pregnancy are unknown.
Design:	Aim 1 and 2: 3-arm (1:1:1) randomized-controlled hybrid-effectiveness trial with Economic Evaluation and Qualitative component to determine implementation support. Aim 3: Cohort study to investigate associations between the presence of lower genital tract organisms in pregnancy and adverse pregnancy outcomes Aim 4: Nested case-control study to investigate the role of the vaginal microbiome in STI treatment outcomes Aim 5: Pilot sub-study investigating repeat *Trichomonas vaginalis* infections and prevalence of 5-nitromidazole drug resistance in pregnant women
Study Population:	HIV negative and HIV positive pregnant women presenting for antenatal care services (ANC)
Sample Size:	3334 pregnant women
Intervention:	Diagnostic testing at a woman's first antenatal care visit using the Xpert® platform with same-day treatment for *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Trichomonas vaginalis* infection with either a test-of-cure three weeks post-treatment (arm 1) or a repeat test at 30-34 weeks gestation (arm 2) compared to the standard of care, i.e. syndromic management (arm 3)
Study Duration	January 2020 – December 2024 (5 years)
Study Aims:	In order to 1) identify optimal, cost-effective screening strategies that decrease the burden of STIs during pregnancy and reduce adverse birth outcomes, 2) provide evidence to update WHO's syndromic management guidelines, and 3) elucidate the role of the vaginal microbiome in STI treatment and pregnancy outcomes, we propose five Specific Aims: 1) Evaluate different screening strategies to decrease the burden of *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Trichomonas vaginalis* among pregnant women and reduce adverse birth outcomes

	2) Evaluate cost per pregnant woman screened and treated, cost of adverse birth outcomes, and cost-effectiveness per STI and disability-adjusted life-year (DALY) averted 3) Investigate associations between the presence of lower genital tract organisms in pregnancy and adverse pregnancy outcomes through a holistic approach 4) Investigate the relationship between the vaginal microbiome and persistent chlamydial infections in pregnant women 5) determine the prevalence and predictors of repeat *T. vaginalis* infections in pregnant women on Arms 1 and 2 of the RCT and the prevalence of drug-resistant T. vaginalis including those with repeat infection
Study Sites:	Five primary healthcare facilities in Buffalo City Municipality, Eastern Cape Province

1 INTRODUCTION

Sexually transmitted infections (STIs) during pregnancy are associated with adverse birth outcomes such as preterm birth, low birth weight, perinatal death, and congenital infections including increased mother-to-child HIV transmission.[1-12] Though STIs are common in pregnant women globally, WHO's and South Africa's current syndromic management guidelines, focusing on symptomatic infections, continue to result in the majority of STIs (most of which are asymptomatic) remaining untreated during pregnancy.[13-18] We conducted a pilot study to examine the benefit, acceptability and feasibility of STI diagnostic screening whereby we integrated point-of-care molecular testing for *Chlamydia trachomatis, Neisseria gonorrhoeae, and Trichomonas vaginalis* into antenatal care (ANC) services (NICHD R21HD084274) for HIV-infected pregnant women in South Africa. We found diagnostic screening and immediate treatment during ANC to be highly acceptable and feasible;[19] 97.8% agreed to be tested and >93% received same-day treatment. Of 430 women screened, 41% had an STI (65% were asymptomatic).[19] Our intervention decreased prevalent STIs at time of delivery by >50% compared to women who received standard-of-care syndromic management.

Though acceptable, feasible and effective, our previous study had its limitations. First, we detected a 9.1% cumulative incidence of STIs between first ANC and delivery, suggesting a single diagnostic screening with appropriate treatment at ANC enrolment may not optimally decrease STIs at time of delivery. Second, our study was underpowered to demonstrate an effect on birth outcomes. Demonstrating the impact of diagnostic screening and treatment, compared to syndromic management, on birth outcomes will provide critical evidence to update WHO's syndromic management guidelines during pregnancy. Third, we found a 26.5% STI positivity at test-of-cure three weeks after the initial baseline visit and targeted treatment for STI detected. Though studies suggest that untreated partners are the primary cause of persistent STI positivity in women, in our study among women with a treated partner, persistent STIs were still high. Consequently, biological factors that increase the risk for STI persistence must be further investigated. Lastly, we did not conduct a cost-effectiveness analysis, which is urgently needed to inform policy design.

Research suggests that the vaginal microbiome and vaginal organisms play a critical role in STI acquisition, persistence, and treatment outcomes. Vaginal community state types (CST) with different concentrations of Lactobacillus (L.) species are associated with increased risk of acquiring STIs.[20-24] In vitro studies revealed certain vaginal bacteria can inactivate metronidazole,[25-27] standard TV treatment, and bacterial vaginosis (BV; CST-4) influenced TV treatment outcomes in HIV-infected women.[28] Vaginal microbiomes dominated by L. crispatus, L. gasseri and L. vaginalis may inhibit CT elementary bodies, while L. iners may increase the risk and duration of CT infection.

RESEARCH STRATEGY SIGNIFICANCE

HIV and STIs among pregnant women in South Africa are a major problem. In 2013, the South African government estimated that 29.7% of women seeking antenatal care (ANC) were HIV-infected,[29] a prevalence that has remained relatively stable since 2007. That high HIV prevalence is compounded by high rates of STIs in women of reproductive age.[30-32] Our recent study using molecular testing found 40.5% of HIV-infected pregnant women at their first ANC visit were infected with CT, NG and/or TV; 65% were asymptomatic (Table 1).[19] Given that most STIs in women are asymptomatic and that the South African government currently only recommends symptomatic screening and syndromic management in line with WHO guidelines, the majority of STIs in HIV-infected South African pregnant women go undiagnosed and untreated.

STIs are associated with adverse birth outcomes and mother-to-child-transmission (MTCT) of HIV. Untreated CT, NG and TV infections during pregnancy are associated with intrauterine growth retardation, low birth weight (LBW), preterm delivery, and premature rapture of membranes.[33-43] Infants in South Africa routinely receive chloramphenicol eye ointment at birth to prevent neonatal bacterial conjunctivitis, most often caused by untreated maternal CT or NG infection.[44] Yet the risks to infants born to HIV-infected mothers are greater than conjunctivitis. A study of HIV-infected women in Tanzania found that in the pre-ART era NG co-infection increased intrauterine HIV transmission by >450%.[2] Our team's prior work in an NICHD HPTN 040 sub-study demonstrated that CT/NG infection increased HIV MTCT by 160% (RR=2.6, 1.1 – 5.8).[9] Prior research in non-pregnant women suggests that STIs in HIV-infected women may augment the risk of HIV transmission by increasing localized inflammatory responses and viral shedding;[45-54] treatment of those STIs reduced HIV transmission.[55,56] Our own study in HIV-infected pregnant women in South Africa documented 34.8% (of 731) with adverse birth outcomes including 17.8% with preterm delivery, 14.8% low birth weight and 4.8% stillbirth (see Preliminary Studies section).[19]

Table 1: Prevalence of Chlamydia trachomatis (CT), Neisseria gonorrhoeae (NG) and Trichomonas vaginalis (TV) among HIV-infected pregnant women in three healthcare facilities in Tshwane District, South Africa (N=430)[19]

	N+	%	95% CI	% Asymptomatic
Any STI (CT/NG/TV)	174	40.5%	36.1% - 45.5%	64.9%
Any CT infection	127	29.6%	25.4% - 34.2%	62.6%
Any NG infection	24	5.6%	3.9% - 8.5%	50.0%
Any TV infection	86	20.0%	16.7% - 24.5%	53.6%

Reproductive tract infections in pregnancy are associated with preterm birth. Preterm birth is a condition with multiple predisposing factors and earlier gestational age at delivery is associated with increased morbidity.[57] According to Romero, et al. "Intrauterine infection (…) is the only pathological process for which a firm causal link with preterm birth has been established."[58] Ascension of organisms from the lower genital tract is considered the main route of infection. Infection might be a sole cause or might act with other risk factors, including multiple infections, through a common pathway of systemic inflammation.[59] Preterm labour also occurs in the absence of intrauterine infection. Inflammatory cytokines play a role in premature activation of inflammation, which may be the common pathway, even if infection has not reached the amniotic cavity.[60] Many genital tract infections, sexually transmitted infections, and the vaginal microbiota have been investigated and implicated in the occurrence of adverse birth outcomes (Figure 1).[61-71] Several of the implicated organisms are pathobionts: vaginal organisms that are non-pathogenic in non-pregnant women, but appear to be pathogenic during pregnancy.

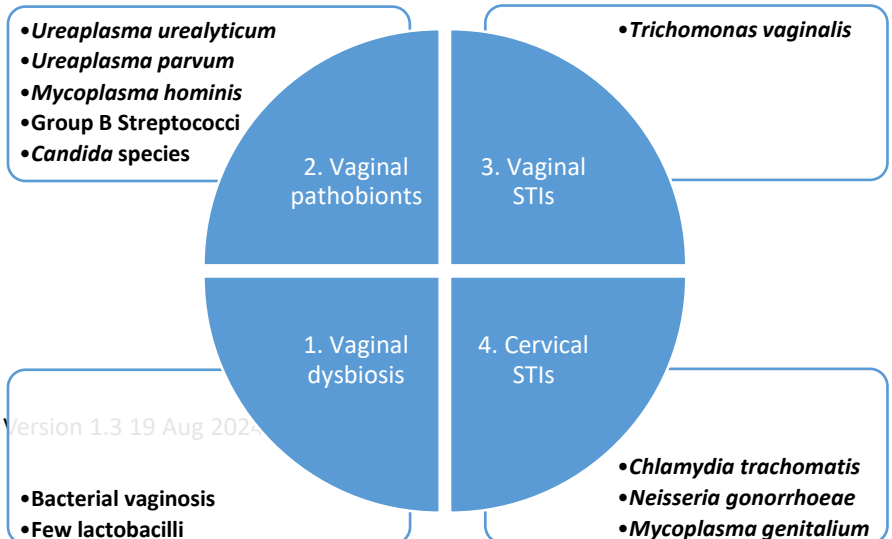


Figure 1 - Four groups of infections and vaginal conditions that have been implicated in preterm birth

Current WHO STI screening recommendations, especially during pregnancy, leave a large burden of disease undetected and untreated. WHO recommends syndromic management of STIs in resource-limited settings due to its low cost and the unavailability of appropriate laboratory infrastructure.[72,73] Syndromic management involves treating STIs based on an algorithm of common symptoms. As our own research[19] (Table 1) and others have shown, most STIs are asymptomatic and go untreated in settings where syndromic management is used.[18,74,75] Major limitations of syndromic management include: 1) non-determination of infectious etiologies, 2) limited specificity, especially during pregnancy, of "symptoms" algorithms, and 3) poor antibiotic stewardship with inappropriate treatment or over-treatment.[75,76] Diagnosis of STIs has traditionally relied on culture and microscopy; even when highly sensitive PCR assays became available, dedicated lab infrastructure and trained laboratory personnel were required.[77-79] However, with the advent of new, rapid, easy-to-use PCR-based 'near-patient' or 'point-of-care' (PoC) technology for the diagnosis of STIs,[80,81] our team has shown in multiple settings like Haiti, Vietnam, Botswana, Peru and South Africa that the implementation of diagnostic screening in variety of clinical settings is now possible.[19,80,82-85] Despite that, optimal models for PoC testing, especially during pregnancy, have not been identified. That is further highlighted by our recent work integrating PoC diagnostic screening for CT, NG and TV into ANC services for HIV-infected pregnant women in South Africa. Specifically, while single PoC screening, treatment and test-of-cure decreased the prevalence of STIs at time of delivery by >50% compared to syndromic management, incident infections were not identified or treated, leaving many women with STIs at time of delivery.

South African and international decision-makers require data on the cost and cost-effectiveness of STI screening and treatment programs. The South African National Strategic Plan for HIV, TB and STIs 2017-2022[81] includes recommendations for the detection and treatment of STIs, including through PoC testing. However, while some efforts are underway to plan for those interventions, to date, no South African study exists to inform those costing and budgeting efforts. Estimates from our proposed study can also inform policy decisions in other low-middle income countries, as well as WHO recommendations for the management of STIs during pregnancy. Ultimately, developing, evaluating and costing STI PoC testing algorithms, especially those implemented during antenatal care, is a very high global health priority (see letter of support from the WHO).

Risk factors associated with persistent STIs must be better understood. Given the increased risks of adverse outcomes from STIs during pregnancy, it is imperative that infections are cleared following treatment. This is especially important amongst HIV-infected pregnant women, where STIs may increase the risk of MTCT of HIV.

Table 2: High frequency of persistent STI positivity following standard treatment at Test-of-Cure (ToC), Pretoria, South Africa

	ToC 1	ToC 2	ToC 3
Any STI	36/136 (26.5%)	14/136 (10.3%)	7/136 (5.1%)

CT	27/102 (26.5%)	10/102 (9.8%)	3/102 (2.9%)
NG	1/16 (6.3%)	0/16 (0%)	--
TV	11/66 (16.7%)	5/66 (7.6%)	4/66 (6.1%)

As part of our recent study integrating molecular screening for CT, NG and TV into ANC services, we performed test-of-cure until a participant cleared their infection, or had a documented birth outcome.[86,87] At the first test-of-cure, 26.5% were persistently positive; a number of women required multiple rounds of treatment before clearing their infection (**Error! Reference source not found.**). Interviews with women suggest that behaviours associated with poor treatment adherence or re-exposure from untreated partners cannot fully explain the high persistent positivity with CT or TV.[88] For those with a positive TV test following treatment, evidence is mounting that clinical treatment failure, rather than organism-specific metronidazole resistance or reinfection, is likely.[28,88-90] Gatski et al.[89] revealed that in HIV+/TV+ women, concomitant BV was significantly associated with metronidazole treatment failure, suggesting that the vaginal environment associated with BV decreased the efficacy of metronidazole. This hypothesis is supported by in vitro studies that have shown that metronidazole can be inactivated by certain bacteria present in the vaginal microbiome.[25-27] Repeat CT positivity following treatment is not well understood; CT antimicrobial resistance is rare.[91] Reports have suggested that heterotypic resistance associated with high organism loads may factor in persistent infections; however, the evidence is limited.[91-94] Given that multiple rounds of repeated test-of-cure testing and treatment are not cost-effective in resource constrained settings, further understanding the biological mechanisms that contribute to persistent infections is imperative.

Inflammatory cytokines to identify STIs and BV. Three cervicovaginal cytokines (IL-1α, IL-1β and IP-10) have been identified which consistently predict the presence of asymptomatic inflammatory sexually transmitted infections and bacterial vaginosis, which have been validated in multiple cohorts of non-pregnant women in different regions in sub-Saharan Africa.[95,96] In addition to identifying asymptomatic inflammation caused by STIs and BV in non-pregnant women, inflammatory cytokine levels may be useful to identify these infections/conditions during pregnancy. This strategy could be implemented in the antenatal setting as a triage tool prior to etiological testing, reducing the number of women needing expensive STI/BV diagnosis. However, as the absolute concentration cut-offs required to classify women as STI/BV positive or negative may differ between non-pregnant and pregnant women,[97,98] it is essential to evaluate these biomarkers in pregnant women and possibly modify the device specifications.

Holistic studies of a wider range of vaginal infections, STIs and vaginal microbiota are needed to improve understanding of their role in preterm birth. The quantity and certainty of evidence implicating different vaginal and cervical organisms in the aetiology of preterm birth is inconsistent and heterogenous. Importantly, Goldenberg has hypothesised that the multifactorial nature of preterm birth "may explain why attempts to reduce preterm birth by attacking 1 risk factor during pregnancy often fail."[99] The reasons for the inconsistency in evidence for a particular organism might be biological, related to factors such as pathogenicity, timing of infection during pregnancy, or biological interactions with other infections. Most studies reviewed examine the association between a single infection found on one occasion and the outcome of preterm birth. Even in studies in which multiple pathogens were detected, results are often reported for single infections.[61] In a review of the role of genital mycoplasmas and ureaplasmas, Capoccia and colleagues noted that the whole vaginal microbiota is of importance and that studies of organisms in combination, and over time, are needed.[63] van de Wijgert has also stressed the importance of co-infections, interactions with disruption of the vaginal microbiota, and the need to consider the quantity as well as types of bacteria.[100,101] Fettweis and colleagues identified patterns of bacterial taxa in the vaginal microbiome that were associated with both inflammatory cytokine levels and preterm birth in women in the USA.[71] Demonstration of the reproducibility and consistency of findings in a different population would add to evidence for a causal role of specific combinations of organisms in preterm birth.

Vaginal microbiota may play an important role in STI treatment outcomes and an important role in genital CT infections.[102-104] Epidemiological studies have demonstrated that BV is associated with an increased risk of acquiring and transmitting HIV and other STIs.[47,105-113] Culture-independent studies of vaginal bacterial communities have revealed that BV is highly associated with vaginal community state types (CSTs) that are deficient in Lactobacillus spp., especially Lactobacillus (L.) crispatus,[21,114-116] and that these CSTs are associated with STIs such as CT and TV.[22,23,117] However, there are little data on the role of the vaginal microbiota on CT treatment outcomes in women.

Women with CT are more likely to have vaginal microbiota dominated by L. iners or diverse anaerobic bacteria.[22] In addition, risk of genital CT increases during BV episodes.[118] Interferon-gamma (IFN-γ), a host pro-inflammatory cytokine known for its anti-chlamydial properties, is an important part of the host immune response to genital CT infection. IFN-γ activates indoleamine[2,3]-dioxygenase in host epithelial cells, which then catabolizes L-tryptophan into N-formylkynurenine. When that happens, the host cell's pool of tryptophan is depleted, which may result in CT eradication by tryptophan starvation. In vitro, genital CT strains have been found to rescue themselves by producing tryptophan from indole using a tryptophan synthase gene when indole is present in the local environment.[102] Indole-producing bacteria (e.g., Prevotella spp,[102] Fusobacterium nucleatum, Propionibacterium acnes, Porphyromonas gingivalis, Escherichia coli, and Enterococcus faecalis) present in altered vaginal microbiota may contribute to genital CT survival by providing a source of indole. It is currently unknown if treatment for genital CT is inactivated by certain bacteria, or if the presence of indole producing bacteria in an altered vaginal microbiome increase the risk for poor treatment outcomes. Consequently, additional research on the role of the vaginal microbiome in genital CT treatment outcomes is urgently needed, particularly in pregnant women where the adverse effects of CT infection are substantial.

The prevalence of repeat *T. vaginalis* infection and drug resistant *T. vaginalis* in pregnant women in Africa is not well understood. Repeat *T. vaginalis* infections are common and have been estimated to range between 5-31% in women across various age ranges and geographical locations .[119-122] This may be due to multiple factors including re-infection from an untreated sexual partner, infection from a new sexual partner, or sexually-associated transmission events (i.e. sharing of infected sex toys, wet wash clothes, bathing water), inconsistent use of soap, or other intravaginal practices. [123] [124] [125,126] [127] If re-infection from all of these entities is ruled out, repeat infection may be due to lack of adherence to prescribed multi-dose therapy (i.e. multi-dose oral metronidazole) [128] or drug resistance[129] should be considered. Depending on the suspected cause of repeat *T. vaginalis* infection, various approaches should be taken including partner treatment, patient counseling regarding the need for 100% adherence to medication, alternative treatment regimens, patient counseling on not sharing wet wash clothes, sex toys, etc. In cases of suspected drug resistance, *T. vaginalis* culture and drug susceptibility testing should be performed. The prevalence of repeat *T. vaginalis* infection among pregnant women is largely uncharacterized, particularly among women in low and middle-income countries. *T. vaginalis* resistance to oral MTZ has ranged from 4.3-9.6% among various studies conducted worldwide; TDZ resistance has been found to be lower at <1% .[130-132] There are minimal data on the prevalence of drug resistance to SEC. A systematic review conducted to review the frequency of MTZ resistance noted high levels of *in* vitro MTZ resistance was rare however there was an overall lack of prospective controlled studies. [133] The prevalence of *T. vaginalis* resistance to SEC is unknown, however several studies note that it may have better in vitro activity compared with MTZ. [134,135] However, the minimal lethal concentration (MLC) of these drugs does not necessarily correlate with clinical outcomes in patients, which may indicate that resistance may be relative rather than absolute. [131] A South African study examining *T. vaginalis* resistance to MTZ in cultured specimens obtained from pregnant women observed a rate of resistance of 9.5% [136], however this study did not examine the prevalence of resistance in cases of reinfection. Thus, additional research on *T. vaginalis* drug resistance in pregnant women is needed.

2 STUDY AIMS

Aim 1: To evaluate different STI screening strategies that aim to decrease the burden of *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis* among pregnant women, and to reduce STI-related adverse birth outcomes. This is established through three objectives:

a) To compare molecular diagnostic testing for *Chlamydia trachomatis, Neisseria gonorrhoeae and Trichomonas vaginalis* (Arms 1+2 Treatment Groups) to the standard of care (i.e. syndromic management; Arm 3 Control) in reducing the frequency of adverse birth outcomes (e.g., preterm delivery, low birth weight, stillbirth/miscarriage).

b) To compare the effectiveness of single point-in-time diagnostic screening plus test-of-cure three weeks post-treatment (Arm 1 Treatment Group) vs. repeated diagnostic screening at 30-34 weeks gestation (Arm 2 Treatment Group) in reducing prevalent and incident STIs at time of delivery

c) To collect process measures to inform future implementation and scale-up.

Aim 2: To evaluate cost-effectiveness of different STI screening approaches to inform guideline and policy design.

a) To conduct a cost-effectiveness analysis including the cost per pregnant woman screened and treated, cost of adverse birth outcomes, and cost-effectiveness per STI and DALY averted.

Aim 3: To investigate associations between the presence of lower genital tract organisms in pregnancy and adverse pregnancy outcomes through a holistic approach. This is achieved through three objectives:

a) To determine the strength of association between the presence of specified vaginal and sexually transmitted organisms, and combinations thereof, and gestational age at delivery

b) To determine the strength of association between the quantified load of specified vaginal and sexually transmitted organisms, and combinations thereof, cytokine concentrations, and gestational age at delivery

c) To explore the combinations of vaginal and endocervical organisms that are associated with earlier gestational age at delivery.

Aim 4: s. This is achieved through two objectives:

a) To determine the impact of vaginal microbiota on *Chlamydia trachomatis* treatment outcomes

b) To explore the natural history of the vaginal microbiome in the context of antibiotic treatment for *Chlamydia trachomatis* infections

Aim 5: To determine the prevalence of *T. vaginalis* infections and the origins of repeat infection in pregnant women and its relation to the prevalence of drug-resistant *T. vaginalis.* This is achieved through two objectives.

a) Examining the prevalence and origins of repeat *T. vaginalis* infections among pregnant women enrolled in Arms 1 and 2 of the parent clinical trial examining STI screening to prevent adverse birth and newborn outcomes

b) Investigate the prevalence of drug-resistant *T. vaginalis* including those with repeat infection by performing 5-Nitromidazole susceptibility testing per modified CDC protocol

PRELIMINARY STUDIES

In Support of Aim 1

Aim 1: Evaluate different screening strategies to decrease the burden of *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis* among pregnant women, and reduce adverse birth outcomes

(All from Medina-Marino/ Klausner NIH R21HD084274):

Acceptability/Feasibility of STI testing among HIV-infected pregnant women, South Africa: We enrolled 845 HIV-infected pregnant women attending ANC. Of 442 eligible women offered *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis* testing using self-collected vaginal swabs, 430 accepted screening (Acceptability= 97.3%).[137] All women had valid test results; >95% received test results within 90 min. Among the 174 women with a positive test result, 92% (n=159) received same-day treatment. Our results demonstrate that integrating diagnostic testing for STIs into ANC services is acceptable and feasible, and that our study team has the capacity and experience to conduct the proposed study with high enrolment and implementation fidelity.

Test of Cure and treatment outcomes: Among 174 STI-positive participants at first ANC, 78% (n=136) returned for a test-of-cure 3 weeks later. Of those, 26.5% (n=36) had any positive result (*Chlamydia*= 26.5%; *Trichomoniasis*= 16.7%; *Gonorrhoea*= 6.3%).[93] Interviews revealed 91.7% of women reportedly disclosed their results to their partner(s), and 64.7% of partners either accepted a partner treatment packet or sought care at a clinic. Interviews suggested behaviours associated with re-infection or poor medication adherence cannot account for the high persistent positivity after treatment.[138] Those findings suggest that a single diagnostic test with immediate treatment may not optimally decrease STIs at time of delivery. Furthermore, biological mechanisms that increase the risk for STI persistence must be further investigated.

STI incidence during pregnancy and prevalence at time of delivery: Among 430 women tested and treated for CT/NG/TV at first ANC, we identified a 9.1% cumulative incidence of STIs between first ANC and delivery. Furthermore, our screening intervention decreased prevalent STIs by >50% compared to women receiving syndromic management (RR = 0.52; Intervention=11.1%, 95% CI: 7.9%-15.5%; Control=21.2%, 95% CI: 16.7%-26.6%).[139] While a single molecular test and treatment approach may decreased prevalent STIs at delivery, it cannot identify incident STIs. Optimal, cost-effective screening algorithms are needed to identify incident infections and decrease the risk of sequel associated with STIs in pregnant women and neonates.

Linkage and utility of national databases for data optimization: We captured unique bar codes of all requested laboratory tests and used this to query the National Health Laboratory Service (NHLS) lab information system (LIS) for maternal syphilis, CD4, HIV viral load, and infant HIV PCR results. Of those tested, we were able to obtain results for 87% of all syphilis tests (1.2% prevalence) and 100% of infant HIV PCRs (0.6% positivity). For those with CD4 and HIV viral load test results not recorded in medical charts, we obtained 85.4% and 80.5% of missing values, respectively, using both the NHLS-LIS and National HIV database (Tier.Net). We will similarly leverage the use of national datasets to ensure completeness of all study variables.

In Support of Aim 2

Aim 2: Evaluate cost per pregnant woman screened and treated, cost of adverse birth outcomes, and cost-effectiveness per STI and DALY averted

Cost-effectiveness modelling for ANC STI interventions (Klausner; P30MH058107): In Botswana, we conducted micro-costing, including time-and-motion studies and provider interviews, to identify capital and recurrent costs of antenatal STI testing interventions, compared to syndromic management. By combining those data with population and epidemiological data from Botswana, and probabilities from the literature, we developed a decision model comparing three approaches for national scale-up of STI testing. Our model revealed that a mixed approach to scale-up, including both PoC and centralized testing, had the lowest cost per STI treated.[140] By extending our model to include health outcomes (i.e., maternal infections at delivery, low birth weight infants, and DALYs averted), our model showed that, diagnostic testing for STIs during ANC services can be cost-effective if policy makers are informed by the WHO Gross Domestic Product / capita threshold. However, identifying the most cost-effective testing algorithms require further research. This work also shows that our study team has the capacity and experience to conduct the proposed study.

In Support of Aim 4

Aim 4: Investigate the relationship between the vaginal microbiome and persistent Chlamydial infections in pregnant women

Pathogenesis of BV in African American women who have sex with women (Muzny; K23AI106957). We followed women prospectively for incident BV (iBV; Nugent score 7-10, at least 2-3 consecutive days) with daily self-collected vaginal swabs for 90 days. For women with iBV or maintaining normal vaginal flora (NVF), we performed 16S rRNA sequencing targeting V4 was specimens for 21 days prior to iBV; raw MiSeq reads processed via DADA2. Species-level taxonomy was assigned to variants using PECAN110 and merged with RDP assigned taxonomy using GreenGenes13_5. Longitudinal microbiome data for BV-candidate bacteria and lactobacilli of interest were analyzed using phyloseq library. Of 31 participants completing the study, 14 (45.2%) developed iBV; 448 specimens were sequenced (14 women with iBV; 8 women maintaining NVF). Relative abundance of G. vaginalis, P. bivia, A. vaginae, and Megasphaera-type1 became significantly higher in women with iBV 4 days before, 3 days before, and day of iBV (A. vaginae and Megasphaera-type 1), respectively.[141] Novel methodologies from this study will be incorporated into Aim 4.

Consequences of the vaginal microbiota on IFN γ-mediated clearance of *Chlamydia trachomatis* (CT) (Taylor; 1R01AI118860-01A1). We are assessing the influence of the vaginal microbiota on the incidence of CT clearance without treatment. Vaginal swabs from women with persistent or spontaneous *Chlamydia* clearance are 16S rRNA gene sequenced, targeting the V4 region, and DADA2 pipeline processed and taxonomy is assigned using the RDP classifier[141] and silva version 128 database.[142] Preliminary results show a prevalence of indole-producing microbiota in the vaginal microbiome of women with persistent *Chlamydia* infection, and a lack of indole-producing microbiota in women who cleared infection without treatment. Those results further support our rationale for studying the vaginal microbiome in pregnant women with persistent *Chlamydia* infections.

Effect of BV on CT organism load and treatment outcomes (Muzny; U19AI113212). We are investigating the relationship of BV with 1) *Chlamydia* organism load, and 2) time to *Chlamydia* DNA clearance after treatment with 1g azithromycin in non-pregnant *Chlamydia*-infected women. To date, 17 *Chlamydia*-infected females have been assessed. We have found a general trend towards a longer median *Chlamydia* DNA clearance time in women with BV (2 days longer, p=0.286); when G. vaginalis and other anaerobic gram-negative rods are seen on Gram stain, 3 days longer (p=0.221); with lactobacilli not seen on Gram stain, 7 days longer (p=0.155); and with a vaginal pH >5, 3.5 days longer (p=0.123).115 Higher vaginal pH correlated with higher baseline log10 *Chlamydia* load (p=0.0352), with a trend in higher Nugent score correlating with higher baseline log10 *Chlamydia* load (p=0.114). Those preliminary data suggest that women with altered vaginal microbiota take longer to clear their *Chlamydia* infection, supporting our aim to investigate the role of the vaginal microbiome in persistent *Chlamydia* infection among pregnant women.

3 METHODOLOGY: AIMS 1, 2 AND 5

3.1 DESCRIPTION OF THE STUDY DESIGN

The study will use an effectiveness-implementation hybrid type 1 three-arm (1:1:1) RCT to address the main study objectives:

1) Evaluate different screening strategies to decrease the burden of *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis* among pregnant women, and reduce adverse birth outcomes

2) Evaluate cost per pregnant woman screened and treated, cost of adverse birth outcomes, and cost-effectiveness per STI and disability-adjusted life-year (DALY) averted

3) Determine the prevalence and origins of repeat *T. vaginalis* infection and drug-resistant *T. vaginalis* among pregnant women in Arms 1 and 2 of the parent RCT

STI screening and treatment for *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis* will be offered to HIV-infected and non-infected women (age >18 years) whom present for first antenatal care services. An effectiveness-implementation hybrid type 1 three-arm (1:1:1) RCT, will be employed to evaluate different screening strategies to decrease the burden of *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis* among pregnant women, and reduce adverse birth outcomes.

Arm 1) Treatment Group: single point-in-time molecular PoC diagnostic screening and treatment for *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis* at first ANC visit and infection-specific test-of-cure 3 weeks post-treatment. Women with a positive test-of-cure will be re-treated. As CT/NG is a combined Xpert test, women who present with an incident infection (newly diagnosed infection) will be treated and managed accordingly. In women with a positive *T. vaginalis* Xpert test, Inpouch cultures will be obtained.

Arm 2) Treatment Group: repeated molecular PoC diagnostic screening and treatment for *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis* at first ANC visit and at week 30–34 gestation. No test-of-cure will be conducted for women with positive test results; however, additional treatment will be provided to women with persistent/recurrent vaginal discharge. In women with a positive *T. vaginalis* Xpert test, Inpouch cultures will be obtained.

Arm 3) Control Group: syndromic management (standard of care) at every ANC visit per South African National Guidelines.[143,144] Participants will be followed up until the postnatal and six-week infant immunization visit to collect pregnancy and birth-outcomes data as well as neonatal health outcomes (morbidities or mortality).

The costs of the different STI screening strategies relative to control will be estimated based on literature review and performance/implementation characteristics and compared, in addition to the costs of managing adverse birth outcomes. Decision analytic modelling will estimate the cost-effectiveness per STI, and DALY averted (Aim 2).

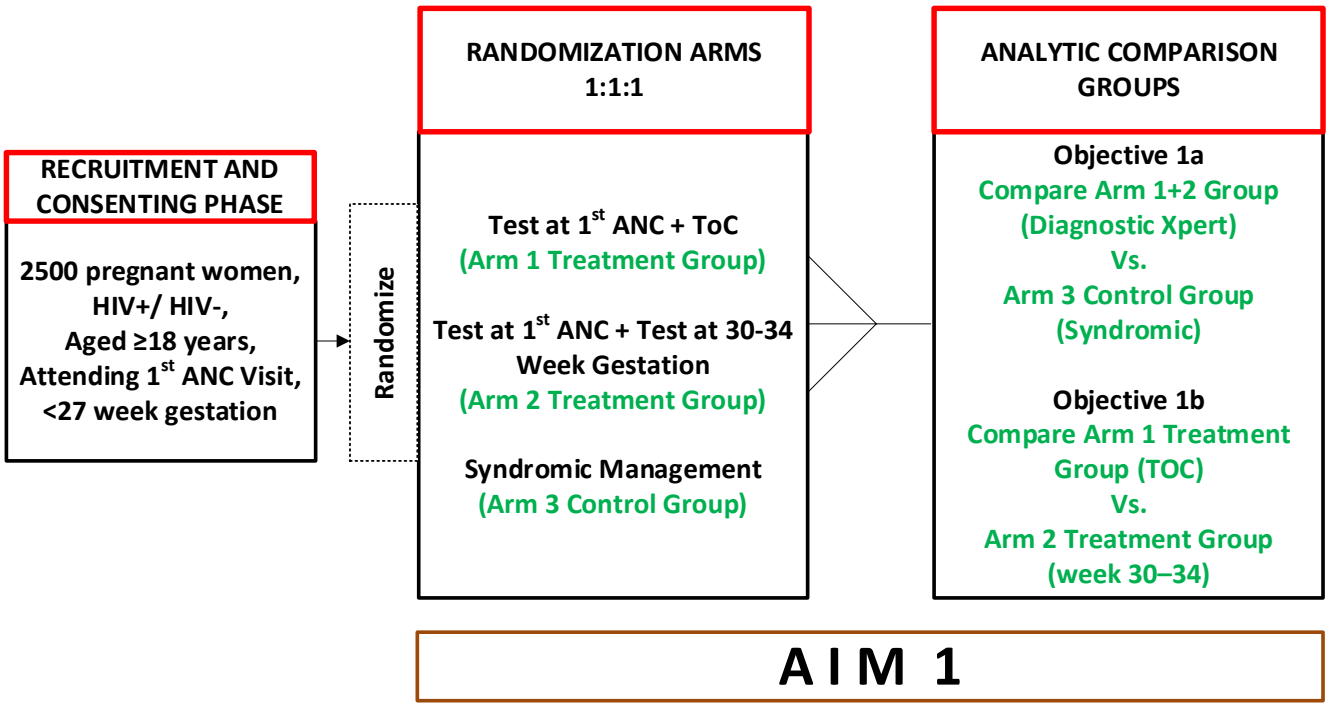


Figure 2 - Study Diagram for Aim 1 - Randomization Arms and Analytic Comparison Groups

3.2 HYPOTHESES

Aim 1: Evaluate three different screening strategies to decrease the burden of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Trichomonas vaginalis* among pregnant women, and reduce adverse birth outcomes

Hypothesis 1: Compared to syndromic management, diagnostic STI screening and targeted treatment will significantly reduce adverse birth outcomes.

Hypothesis 2: Compared to diagnostic screening with follow-up test-of-cure (ToC) three weeks post-treatment, repeat screening and treatment without any ToC at 30-34 weeks gestation will significantly decrease STIs at delivery

Aim 2: Evaluate cost per pregnant woman screened and treated, cost of adverse birth outcomes, and cost-effectiveness per STI and disability-adjusted life-year (DALY) averted

Hypothesis 1: Compared to syndromic management, STI diagnostic screening will cost-effectively avert STIs at delivery, and reduce adverse birth outcomes.

Aim 5: Determine the prevalence and predictors of repeat *T. vaginalis* infections in pregnant women on Arms 1 and 2 of the RCT and the prevalence of drug-resistant *T. vaginalis* including those with repeat infection

Hypothesis 1: The prevalence of repeat *T. vaginalis* infections in South African pregnant women will be high at 10% (either due to re-infection or drug resistance).

Hypothesis 2: The prevalence of *T. vaginalis* drug resistance to 5-nitroimidazoles (MTZ, TDZ, and SEC) will be between 5-10% based on studies conducted in the U.S. [130]

3.3 DESCRIPTION OF INTERVENTIONS

In order to evaluate different screening strategies to decrease the burden of *Chlamydia, Gonorrhoea* and *Trichomoniasis* among pregnant women, participants will be randomized at first ANC on an individual level within each clinic, into one of the following arms:

Arm 1: Treatment Group

Single point-in-time molecular PoC diagnostic screening and treatment for *Chlamydia, Gonorrhoea* and *Trichomoniasis* at first ANC visit and infection-specific test-of-cure 3 weeks post-treatment. Women with a positive test-of-cure will be re-treated.

Arm 2: Treatment Group

Repeated molecular PoC diagnostic screening and treatment for *Chlamydia, Gonorrhoea* and *Trichomoniasis* at first ANC visit and week 30–34 gestation. No test-of-cure will be conducted for women with positive test results.

Arm 3: Control Group

Participants will be provided standard of care syndromic screening and management for STIs per South African National Guidelines.[145,146]

T. vaginalis InPouch cultures will be obtained from pregnant women who test positive for *T. vaginalis* at study enrollment and at follow-up visits per the GeneXpert *T. vaginalis* NAAT in both Arms 1 and Arms 2 of the parent RCT.

3.4 STUDY SETTING

Aim 1 and 2 study components will be conducted in four ANC clinics in Buffalo City Municipality (BCM), Eastern Cape Province. BCM has approximately 700 000 inhabitants. The general population HIV prevalence in the district is 14% and antenatal HIV prevalence of 31.2%, above the national average of 29.7%. The Foundation for Professional Development's research unit is currently head-quartered in East London.

3.5 METHODS AND PROCEDURES

3.5.1 OVERVIEW STUDY VISIT AND PROCEDURE SCHEDULE

Participants will remain in the study until the six-week infant immunization visit post-delivery. Depending on the randomization arm, participants will be scheduled to be seen various times throughout pregnancy by the study team; ANC visits will be conducted in line with national policy. All post-partum mothers and infants will be asked to be seen at the first post-delivery clinic visit. Table 3 below shows the STI testing time-points for the different arms.

Table 3: STI Testing Schedule Per Randomization Arm

Clinic Visit	Participant	Specimen Collected	CT, NG and TV Testing

First ANC Visit	**All Pregnant Women**	**Vaginal Smear** **Vaginal Swabs** **Urine**	**Arm 1 and 2 Only**
ToC 3-Weeks Post-treatment	**Arm 1 Only**	**Vaginal Smear** **Vaginal Swabs**	**Arm 1 Only**
30 – 34 Weeks Gestation	**Arm 2 Only**	**Vaginal Smear** **Vaginal Swabs**	**Arm 2 Only**
First Post-delivery Clinic Visit	**All Post-partum Mothers**	**Vaginal Swabs**	**All Post-partum Mothers**
First Post-delivery Clinic Visit	**All Infants**	**Nasopharyngeal Swabs** **Conjunctival Swabs**	**All Infants***

* Post-delivery infant swabs will only be tested if the maternal swab is positive

3.5.2 RECRUITMENT AND ENROLMENT

Women presenting for antenatal care services at the four ANC clinics selected for Aim 1 and 2 study activities will be approached to participate in the study. All pregnant women will be screened for eligibility by study staff following standard HIV testing per South African National Guidelines. Both HIV-infected and un-infected pregnant women will be invited to participate into the study. Staff will read all interested women a brief study description. Eligibility will be determined based on a screening tool comprised of an eligibility criteria checklist (further detail follows below). Interested eligible women will then be read together with the study staff, in their preferred language, the study consent form and will be invited to participate. Those providing informed consent will be enrolled and randomized into one of the 3 study arms; randomizations will be allocated in blocks of 15 with a 1:1:1 randomization into the 3 study arms.

Recruitment and enrolment of participants for STI study will be done by STI Test Counsellors and Research Nurses based at the clinic.

Study staff will record reasons for ineligibility/refusal, if provided.

Figure 3 shows the participant recruitment and enrolment flow:

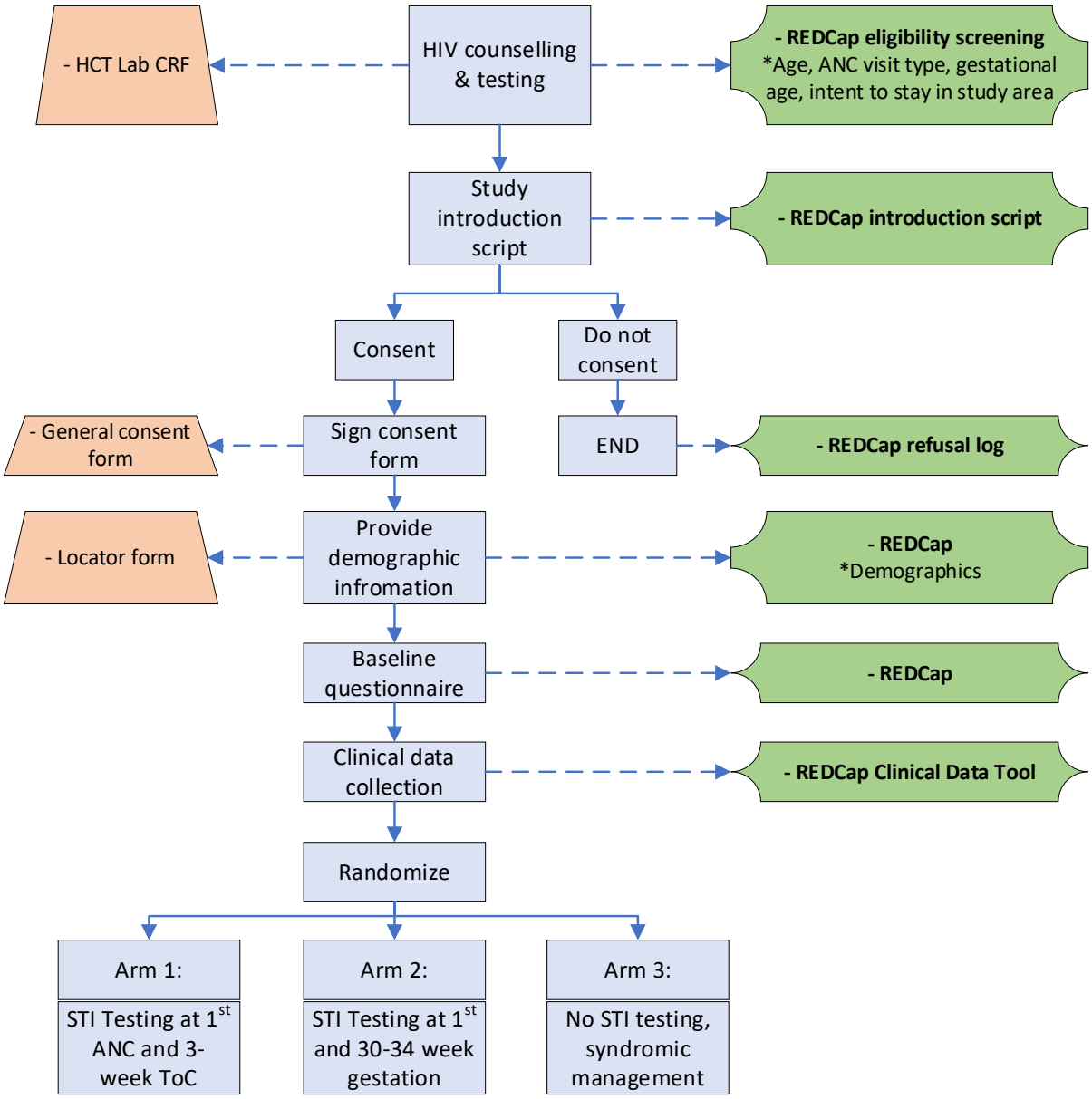


Figure 3 - Participant Recruitment and Enrolment Flow diagram (Aim 1 and 2)

3.5.2.1 Eligibility Screening and Inclusion/Exclusion Criteria

Women attending their first antenatal care visit will be identified by study staff (e.g. whilst attending the health talk). A STI Counsellor will assist with mobilizing women from the waiting area. Women who know their HIV status at first ANC as per HCT guidelines will be referred to study staff in a private space within the clinic. A brief study description will be read to the potential participant, to determine interest in the study. Interested women will undergo a brief peer-administered eligibility screening tool. Specifically, study staff will ask participants about the presence or absence of inclusion and exclusion criteria listed;

Inclusion criteria

To be eligible for participation in the study, women must meet all of the following criteria:

1. Age≥18 years
2. Currently pregnant
3. Attending first ANC visit for current pregnancy
4. Gestational age <27 weeks*
5. Agreeing to nurse-collected specimens
6. Intent to deliver in one of the four MOU's in BCM

Exclusion Criteria

Women will be excluded from the study if they meet any of the following criteria:

1. Planning to relocate during pregnancy or deliver in an MOU outside of BCM
2. Currently participating in another ANC/HIV study
3. When the ultrasound confirms >26 weeks 6 days gestation at first ANC*

*All women with a <27-week gestation calculated by LNMP (last menstrual period) will undergo a confirmatory ultrasound to confirm gestation and eligibility. The cut-off of <27 weeks gestational age at first ANC has been chosen as an inclusion/exclusion criterion. Even though WHO and NDOH guidelines recommend that pregnant women attend their first antenatal care before 20 weeks to achieve optimal pregnancy and PMTCT outcomes, a considerable proportion of pregnant women only come for their 1st ANC visit after 20 weeks of gestation. We do not want to exclude these but decided a cut off of 26 weeks 6 days to be able to include stillbirth as an outcome. This cut-off is also in line with other current studies[147] which will allow to compare results and conduct meta-analysis at a later stage.

The eligibility screening tool will be electronically administered on a RedCap platform and will allow the system to flag individuals as eligible or ineligible in real-time. Written informed consent will be sought from all participants prior to randomization and administration of questionnaire.

3.5.2.2 Enrolment of participants

Study staff will seek informed consent from participants whom were flagged as eligible. Interested women will then read the study consent form together with study staff, in their preferred language (i.e. English or isiXhosa). As part of the informed consent process the staff member recruiting the participant. The consent form will explain to participants what would be expected from them during the study, and possible risks if they choose to participate. It will be emphasized that participation is voluntary and that the standard care that they receive from the facility, will not be affected should they decide to participate or not. Also, it will be made clear that participants may withdraw from the study at any given time and without providing specific reason.

3.5.3 RANDOMIZATION

Those providing informed consent will be enrolled and randomized into one of the 3 study arms; randomizations will be allocated in blocks of 15 with a 1:1:1 randomization into the 3 study arms across the participating facilities. Study staff is blinded to the randomization blocks and status. Automated randomization will be done on RedCap, ensuring allocation concealment.

3.5.4 SAMPLE SIZE

Aim 1 analyses will explore intervention effects on reducing probabilities for adverse birth outcomes and STI prevalence at time of delivery. Based on a total sample size of ~2500 participants (~834 participants in each study arm), calculations show that we will have at least 80% power to detect study arm absolute differences of

approximately 10% or larger in the frequency of adverse birth outcomes. We conducted two sets of calculations. 1) Calculations for the probability of an adverse birth event were conducted in PASS 2008 software (https://www.ncss.com/) for differences in proportions at a single time point (i.e., at birth). Calculations were run for a range of base rates ranging from 30% to 50%; this is in line with base rates from preliminary data (~40%). 2) We calculated changes in STI prevalence based on two time points (i.e., first ANC visit and birth) and conducted simulation studies in two steps. First, we simulated STI data from a binomial distribution with parameter values based on preliminary data. Preliminary results gave pregnancy STI rates around 40%; simulations used a range of pregnancy STI rates from 30% to 50%. Based on preliminary data, we anticipate that the intervention will reduce STI rates by 20% (absolute). We assumed an attrition rate of 15%.

3.5.5 DATA COLLECTION

3.5.5.1 Enrolment/First ANC

3.5.5.1.1 RedCap Questionnaire

Trained study staff will administer a questionnaire to all participants. The questionnaire, adapted in part from measures used by our team in previous and current STI screening and maternal-child health studies, or documented in the literature, will include participant:

a) demographics and socio-economic status
b) obstetric, gynaecological and sexual health history
c) sexual behaviours, sexually-associated practices (i.e. sex toy use, wet washcloth use), intra-vaginal practices, risk factors and self-perception of risk for HIV and STI acquisition before and during pregnancy
d) partner characteristics and HIV status
e) knowledge and previous history of STIs

Interviews will be designed to take no more than 45 minutes and will be administered in a private space. Research staff will be available to assist participants should they require clarity during the interview process or encounter any technical issues.

3.5.5.1.2 Clinical Data Abstraction

Staff will abstract additional clinical history from each participant's maternity case record, including HIV status, date of diagnosis, and immunological characteristics associated with HIV infection (e.g., CD4 T-cell level, HIV viral load, type of ART, ART use/duration). The maternity case record is used from the day of first ANC consultation to record clinical information throughout the duration of the pregnancy. Staff will verify self-reported and medical record-abstracted HIV-related information with data from the South African national HIV database, Tier.net, and the NHLS LabTrack/Webtrack system, both of which contain individual-level heath data.

3.5.6 SYNDROMIC MANAGEMENT AND DATA EXTRACTION

Participants will be seen by the study nurse at first ANC. As all participants are seen by clinic staff for their follow-up ANC appointments, any syndromic management given during ANC, should be indicated on the ANC charts by the clinic nurses. Study staff will maintain a close relationship with clinic staff to extract any management information and treatment given for STIs during ANC. One of the two STI counsellors placed at the study site will

be dedicated to assist with data extraction activities, including data extraction ANC charts to determine syndromic management outcomes for participants.

3.5.6.1 Postnatal Clinic Visit

Data will be collected on pregnancy and birth outcomes from all study participants via abstraction of labour/postnatal ward clinical records and face-to-face interviews with participants during the first postnatal clinic visit. All clinical data relating to labour, delivery and birth/neonatal outcomes are recorded on a discharge summary; women are given a copy of discharge summaries when they leave an MOU (a carbon copy is kept in the labour ward).

Additional data will be abstracted from the infant health record, known as the Road-to-Health card, which is issued to all infants born in South African facilities. Staff will collect information on fetal loss, preterm labour, preterm birth, birth weight, the calculated small-for-gestational-age status, and infant mortality. Information on potential confounding variables such as maternal history of chronic illness (e.g., hypertension, diabetes), other infections during pregnancy (e.g., urinary tract infections, syphilis), antibiotic use during pregnancy, and pregnancy complications (e.g., premature rupture of membranes, maternal fever, chorioamnionitis, and pre-eclampsia) will also be collected.

HIV PCR results from routine at-birth testing of HIV-exposed infants will be collected via clinical records and verified using the NHLS/LabTrack system.

The birth outcomes are defined and measured as follows:

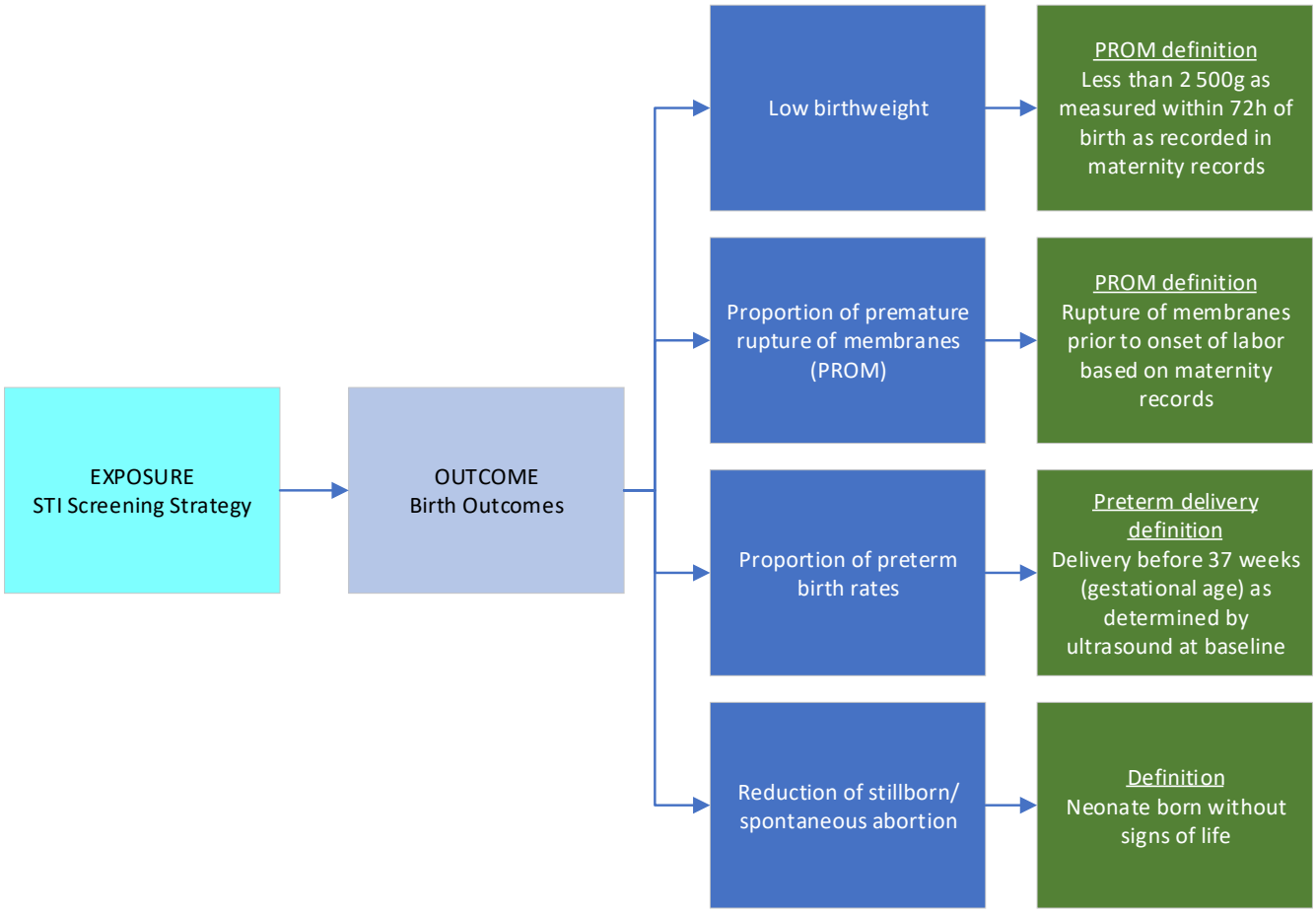


Figure 4 Birth Outcomes

3.5.6.2 Six-week follow-up visit aligned with infant immunization visit

At the routine 6-week immunization visit, data will be collected on neonatal health outcomes and morbidities (or mortality) (i.e., admission for respiratory distress, conjunctivitis, sepsis) via maternal interviews and patient medical records. Should a mother-infant pair not present for a scheduled 6-week follow up visit, research staff will make repeated attempts to contact her to attend clinic. If neonatal mortality is identified, a verbal autopsy will be performed, and cause of death will be confirmed via medical records. A study supervisor will perform weekly reviews to ensure data completeness and validity; discrepancies will be resolved using information that will be collected from the delivery register. If the swab taken from the mother at the post-natal visit is positive for one of the STIs, the infant's swabs will also be tested and treatment is given where needed at the six-week follow-up visit.

3.5.6.3 Process Evaluation

The Reach-Effectiveness-Adoption-Implementation-Maintenance (RE-AIM) model will be used as a conceptual framework[148-150] to guide the collection of valuable information during our effectiveness trial. Per Table 4, a mixed methods approach will be used to collect process measures such as recruitment rates, refusal characteristics, perceived and experienced barriers and facilitators to optimal implementation, intervention costs, impact of intervention on patient outcomes, perceived health system readiness to implement our interventions, and to assess modifications that can be made to maximize future implementation success.

We will extract quantitative measures from implementation tracking tools, recruitment/refusal logs, participant demographic data, and participant tracking/retention tools.

Qualitative data will be collected during interviews with different stakeholders, including participants, research and clinic staff, facility managers, and the South Africa NHLS and National Department of Health (NDoH).

Table 4: Process Evaluation

Element	Questions	Measures	Data Sources/Tools
Reach	1) What % of eligible patients consented to receive the intervention? 2) Do those that consent differ significantly from those that do not?	1) Recruitment rates 2) Socio-demographics of all eligible participants stratified by consent/refused	1) Enrolment tracking sheets 2) Enrolment tracking sheets
Effectiveness	What is the effect of the intervention on patient outcomes?	Main study outcomes comparing interventions & Control	Study datasets
Adoption	1) What are the main barriers/facilitators to adopting the intervention? 2) What systems need to be in place for the health system to adopt intervention?	1) Perceptions of research/clinic staff, facility management, NHLS & NDoH	1) Staff observational logs and post-intervention interviews 2) Post-intervention interviews clinic and national stakeholders

			3) Interviews with Philani participants
Implementation	1) What does the intervention cost? 2) What support and tools are needed for consistent delivery of intervention? 3) How does the implementation of the intervention compare across each site? 4) What were the major changes/adaptations needed during implementation?	1) Cost/Cost-effectiveness data 2) Perceptions of study and clinic staff, NHLS and NDoH	1) Study datasets 2) Post-intervention interviews w/ implementing staff, clinic & national stakeholders 3) Study site observations
Maintenance	1) What resources will be needed for the intervention to be sustainable? 2) What adaptions are needed to integrate intervention into current practices? 3) How do the outcomes compare to STI/PHC health goals?	1) Perceptions of research staff, facility managers, NHLS and NDoH	1) Research staff observation logs, post-intervention interviews 2) Post-intervention interviews clinic and national stakeholders 3) Research staff interviews 4) Key informant interviews/expert panel discussion

3.5.6.3.1 Details of qualitative study sample:

All healthcare practitioners and managers based in BCM directly and indirectly involved in the project are eligible to participate in this qualitative evaluation if of adult age (≥18 years). Policymakers and provincial representatives from the Eastern Cape will be eligible to participate in in-depth interviews or an expert panel discussion to expand on geographic location and feedback for STI POCT.

Philani Ndiphile participants enrolled in the parent study are eligible based on the provision of separate informed consent for in-depth interviews.

3.5.6.3.2 Recruitment and sampling:

For each activity, the study will seek voluntary consent. Following written informed consent, participants are registered and allocated a participant identification number for further identification. Informed consent will be sought for participants in person, for key informants this will either be requested in person or electronically via a secured FPD-study link that will allow for consent captured on the FPD-REDCap study database, that is password-protected and only accessible to relevant staff. Eligible participants will be invited to an interview that will inform the adoption, implementation, and maintenance of diagnostic STI screening and care. Provincial stakeholders will be approached via e-mail or regional meetings relevant to the study to determine interest.

Philani participants will be approached post-intervention at the healthcare facility in a private space to determine interest in sharing study-related experiences. Insights from participants will inform screening preferences, values, and needs to inform future management of STIs at primary healthcare.

3.5.6.3.3 Survey questions and qualitative interviews

Basic demographic data will be requested from interviewed key informants such as age, gender, geographic location, occupation, clinic experiences, and responsibilities including STI management. For Philani participants, a few survey questions have been included to determine access to care, and STI clinical care.

The RE-AIM framework as per Table 5, will be used as a conceptual framework to guide the collection of valuable information during key informant interviews and focus group discussions during our effectiveness trial. The qualitative data collection will particularly focus on the Adoption, Implementation, and Maintenance of the framework. The study will collect information involving barriers, and facilitators to optimal implementation, perceived health system readiness to implement our interventions, and to assess modifications that can be made to maximize future implementation success. In-depth interviews and focus-group discussions will be conducted in the BCM health district at local venues that are easily accessible and offer participant discretion such as health facility meeting rooms or community halls. Expert panel meetings or interviews with provincial key informants will either be conducted in-person or via TEAMs depending on the geographic location of participants. Recruitment and interviews will be conducted by trained qualitative research staff. Interviews will be audio-recorded, translated, and transcribed for analysis.

Key focus areas and common measures as per the 'AIM' framework:

Adoption	**Proportion and representativeness of settings or organizations that implement the program** Measure: Eligible settings that agree to implement the program and the characteristics of adopting settings compared to non-adopting settings including potential barriers and facilitators
Implementation	**Evaluates the fidelity and consistency with which the intervention is delivered as intended** Measure: Qualitative measures such as observations, and interviews with staff, participants and key informants to understand preferences and needs.
Maintenance	**Sustainability of the intervention over time (e.g., delivery and effects)** Measure: Prospects for program continuation, and the integration of the STI POC into routine practice or policy

3.5.6.4 Sample size

We aim to approach up to 90 key informants at varying levels at district and provincial health. The proposed sample includes the following:

Key informant	Sample	Type if interview	Element(s)
Philani Participants	15-25	Individual in-depth interviews	Values and preferences that inform adoption

Philani study staff	10-20	2-3 focus group discussions	Adoption and Implementation, Maintenance
Clinic staff, health practitioners at varying levels e.g., focal nurses and operation managers	10-20	2-3 focus group discussions	Adoption and Implementation, Maintenance
District and provincial DOH, NHLS representatives policymakers, key opinion leaders	15-25	Expert panel discussion or individual in-depth interview	Maintenance

We expect that the key informants directly and indirectly involved in this project who are willing to contribute to the evaluation will be sufficient to reach saturation of data. This will include staff that is on the ground (e.g., study and clinic staff) and managerial staff (e.g. operation managers, district leaders and laboratory staff). Study staff will be able to reflect on the intervention to inform the implementation and adoption including step-by-step processes and resources needed. Intervention experiences will inform differences by site, adaptations over time and lessons learnt. Study observations may further inform implementation. We will be able to triangulate the data approaching key informants at varying levels using different qualitative approaches.

3.5.6.4.1 Qualitative Informed Consent

Participants do not have to answer any question that they do not want to and can stop answering the questions at any time. Additionally, skip patterns will be used to ensure to only ask more detailed questions to participants for whom these questions are relevant. Qualitative participants will not be pressured to disclose personal details they are not comfortable sharing. Philani participants will be informed by trained study staff that refusal or withdrawal from this study will have no impact on their ability to access any services provided at the antenatal/postnatal clinics or as offered by the Philani Ndiphile study.

3.5.6.5 Cost-Effectiveness from The Societal (Government Provider and Patient) Perspective

While Aim 1 will determine the efficacy of our screening interventions in improving birth outcomes for pregnant women, it is also crucial to determine whether the monetary costs of our interventions are cost-saving or cost-effective (Aim 2). This analysis will take into account the costs of each intervention, costs averted and the overall cost-effectiveness using a societal (government provider and patient) perspective.

The Provider Perspective: We will assess the full economic costs of each study arm and the full economic costs of adverse birth outcomes. A full economic costing approach includes financial and opportunity costs and is necessitated by the reality of severely constrained capacity within the South African and similar low/middle-income country health systems. Our approach to costing establishes the utilization of health services (e.g.

diagnostic and treatment visits), diagnostic tests, and medication directly from trial data specific to each arm. Within a decision analytic modelling framework, those utilization estimates are multiplied by the full economic or unit cost of each service, diagnostic test or medicine. Unit costs are computed using a micro costing with bottom-up and step-down allocation approaches, as appropriate. For example, for diagnostic visits, bottom-up costing captures staff time for diagnosis (using time and motion tools), while step-down approaches are used to apportion shared costs within the facility such as managerial, clerical, cleaning and security staff, and utilities. For diagnostic tests, bottom-up costing is used to capture the costs of the test cartridges and GeneXpert machines (appropriately annuitized). Similarly, the costing of adverse pregnancy or birth outcomes entails the bottom-up costing of clinical staff, infrastructure and equipment within the facility where care is provided (e.g. neonatal ICU), together with a step-down allocation of shared costs such as overheads within the hospital. When valuing resources within the cost analysis that are paid from the research budget, we will use routine public sector 'prices' for staff and medication and will seek to cost GeneXpert machines and cartridges at a level commensurate with a potential public sector scale-up. Care will be taken to exclude any costs that are incurred only as part of research activities.

The Patient Perspective: We will collect demographic, socio-economic, patient cost and household income data. Data will be collected at each interview unless the variable is expected to stay constant over the study period (e.g. educational status). Socio-economic status will be computed via a multiple correspondence analysis on household type, assets, and access to services following established methodology.[145,146] Patient costs will include transport costs, opportunity costs of travel, waiting and visit times, and other out-of-pocket payments, such as user fees (applicable for public inpatient care in South Africa but not for ANC). Productivity gains or losses will not be included, as the study population includes pregnant women and their babies. To increase response rates, questions about household income will include quantitative and categorical approaches.[145] The categorical income variable will be transformed into a quantitative variable using a regression methodology, where household income can be predicted as a function of demographic and socioeconomic status. Per capita household income will be computed as total household income divided by total number of household members, with appropriate adjustments for children. The opportunity cost of time can be valued using wages/salary earnings foregone.[151] In order to value these costs equitably, the mean per capita household income reported at the baseline interview will be used as a proxy of this opportunity cost. In contrast, time, travel and user fee costs will be compared to the mean per capita income of the respondent's own household in order to assess the share of per capita household income spent on these costs.

3.5.7 DATA MANAGEMENT

3.5.7.1 Participant Identification

Each potential participant screened for eligibility will be assigned a unique survey identification number (Redcap #). This will not include any personal identifying information; it will include the eligibility screening criteria only. Eligible participants who sign a consent form will be assigned a unique participant identification number (PIN) . PINs will be placed on paper copies of participant records and on specimen collection containers. Laboratories supporting the study will be asked to use the PIN as an identifier when capturing test results on their laboratory information systems. Study staff will use this to search and link results to other participant data.

3.5.7.2 Data Storage

Data will be stored in a secure, password-protected, web-based database which will only be accessible to authorized project staff. Tablet computers used for interviews and extraction of data from medical records will be password protected and will be stored securely at FPD offices in the study districts. Paper records of participants will be kept in lockable filing cabinets at FPD offices; forms with identifiers will be kept separately from demographic, clinical and other data. Paper records, excluding informed consent forms, will only contain PINs. A separate, access controlled, link log database will be developed and maintained by the data manager. The link log will be stored separate from the rest of the study data and will only be accessed when it is an absolute necessity. Lab case report forms will be stored seperately from any other dcuments that contain identifiable information. Qualitative data including audio files and password-protected transcripts will be stored on a secure, access-controlled cloud-based database (Sharepoint).

3.5.7.3 Data Quality

Double-data entry of results and clinical data will be employed when capturing participant data onto RedCap. Automated data quality checks and skip patterns will be built into RedCap. STI Test Counsellors and Research Nurses will conduct onsite data quality checks daily under the supervision of a field coordinator. Data administrators based at the FPD head office will conduct data quality checks weekly and flag any inconsistencies for field-based staff to rectify. Data will also be checked against hard-copy source documents for consistency. Interviewers will be trained by a qualitative research expert; initial interviews will be facilitated collaboratively for training purposes and interviewers will receive feedback on their initial solo interviews before proceeding with more. Each qualitative transcript will be checked for grammar and accuracy upon receipt by the interviewer or study coordinator.

All research study personnel will meet weekly to review study enrolment, specimen collection, processing, test turn-around-time, data management, and treatment outcomes. Meetings will discuss descriptive study results to date, problems encountered and remedial actions to be taken. Field-based staff will be invited to monthly team meetings to discuss the above and/or take the form of a refresher training where needed to ensure study protocol compliance.

Scheduled and unscheduled data quality inspections will be carried out by the data quality assurance personnel in order to ensure high data quality standards. The Principal Investigators, or their designate, will randomly select 10% of all participant files for inspection. This will be done every 3 months. An external study monitor will also be consulted at three time points during the study to conduct an external audit of source documents as well as the Regulatory study binder.

3.5.8 SPECIMEN COLLECTION AND TESTING PROCEDURES

All specimen collection will be done by research nurses and GeneXpert testing may either be done by the research counsellor or research nurse. A vaginal swab will be used to inoculate an InPouch culture from women with a positive T. vaginalis NAAT in Arms 1 and 2 of the parent RCT. This InPouch culture will be immediately incubated at 37°C and read 3+ times over a 5-7 day time period for the presence of motile trichomonads. Positive trichomonas cultures will be frozen at -80°C until ready for 5-nitroimidazole drug susceptibility testing in the near future (to metronidazole, tinidazole, and secnidazole). Specimens will be labelled with barcoded specimen identification numbers before storage and shipping to laboratories for biobanking.

3.5.8.1 Specimen Collection

After enrolment, research nurses will collect up to four swabs from the participant: 1 swabs for STI testing, 1 swab for microbiome analysis , 1 swab for microbiological profiling and 1 swab for cytokine analysis (NOTE: all pregnant women from our recently completed study found it acceptable and feasible to collect up to three vaginal swabs at a visit) and two inoculation loops will be used to prepare a smear on glass slide for microscopy. Mid-stream urine collected from participants as part of normal practice prior to the confirmatory ultrasound test at baseline visit will be used for urinary dipstick to detect haematuria, leukocyturia and nitrite

GeneXpert - Nurses will use the GeneXpert Vaginal/ Endocervical Specimen Collection kit [Cepheid, Sunnyvale, CA] for vaginal swab specimen collection from participants.

Microbiome - For vaginal microbiome analysis, nurses will use a Dacron swab [Qiagen, Digene] for specimen collection from participants, with subsequent storage in DNA AssayAssure® [Sierra Molecular, Incline Village, Nevada] at ambient room temperature.

Microbiological Profiling - For specimen microbiological profiling nurses will use a dry FLOQswab® [COPAN, Murrieta, CA] for specimen collection, with subsequent storage in a sterile tube. This swab will be collected for targeted microbiological profiling of genital infections at the Department of Medical Microbiology, University of Pretoria.

Cytokine Analysis - For collection of specimen for cytokine analysis nurses will use a dry FLOQswab® [COPAN, Murrieta, CA], with subsequent storage in a sterile tube. This swab will be stored in a freezer and processed for cytokine analysis at the Immunology Laboratory at the division of Medical Virology, University of Cape Town.

Inoculation Loops – Two vaginal loop specimens will be collected from participants by study nurses. Nurses will smear the collected specimen along a glass side which will then be airdried. Specimens will be subsequently stored in a box for glass smear microscopy to identify bacterial vaginosis and dysbiosis as well as candidiasis.

Midstream urine – Women will be asked to urinate prior to ultrasound for gestational age determination. We will ask these women to collect midstream urine for the urinary dipstick to detect haematuria, leukocyturia and nitrite.

Nasopharyngeal swabs /Conjunctival swabs– For nasopharyngeal specimens, collected from the infant's nose, nurses will use the GeneXpert Nasopharyngeal Specimen Collection kit for STI testing and a dry FLOQswab [COPAN, Murrieta, CA] for biobanking, with subsequent storage in a sterile tube. Infants will only be tested for the mother's infection detected post-partum.

***T. vaginalis* InPouch™ culture –** For women with positive GeneXpert *T. vaginalis* NAAT on enrollment, ToC visit (Arm 1), and 30-34 week visit (Arm 2), a *T. vaginalis* InPouch culture will be obtained.

3.5.8.2 Vaginal PH-strips

Vaginal pH of participants will be measured on pH strips using vaginal secretions collected from a swab used for STI testing; pH strips will be interpreted using the manufacturer's chart.

3.5.8.3 Specimen Labelling

Staff will handle specimens and label with a unique study barcode to link a participant's STI test results, medical chart and questionnaire data (see 3.5.5 DATA COLLECTION).

3.5.8.4 Specimen Storage

Specimens collected for additional testing will be stored at 2-8°C and transported to the microbiology laboratory on a bi-weekly basis according to Good Laboratory Practice. Specimens will be flash frozen and stored at -20°C for bio-banking. Frozen specimens will be shipped quarterly for microbiome processing and analysis to University of Cape Town.

3.5.8.5 Diagnostic Testing

3.5.8.5.1 Chlamydia trachomatis, Neisseria gonorrhoeae and Trichomonas vaginalis testing

Vaginal specimens collected from participants will be tested for *Chlamydia, Gonorrhoeae* and *Trichomoniasis* using the Xpert® CT/NG and Xpert® TV assays [Cepheid, Sunnyvale, CA]. Trained staff (STI Test Counsellors and Research Nurses) will conduct the PoC testing at each of the clinical sites. Once collected, research staff will follow test kit instructions for swab preparation and testing. Xpert® CT/NG provides 90-minute detection and differentiation of *Chlamydia* and *Gonorrhoeae*, while Xpert® TV provides 60 min detection of *Trichomoniasis*; both test cartridges have high sensitivity and specificity[152] and function well in resource-constrained environments and clinical settings such as those proposed here. Should a participant test positive for *Neisseria gonorrhoeae*, and/or *Trichomonas vaginalis*, dependent on the clinical history, we will obtain a specimen for culture and susceptibility testing.

Each test includes a sample processing control (SPC) to ensure correct cell lysis/DNA extraction of the sample, a sample adequacy control (SAC) which ensures adequate human DNA in the specimen and a probe check control (PCC). The PCC monitors reagent rehydration, reaction-tube filling, probe integrity, and dye stability.

If testing cannot be conducted due to power failures, errors, or testing delays, specimens will be stored at 2-4°C in a secure storage area for up to 24 hours until tested.

3.5.8.6 Microbiological Profiling

The dry FLOQswab will be collected from participants for targeted microbiological profiling of genital infections at the Department of Medical Microbiology, University of Pretoria. These include the following characterisations: prevalence determination (e.g. Mycoplasma genitalium, various other Mycoplasma and Ureaplasma species, Group B streptococcus), molecular epidemiology and antimicrobial resistance determination (e.g. for *Mycoplasma genitalium*, *Neisseria gonorrhoeae* and Trichomonas vaginalis). Determination of prevalence of specific microorganisms will be done using targeted real-time PCR assays on the LightCycler Instrument following DNA extraction; these tests can be validated in-house tests or commercially available laboratory tests that specifically detect the targeted pathogen. Molecular typing will be done using established methods such as multilocus sequence type (MLST) analysis, culture-free multi-antigen sequence type (MAST) analysis, restriction fragment length polymorphism (RFLP) analysis and enriched whole-genome sequencing (WGS) analysis.

Determination of presence of antimicrobial resistance genes may be done using targeted next-generation sequencing methods, melting cure analysis and/or commercial assays with the specific aim of resistance profiling.

3.5.8.7 Cytokine Analysis

The dry FLOQswab collected from participants for cytokine analysis will be stored in a freezer and analysed at the Immunology Laboratory at the division of Medical Virology, University of Cape Town. Levels of Cytokine-IL1-alpha, interferon γ-induced protein (IP-10) and possibly further cytokines will be determined using an immunoassay.

3.5.8.8 Urine Testing

As per good clinical practice, women will be asked to urinate prior to ultrasound for gestational age determination. We will ask these women to collect their mid-stream urine for the urinary dipstick to detect haematuria, leukocyturia and nitrite.

3.5.8.9 STI Reporting and Treatment

The GeneXpert systems consist of an instrument, computer, and preloaded software for running tests and displaying results. STI Test Counsellors will report all test results to the ANC Research Nurse embedded within study clinics. Research nurses provide test results notification, treatment, partner treatment counselling and treatment packets to STI-infected participants per National STI treatment protocols.[153,154] Arm 1 and 2 participants will be provided same day results and immediate targeted treatment for their infection. Arm 3 participants will receive syndromic management as per standard of care and South African guidelines.

3.5.8.10 Partner Treatment

Women testing positive for an STI will be counselled on safe disclosure to their partners, assessed for potential intimate partner violence related to disclosure, and given a notification slip(s) to request their partner(s) present to a clinic for treatment.

3.5.8.11 Testing Schedule During Pregnancy

Arm 1 Specific Activities: Per Table 3, at first ANC visit, participants randomized to Arm 1 - nurses will collect vaginal swab specimens and 2 loops as described above (Specimen Collection). One specimen will be used for *Chlamydia, Gonorrhoea* and *Trichomoniasis* testing, and one for microbiome analysis, one for microbiological profiling and one for cytokine analysis. Mid-stream urine for ultrasound gestation confirmation will be used for urinary dipstick to detect haematuria, leukocyturia and nitrite . Test of Cure (ToC): Participants treated for an STI infection at first ANC will be asked to return 3 weeks post-treatment for a targeted ToC (i.e., women will only be tested for the STI for which they were treated). At the ToC visit, nurses will again collect the same specimen as described above. Women with positive ToC will again be treated. If women are positive for *T. vaginalis*, an additional vaginal specimen will be collected to inoculate an InPouch culture which will be incubated at 37°C and read over a period of 5-7 days; positive cultures will be frozen at -80°C until ready for drug susceptibility testing. Drug susceptibility testing to 5-nitroimidazoles will later be performed. During routine 30-34 weeks ANC visit we will collect additional clinical information through a short questionnaire and the same specimens as described above for.

Arm 2 Specific Activities: Per Table 3, at BOTH first ANC visit and during an ANC visit occurring between 30-34 weeks gestation, participants randomized to Arm 2, nurses will collect four vaginal swab specimens; one for *Chlamydia, Gonorrhoea* and *Trichomoniasis* and one for microbiome analysis, one for microbiological profiling and one for cytokine analysis in addition to two loops. Mid-stream urine for ultrasound gestation confirmation will be used for urinary dipstick to detect haematuria, leukocyturia and nitrite . No ToC activities will be

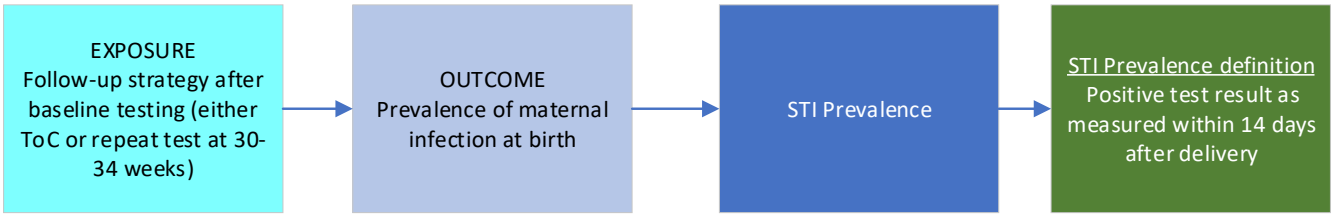
performed for Arm 2 participants. At the 30-34 weeks study visit specimens collected for STI testing, point-of-care testing will be done and women with a positive result will be treated. If women are positive for *T. vaginalis*, an additional vaginal specimen will be collected to inoculate an InPouch™ culture which will be again incubated, read over a period of 5-7 days, and then frozen for future drug susceptibility testing. Drug susceptibility testing will subsequently be performed.

Arm 3 Specific Activities: Per Table 3, at first ANC visit participants randomized to Arm 3 will be asked for four vaginal swab specimens as described (*Specimen Collection* section). All specimens will be immediately bio-banked. Women recruited in the syndromic arm will also collect mid-stream urine to be tested for urinary dipstick to detect haematuria, leukocyturia and nitrite before ultrasound testing. Arm 3 participants will be provided standard of care syndromic screening and management for STIs per South African National Guidelines.[143,144] To determine infection status and prevalence of *Chlamydia, Gonorrhoeae* and/or *Trichomoniasis* at first ANC, specimens collected from Arm 3 participants will be tested at a later date using GeneXpert as previously described. During routine 30-34 weeks ANC visit we will collect additional clinical information through a short questionnaire.

3.5.8.12 Post-partum and Infant Specimen Collection

During the first postnatal visit (typically 3-6 days post MOU discharge), four vaginal swabs will be collected from all post-partum women to provide a proxy for STI infection at time of delivery, and two nasopharyngeal (NP) swabs and two conjunctival swab specimens will be collected from all infants. Specimens will be labelled with random specimen IDs that link to participant IDs. Specimens will be transported to the Univ. of Pretoria and stored as previously described. One vaginal and infant swab (only when the maternal swab is tested positive) will be batch tested using Xpert® CT/NG and Xpert® TV assays, the other swabs will be stored for microbiological profiling, microbiome and cytokine analysis. Test results will be used for treatment where needed at the six-week infant immunization visit.

Post-partum STI prevalence will be defined as follows:



Specimen Collection Time Points

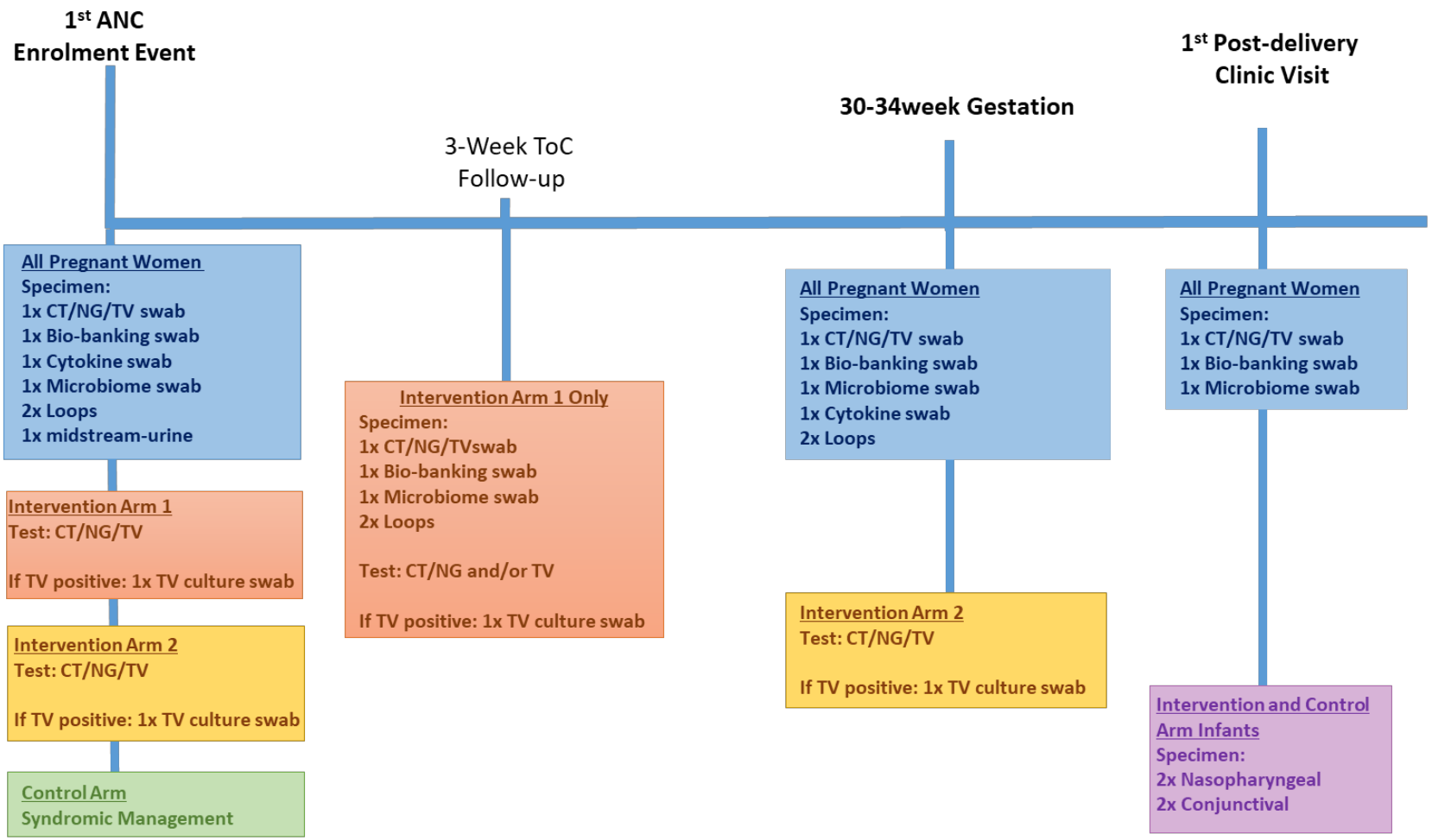


Figure 5 (below) shows the type of specimens that will be collected at the different study timepoints (I.e. Baseline/Enrolment, Test-of-Cure, 30-34 weeks gestation and at the first post-delivery clinic visit):

Specimen Collection Time Points

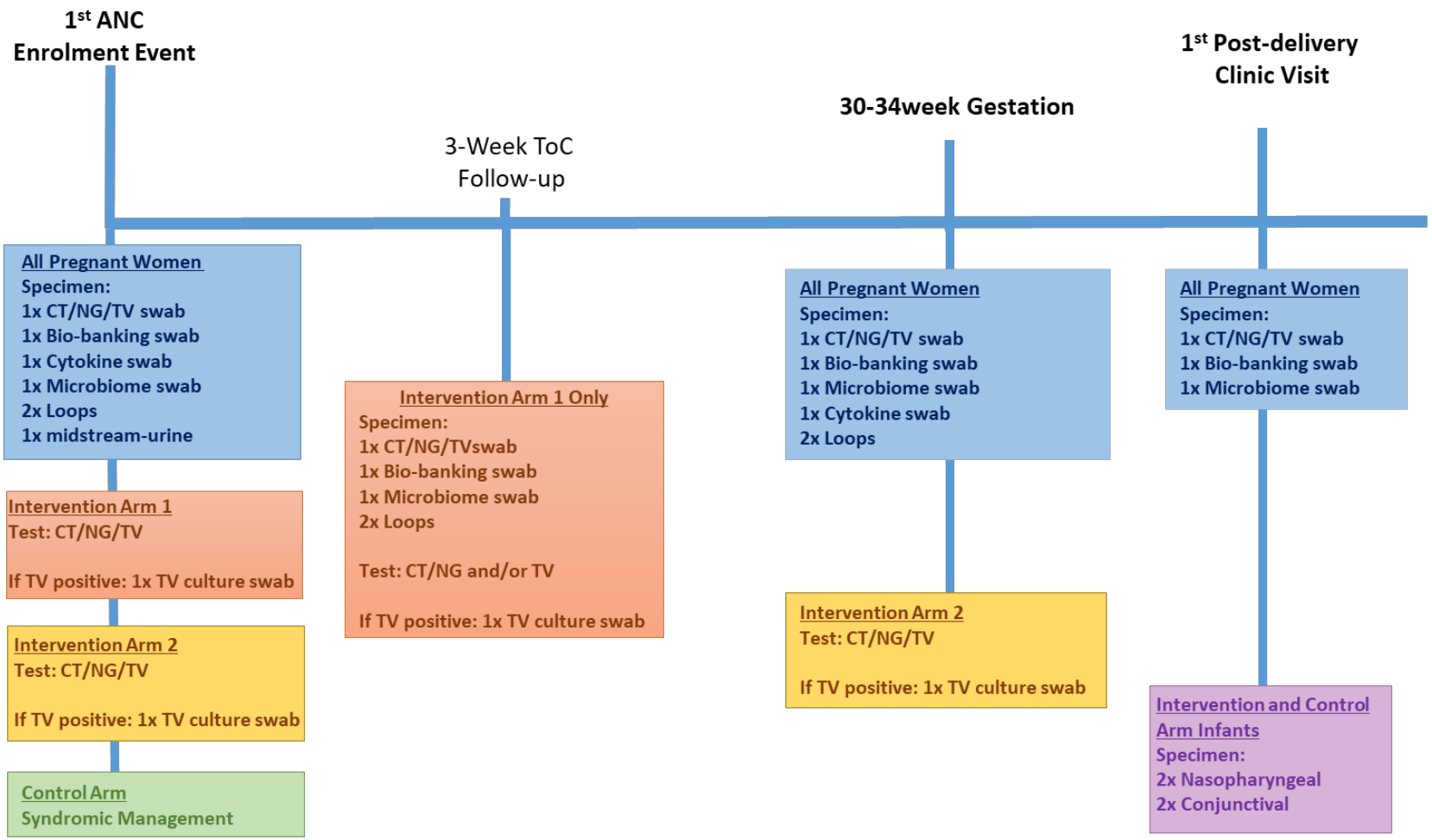


Figure 5 – Figure 5 - Specimen Collection Time Points - R01 STI Project

4 METHODOLOGY: AIM 3 AND 4

4.1 DESCRIPTION OF THE STUDY DESIGN

This study will use a cohort design with a nested case-control study to address the main study aims:

Aim 3) Investigate associations between the presence of lower genital tract organisms in pregnancy and adverse pregnancy outcomes through a holistic approach.

Aim 4) Investigate the relationship between the vaginal microbiome and persistent chlamydial infection in pregnant women.

Cohort study: STI screening and treatment for *Chlamydia* and *Gonorrhoea* will be offered to HIV-infected and non-infected women (age >18 years) with a gestational age of less than 27 weeks at the enrollment visit. The cohort study will use vaginal specimens collected at the enrollment and third trimester ANC visits to investigate associations between the presence different of lower genital tract organisms in pregnancy and adverse pregnancy outcomes through a holistic approach. The study procedures are the same as for aims 1 and 2, except for postnatal STI testing, but includes additional detection of vaginal pathobionts and vaginal microbiome profiling.
Nested chlamydia case-control study: If the Xpert® test for *C. trachomatis* is positive at the enrollment visit, the woman is invited to participate in the nested chlamydia case-control (1:2) study to investigate the relationship between the vaginal microbiome and persistent chlamydial infection (cases: persistent infection defined by positive test at three weeks post treatment; controls: all women with negative test result three weeks post treatment). Chlamydia-infected pregnant women are invited to return to the clinical site twice weekly between enrollment, the 3-week TOC visit and 6-week TOC visit (should participants test positive again for CT on the GeneXpert machine at 3-week TOC) following the provision of 1 gram of oral azithromycin. At these visits, study staff will collect vaginal specimens for Nugent score and 16S sequencing. If *N.* gonorrhoeae is present women will also be treated with 500mg of ceftriaxone.

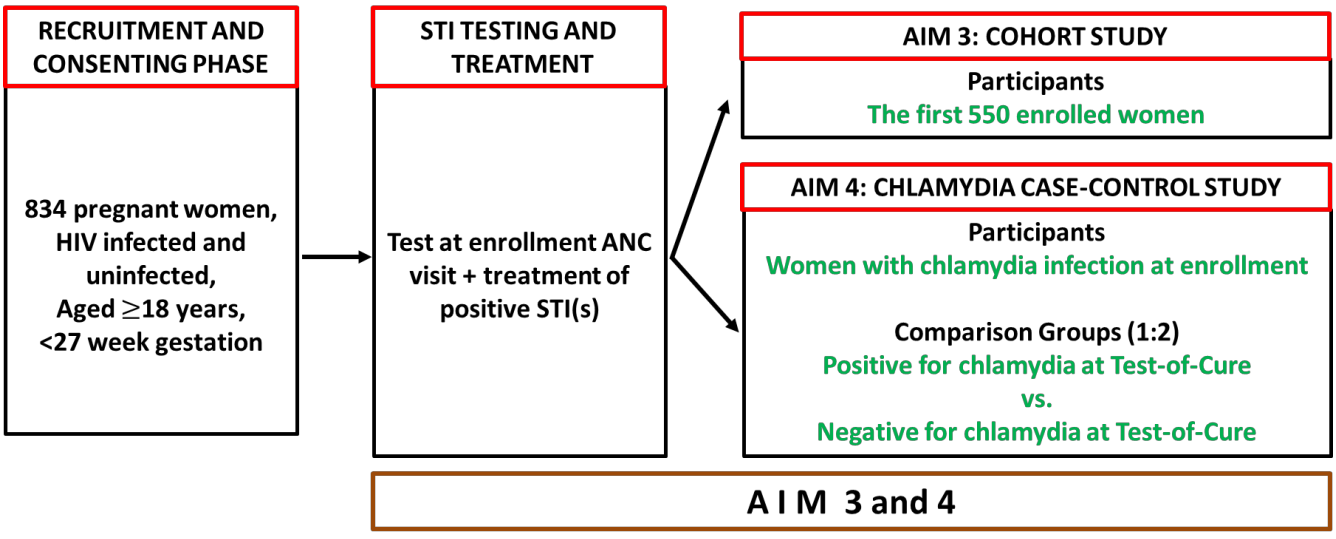


Figure 6 - Aim 3 and 4 - Microbiome study

4.2 HYPOTHESES

Aim 3: Investigate associations between the presence of lower genital tract organisms in pregnancy and adverse pregnancy outcomes through a holistic approach.

Main hypothesis: Identifiable combinations of vaginal and sexually transmitted organisms in pregnancy are associated with earlier mean gestational age at delivery.

Aim 4: Investigate the relationship between the vaginal microbiome and persistent chlamydial infection in pregnant women.

Hypothesis: Chlamydia-infected pregnant women with BV-associated vaginal microbiota CSTs will be significantly more likely to have persistent infections at a 3-week test-of-cure (TOC) visit compared to women with non-BV associated CSTs.

4.3 STUDY SETTING

This study will be conducted at a fifth, separate ANC clinic in Buffalo City Municipality (BCM), Eastern Cape Province.

4.4 METHODS AND PROCEDURES

4.4.1 PILOT STUDY

An internal pilot phase will precede the full implementation of Aim 4 to determine the acceptability and feasibility of collecting specimens daily, we will initially recruit 20 pregnant women and collect specimens from these women daily for a 3-week period. After this 3-week follow up period, the study team will determine the acceptability and feasibility of the approach to optimize the specimen collection procedures and schedule before continuing to the full sample size described in the sections below.

4.4.2 STUDY VISIT AND PROCEDURE SCHEDULE

Participants are enrolled at the ANC clinic of the participating facility. Following informed consent, a baseline questionnaire is completed, and specimens are collected for on-site STI testing (*C. trachomatis/N. gonorrhoeae*), laboratory microbiological profiling (e.g. mycoplasmas) and microbiome analysis. A repeat visit at 30-34 weeks is scheduled at which clinical and obstetric information as well as specimens are collected (Table 5). Finally, data on pregnancy outcomes are collected at the first post-natal visit and from the obstetric registers, as described in aims 1 and 2.

Should the participant test positive for *C. trachomatis* or *N. gonorrhoeae* at the baseline visit, she will immediately be provided with antibiotic treatment according to South African national guidelines and a partner notification slip. Women who tested positive for *C. trachomatis* will then be invited to participate in the nested chlamydia case-control study. In that study, she will have a baseline Nugent score and vaginal specimen collected for 16S sequencing. Participants will be asked to return to the clinical site twice weekly between enrolment and the 3-week TOC visit. At these twice weekly visits, study staff will collect vaginal specimens for Nugent score and 16S sequencing. When the participant returns to the facility for a test-of-cure visit, clinical data will be collected and a repeat Xpert® test for *C. trachomatis* is performed. Additionally, at the 3-week TOC visit, participants will continue to have vaginal specimens collected for Nugent score and 16S sequencing. Should participants have a repeat positive CT test at the week 3 TOC visit, they will be re-treated and continued in this microbiome study through a 6-week TOC visit, including the same twice weekly visits for Nugent score and 16S sequencing. At the 6-week TOC visit, participants will have an additional Nugent score and vaginal specimen for 16S sequencing collected. Clinical data will be collected and a repeat Xpert® test for *C. trachomatis* is performed at the 6-week

TOC visit. Any participant with a positive Xpert test at 6-week TOC will be retreated. If *N.* gonorrhoeae is detected at any of these visits women will also be treated with 500mg of ceftriaxone. .

Table 5 below shows the STI testing time-points at the clinic for Aim 3 and 4 participants.

Table 5: Clinic Visit and STI Testing Schedule

Clinic Visit	Participant	Specimen Collected	CT, NG Testing
Enrollment Visit	**All Pregnant Women**	**Vaginal Smear** **Vaginal Swabs**	**All Pregnant Women**
ToC 3-Weeks Post-treatment	***C. trachomatis*-positive Pregnant Women at 1st ANC**	**Vaginal Smear** **Vaginal Swabs**	**Specific Test for STI Treated**
ToC 6-Weeks Post-treatment	***C. trachomatis* positive Pregnant Women at the week 3 TOC visit**	**Vaginal Smear** **Vaginal Swabs**	**Specific Test for STI Treated**
3rd Trimester Visit (30-34 weeks of gestation)	**All Pregnant Women**	**Vaginal Smear** **Vaginal Swabs**	**All Pregnant Women**
Post-delivery visit	**All post-partum mothers and infants**	**No specimen taken**	**No testing**

4.4.3 RECRUITMENT AND ENROLMENT

Enrollment Visit. Written informed consent will be obtained from eligible participants prior to initiating any study-related activities. Exclusion criteria: women that used intra-vaginal or oral antibiotics within 14 days prior to study enrollment will not be eligible to enroll. Trained study staff will subsequently administer a questionnaire to all enrolled participants at this visit. The questionnaire will include questions pertaining to: 1) participant demographics and socio-economic status, 2) obstetric, gynaecological and sexual health history, 3) sexual behaviours, risk factors and self-perception of risk for HIV and STI acquisition before and during pregnancy, 4) partner characteristics and HIV status, 5) knowledge and previous history of STIs, and 6) screenings for depression, substance abuse, interpersonal violence and social support. Staff will abstract additional clinical history from each participant's maternity case record, including HIV status, date of diagnosis, and immunological characteristics associated with HIV infection (e.g., CD4 T-cell level, HIV viral load, anti-retroviral therapy use/duration). Staff will verify self-reported and medical record-abstracted HIV-related information with data from the South African national HIV database, Tier.net, and the South African NHLS corporate data warehouse,

both of which contain individual-level heath data. Specimens will be taken as outlined in Figure 9 and section 4.4.7.1. If the Xpert® test for *C. trachomatis* and/or *N. gonorrhoeae* is positive, or the woman reports symptoms, she will receive directly observed antibiotic treatment according to South African national guidelines and a partner notification slip will be handed to the participant. If the woman tests positive for *C. trachomatis* she will be invited to participate in the nested chlamydia case-control study (section 4.4.2).

***Participants of the nested chlamydia case-control study only*:** Women will be treated with 1 gram of oral azithromycin by directly observed therapy for her chlamydial infection.. Women will then be asked to return to the clinical site twice weekly between enrolment and the 3-week TOC visit. At these twice weekly visits, study staff will collect vaginal specimens for Nugent score and 16S sequencing. These include 2 vaginal swabs and 2 loops for vaginal gram stain slides. See section 4.4.7.1.1 for detailed instructions on collection of these specimens. Women enrolled in the nested chlamydia case-control study will be asked to return to the clinic 3 weeks post-treatment for a repeat Xpert® test to determine persistence or clearance of *C. trachomatis* infection. At this visit, three vaginal specimens (1 for chlamydial ToC and 2 for future vaginal microbiome analysis) and 2 Loops for vaginal Gram stain slide will be collected. Women with a positive TOC at this visit will be provided directly observed therapy with 1 gram of oral azithromycin. Participants who are repeat CT positive at week 3-TOC will be re-treated and continued in this microbiome study through a 6-week TOC visit, as described for the 3-week TOC. At the 6-week TOC visit, participants will have an additional Nugent score and vaginal specimens taken for 16S sequencing (2 vaginal swabs, and 2 loops).

3rd trimester visit for all participants. During the routine 30-34 weeks ANC visit, we will collect additional clinical information through a short questionnaire and specimens, as outlined in Figure 9.

Postnatal visit for all participants. The postnatal visit will follow the same procedures as described in Aim 1 and 2 (please see **3.5.6.1 Postnatal Clinic Visit**) except of that no specimens will be collected.

4.4.4 SAMPLE SIZE

The cohort study and the nested chlamydia case-control study will be aligned to start and end at the same time. The cohort study needs longer to complete with a 3rd trimester visit and collection of birth outcome than the nested case-control study, which ends after the 3-week or 6-week test of cure visits if repeat positive. We expect 834 women to be eligible for the cohort and the nested chlamydia case-control study, based on attendance figure at the study clinic. We will enroll 550 into the cohort study to align project time.

4.4.4.1 Cohort study

Our sample size calculation takes into account the following considerations: 1. we base the total sample size calculation on univariable comparisons, allowing for multiple hypothesis testing; 2. the outcome variable (gestational age at delivery) is treated as continuous, rather than a dichotomised outcome (with cut-off at 37 weeks), because this is statistically more efficient and because morbidity may occur at all gestational ages before term;[57] 3. the analysis of combinations of organisms that are associated with gestational age at delivery using classification and regression tree analyses are not amenable to standard sample size calculation.

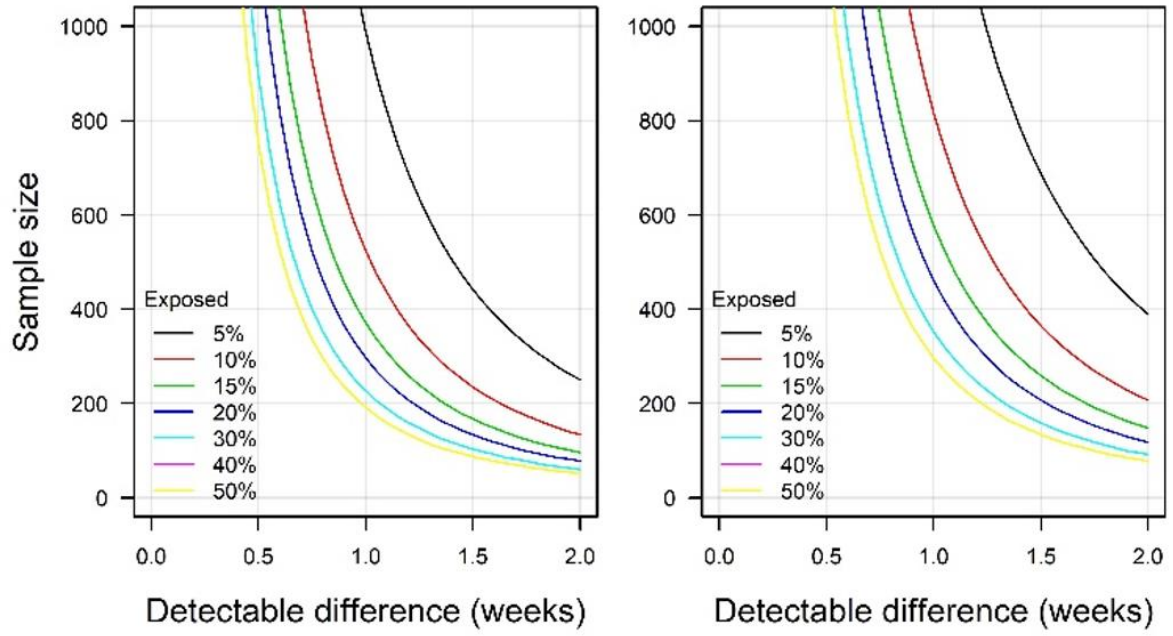


Figure 7 - Sample size requirements at different levels of exposure prevalence for 90% and alpha 1%. Left-hand panel, standard deviation 2, right-hand panel, standard deviation 2.5. Curve is symmetrical around a prevalence of 50%

Figure 7 shows that, for any vaginal or sexually transmitted organism, or vaginal microbiota type that has a prevalence of 10% or more among all enrolled women, about 500-600 patients would provide adequate power (90%) to detect a one week difference in mean gestational age between the two groups (with standard deviation 2). Specifying alpha 1% allows for multiple hypothesis testing (5 hypotheses, using a Bonferroni correction).

We therefore plan to enrol 550 women, and aim to have complete follow up and outcome data on 500 women.

4.4.4.2 Nested chlamydia case-control study

Based on 834 pregnant women, and a 30% chlamydia prevalence among pregnant women, approximately 246 chlamydia-infected women will be included in this study. Considering 26.5% of chlamydial-infected women remain positive after treatment at a 3 week TOC visit, we anticipate approximately 65 “cases” and 130 “controls” will be included (1:2 match). Assuming 65 cases and 130 controls provide daily or every other day vaginal swab specimens over a period of at least 3 weeks, longitudinal study of the temporal dynamics of the vaginal microbiome over time in the setting of persistent chlamydial infection will be investigated. Given the repeated observations that will occur within an individual participant in this study, the non-independence of observations within a subject must be accounted for in the sample size calculation. Assuming an intra-class correlation coefficient of 0.20, 200 women with 4 repeated observations provide 85% power to community state prevalence of 33% among non-responses as compared to 20% among responders using a two-tailed Type I error rate of 0.05. This effect size equates to a risk ratio of 1.65, and an odds ratio of 1.97.

4.4.5 DATA COLLECTION

The same participant data will be collected for participants recruited in Aims 1 and 2. Data that will be collected throughout pregnancy and post-partum has been described in section **3.5.5 Data Collection.**

4.4.6 DATA MANAGEMENT

The same data management standards will be adhered to for Aim 3 and 4, as is described in Aim 1 and 2. This includes participant identification, data storage, and data quality. Please see **3.5.7 Data Management** for more information. All participant data from each Aim will be managed and stored under the same RedCap project platform.

4.4.7 SPECIMEN COLLECTION AND TESTING PROCEDURES

4.4.7.1 Specimen Collection

Specimen collection will follow the same procedures as described in Aim 1 and 2 (please see **3.5.8.1 Specimen Collection)** but 1 additional swabs will be taken for microbiome quantitative PCR (qPCR).

Microbiome bacterial DNA load analysis – same procedure as for microbiome described in Aim 1 and 2 (please see **3.5.8.1 Specimen Collection**).

4.4.7.1.1 Nested chlamydia case-control study

Participants will return to the clinical site twice weekly for 21 days or 42 days (ToC positive) for study staff to collect vaginal specimens (using a cotton-tipped swab) and loops for smear vaginal microbiome analysis.

Instructions for vaginal Gram stain slide collection and preparation. (1) Remove a cotton swab from the packaging material by tearing off the top of the packaging. Be careful not to touch the tip of the swab. (2) Using the non-dominant hand (i.e. not the writing hand), the vaginal labia will be opened to allow entrance of the swab into the vagina. (3) The swab will be inserted 2 inches into the vagina, with care taken to not touch the tip of the swab elsewhere in the vaginal area. (4) The swab should be twisted several times while inside of the vagina. (5) The swab should be removed from the vaginal in the same manner that it was inserted. Care should be taken to not touch the tip of the swab outside of the vagina. (6) The swab should be rolled across the length of the Gram stain slide from the left to the right several times and then discarded. (7) The Gram stain slide should be placed into the slide carrier and the top of the slide carrier should be closed. (8) The Gram stain slide carrier should be placed in a small biohazard bag for transport.

Instructions for vaginal swab specimen collection. (1) Remove the Dacron swab from the packaging material by tearing off the top of the packaging. Care should be taken to not touch the tip of the swab. (2) The second vaginal swab should be collected in the same way as the first swab for vaginal Gram stain preparation. (3) This swab should be placed into a colour-capped specimen tube filled with DNA Assay Assure for future vaginal microbiota analysis. (4) The handle of the swab should be broken off at the score line and the handle should be discarded. (5) The top of the collection tube should then be put on the sample and screwed on firmly. (6) This tube should then be placed in a small biohazard bag and on ice in a cooler for transport.

4.4.7.2 Specimen Labelling

Vaginal Gram stain slides and swab specimen tubes should be labelled with a combination of the unique subject ID number and day of specimen collection (i.e. day 1 (D01), visit 1 [V01]). Leading 0's should be used in the subject ID number and day of collection up to the number of digits anticipated. For example, if <1,000 subjects are enrolled, subject #1 should use the identifier 001. If ≥1,000 subjects are enrolled, subject #1 should use the identifier 0001. Similarly, if >9 samples are obtained per subject, then the first sample from each subject should use the identifier 01. If >100 samples are obtained from a given patient, then sample number 1 should use the identifier 001. Examples: S0007-D07 <- This sample is from Subject #7 and is the 7th sample in the series. S0081-D15 <- This sample is from Subject #81 and is the 15th sample in the series. All subject IDs should be de-identified

from personal identifiers. The specimen tubes should be labelled with the subject ID number and day of collection on both the cap and the side of the tube using a permanent marker.

4.4.7.3 Specimen Transport and Storage after collection

All vaginal Gram stain slides and vaginal swab specimens should be transported in slide carriers at room temperature (vaginal Gram stains) and on ice (specimen collection tubes) from the clinic site or the participant's home on the day of self-collection to the Laboratory. Vaginal Gram stains will be stored at room temperature in the plastic slide carriers until the Gram staining procedure is performed. Vaginal swab specimens for future microbiota analysis are placed in preservation medium to decrease the likelihood of sample degradation and changes in the composition of the vaginal microbiota. Specimens will be stored in the -20° freezer until they are used to isolate DNA. Ideally, isolation of DNA should be performed within 2 weeks of specimen collection to avoid alterations of the composition of the vaginal microbiota.

For National shipping of Vaginal Swab Specimens, aliquots of extracted DNA from vaginal swab specimens will be shipped to the University of Cape Town (UCT). The front of the box will be labeled properly. A hard copy specimen manifest will accompany the specimens in the shipment for cross-checking purposes. A second copy of the shipping manifest will be emailed to UCT. The manifest will include each of the sample IDs and dates of sample collection in addition to the plate layout as well as the original and normalized concentration of each specimen. Once the specimens are received at UCT, they will send an email confirming their arrival. The specimens will be stored in a -80°C freezer at UCT until 16S rRNA gene library preparation and sequencing.

In case international shipping of vaginal swab specimens is needed for further analysis, material transfer agreements, customs and regulatory declarations and approvals will be obtained followed by shipment of aliquots of extracted DNA from vaginal swab specimens to LSUHSC in a dry ice box to the following address: Meng Luo, PhD, Pulmonary and Critical Care, School of Medicine, Louisiana State University, Health Science Center, 1901 Perdido Street, Suite 3205, MEB, New Orleans, LA 70112. The front of the box should be labeled properly. A hard copy specimen manifest should accompany the specimens in the shipment for cross-checking purposes. A second copy of the shipping manifest should be emailed to Drs. Luo and Taylor. The manifest should include each of the sample IDs and dates of sample collection in addition to the plate layout as well as the original and normalized concentration of each specimen. Once the specimens are received at LSUHSC, Dr. Luo will send an email confirming their arrival. The specimens will be stored in a -80°C freezer at LSUHSC until 16S rRNA gene library preparation and sequencing.

4.4.7.4 Diagnostic Testing

Vaginal specimens collected from participants will be tested for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in the same way as described for for Aim 1 and 2 (please see **3.5.8.5 Diagnostic Testing**).

4.4.7.5 Microbiological Profiling

Microbiological Profiling will follow the same procedures as described in Aim 1 and 2 (please see **3.5.8.6 Microbiological Profiling)**. For Aim 3, *Trichomonas vaginalis* will also be tested for at the laboratory of the Department of Medical Microbiology, University of Pretoria and not on site at the clinic. Additionally, molecular detection of *Candida* species will be done with well-established molecular essay following enhanced DNA extraction.

4.4.7.6 Cytokine Analysis

Cytokine Analysis will follow the same procedures as described in Aim 1 and 2 (please see **3.5.8.7 Cytokine Analysis**).

4.4.7.7 STI Reporting and Treatment

STI Reporting and Treatment will follow the same procedures as described in Aim 1 and 2 (please see **3.5.8.9 STI Reporting and Treatment**).

4.4.7.8 Partner Treatment

Partner Treatment will follow the same procedures as described in Aim 1 and 2 (please see **3.5.8.10 Partner Treatment**).

4.4.8 MICROBIOME SPECIMEN PREPARATION AND ANALYSIS

4.4.8.1 Selection of Stored Vaginal Gram Stain Slides and Vaginal Swab Specimens for Nugent Scoring and Vaginal Microbiota Analysis for the nested chlamydia case-control study:

"Cases" will subsequently be defined as participants who test positive for chlamydia by the Cepheid GeneXpert NAAT test at their enrollment visit (week 0) and at their first TOC visit (week 3; 'no clearance'). "Controls" will subsequently be defined as participants who test positive for chlamydia by the Cepheid GeneXpert NAAT test at their enrollment visit (week 0) but test negative at their first TOC (week 3; 'clearance'). Stored vaginal Gram stain slides and vaginal swab specimens (weeks 0-3) from cases and controls will be selected for Nugent scoring and vaginal microbiota analysis. Additional twice weekly stored vaginal Gram stain slides and vaginal swab specimens from "cases" who remained persistently positive for chlamydia by the Cepheid GeneXpert NAAT test after their 3-week TOC or 6-week TOC visit will also be selected for vaginal microbiome analysis (i.e. vaginal specimens collected after week 3).

4.4.8.2 Gram Stain Procedure and Nugent Score Determination for Vaginal Slide Specimens

Vaginal Gram staining will be performed in Dr. Remco Peter's South African laboratory for Nugent score and hyphae detection. Gram staining procedures: Crystal violet stain should be added to the specimen portion of the slide. Let stand for 10 to 60 seconds. Pour off the stain and gently rinse the excess stain with a very small stream of water from a faucet. Note that the objective of this step is to wash off the stain, not the fixed specimen. Add iodine solution on the smear, enough to cover the specimen portion. Let stand for 10 to 60 seconds. Pour off the iodine solution and rinse the slide with a very small stream of water from a faucet. Shake off the excess water from the surface of the slide. Add a few drops of decolorizer so the solution trickles down the slide. Rinse this off with water after 5 seconds. The exact time to stop is when the solvent is no longer colored as it flows over the slide. Further delay will cause excess decolorization in the gram-positive cells, and the purpose of staining will be defeated. Counterstain with basic fuchsin solution for 40 to 60 seconds. Wash off the solution with a stream of water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may gently be shaken to remove most of the water and air-dried. Instructions for Nugent score determination: The Nugent score is calculated by assessing for the presence of large Gram-positive rods (Lactobacillus morphotypes; decrease in Lactobacillus scored as 0 to 4), small Gram-variable rods (Gardnerella vaginalis morphotypes; scored as 0 to 4), and curved Gram-variable rods (Mobiluncus spp. morphotypes; scored as 0 to 2). A Nugent score of 0–3 is considered negative for BV, 4–6 intermediate for BV, and 7-10 indicative of BV. At least 10–20 high power fields (hpf) (1000× oil immersion) are counted on the Gram stain slide and an average number of the following criteria is calculated to determine the Nugent score:

Lactobacillus morphotypes - average per hpf (1000× oil immersion).	*Gardnerella/Bacteroides* morphotypes - average per hpf (1000× oil immersion).	Curved Gram variable rods - average per hpf (1000× oil immersion).
Score 0 for >30 Score 1 for 5-30 Score 2 for 1-4 Score 3 for < 1 (this is an average, so results can be >0, yet <1) Score 4 for 0	Score 0 for 0 Score 1 for <1 (this is an average, so results can be >0, yet <1) Score 2 for 1–4 Score 3 for 5–30 Score 4 for > 30	Score 0 for 0 Score 1 for 1-4 Score 2 for >= 5

Nugent scores and hyphae observation will be recorded in a laboratory-based data system (REDCap) and linked to a participant's metadata via their unique study ID.

4.4.8.3 DNA Extraction from Vaginal Microbiome Swab Specimens

This will be performed in Dr. Remco Peters' South African laboratory. For vaginal microbiome DNA isolation from vaginal swab specimens, any of the following kits can be used:

- Qiagen: DNEASY POWERSOIL KIT: Cat No./ID: 12888-100.
- Nvitrogen: PureLink™ Microbiome DNA Purification Kit: Cat. No. A29790.
- Microbiome DNA Isolation Kit (Norgen) from Canada.

All DNA isolation kits should be stored per the manufacturer's instructions. If any chemical pellets appear in the reagents, these need to be resolved by either shaking or the use of heat. RNase should be used to remove total RNA during DNA isolation. Agents to remove host DNA or PCR inhibitors are not necessary and increase kit costs. A DNA isolation control should be prepared from an unused vaginal swab specimen during this process. Each specimen prepared for international shipping should contain 50ng of extracted DNA with a total volume of 28 µL (no more than 35 µL) to allow for repeat library preparations, if the first library preparation fails. The DNA concentration can be measured by using either Qbit (fluorometer) or NanoDrop. Both can be used for RNase-treated DNA, however, only Qbit can be used for DNA samples without RNase treatment. All DNA samples should be normalized to the same concentration. You can dilute or concentrate to either 10 ng/µl, or 5 ng/µl, or 2 ng/µl depending upon which concentration is easiest to obtain. All concentration-normalized samples should be placed in skirted 96-well plates, with the study name ("i.e. South Africa R01"), date, specimen ID number, and normalized DNA concentration labelled on the side of the plate. The plate should be labelled column by column (i.e. A1 to H1) and not row by row (i.e. A1 to A12). The last two wells on the plate (i.e. G12 and H12) should be left empty as they will be used for the direction of the plate layout and negative/positive PCR controls. The plates should be sealed using an adhesive film such as Microseal B Sealer (BioRad).

4.4.8.4 Vaginal Microbiome Sequencing

To provide high quality sequencing reads, two steps of PCR methods will be used to prepare amplicon libraries. The primers for the first specific PCR are designed based on the 16S rRNA gene V4 region:
16SV4F 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA 3'; 16SV4R 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT 3'.

The second index PCR will use the Illumina Nextera XP index system. High-fidelity PCR DNA polymerases and Illumina-suggested PCR programs are used to perform the two PCRs. Typically, approximately 300 samples including 2 controls are included in each sequencing run. Amplicon concentrations for all sample libraries are measured and normalized to form a mixed loading library. Two QA/QC checks are performed during library preparation. Paired-end sequencing of 250 bp on each end of the V4 amplicons is conducted using the sequencing reagent kit V2 (500 cycles) and Illumina MiSeq NGS sequencer. The amount of high-quality reads provided is approximately 15,000 bp per sample.

To quantify the vaginal microbiome bacterial DNA load, we will use a broad-coverage qPCR targeting the V3-V4 region, which measures the 16S rDNA gene copies per swab.

4.4.8.5 Bioinformatics Analysis of Sequencing Data

Sequencing data will be stored in a RAID protected disk array at LSUHSC and UCT and backed up to external hard disks for additional redundancy. Sequencing data will later be quality controlled and analyzed using the DADA2 processing pipeline to produce high quality sequence variant tables. Phyloseq within the R statistical computing environment will be used to process secondary analysis and produce visualizations. Taxonomic classification will be performed using the RDP classifier and Silva version 128 database for precise assignment of taxonomy. Phyloseq and QIIME analysis packages will be used to assess taxonomic composition, and alpha and beta diversity of vaginal microbiome communities. Vaginal CSTs will be formed using the Phyloseq package based on hierarchical clustering of samples using the Bray-Curtis distance. Produced sequencing data will be deposited into the NIH Short Read Archive to provide accession numbers for publication of manuscripts.

4.4.8.6 Vaginal Microbiome Sequencing Data Transfer to Study Statistician

Sequencing data will be sent by Dr. Taylor at LSUHSC to statistician Dr. Redden at UAB as an excel document via LSUHSC's encrypted LSU Health FileS service which produces an encrypted email link. Data will be delivered as sequence variant tables containing sample names in the rows, sequence variants in the columns, and a count of the number of times a given sequence variant was found in a sample entered into each respective cell of the table.

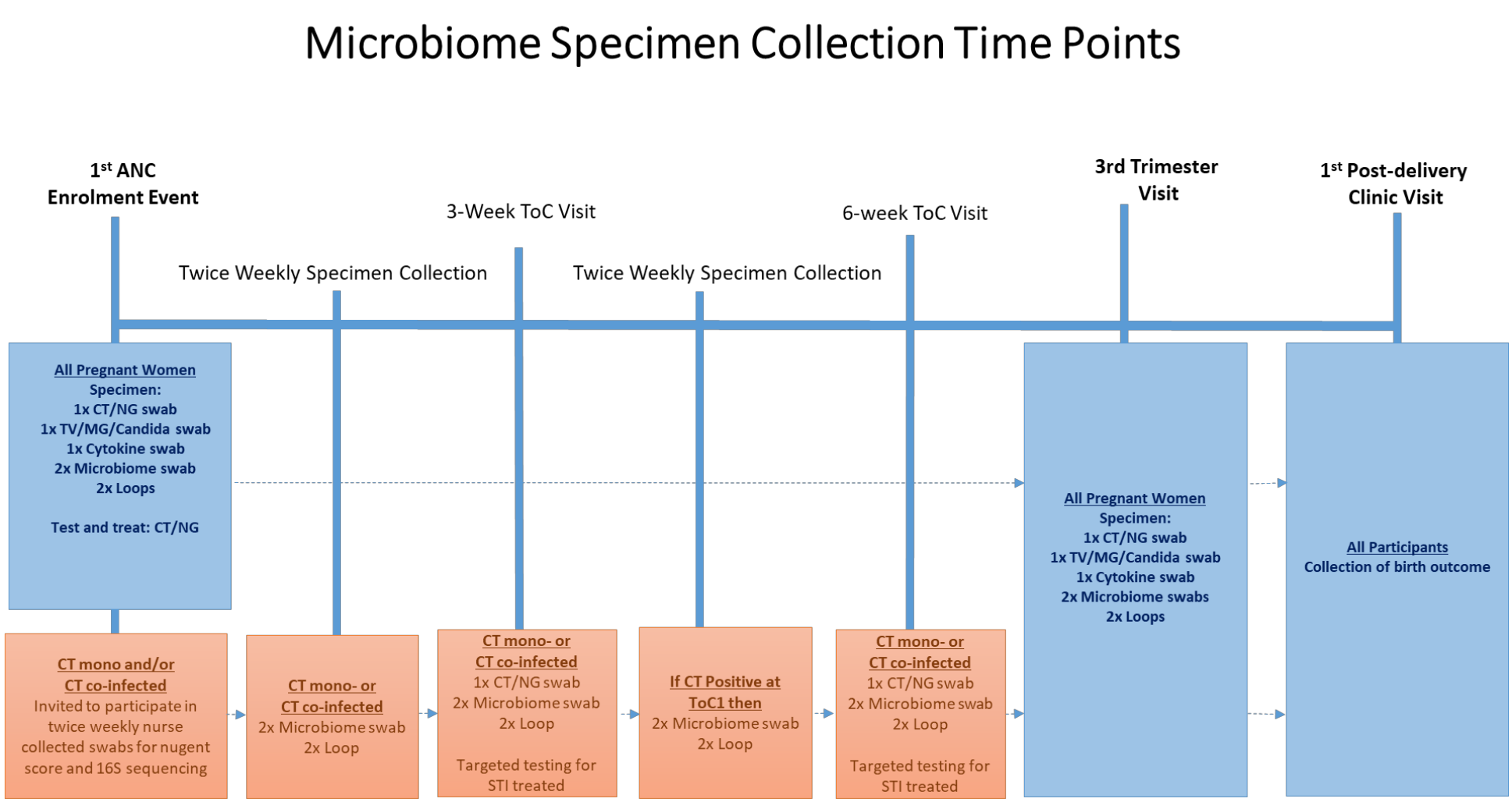


Figure 8. Aim 3 and 4 - Microbiome Specimen Collection Timepoints

Figure 9 - Aim 3 and 4 - Microbiome Specimen Collection Timepoints

4.4.8.7 Specimen Collection, Handling and Shipping

Vaginal specimen collection for microbiome analysis, glass slide smearing for Nugent scoring and specimen bio-banking will occur twice weekly. At ToC1 and ToC2 (i.e., week 3 and week 6 respectively), participants will be re-tested for *Chlamydia* and any other co-infections previously treated (i.e., TV and/or NG). Those with a positive ToC for *Chlamydia* (with or without co-infection) will again be provided targeted treated. We will also provide them with referral letters for their partners.

4.4.8.8 Nugent Scoring for BV

Air-dried slide smears will be heat-fixed, and Gram stained per standard procedure.[155] Nugent scores (0-3: normal, 4-6: intermediate and 7-10: BV flora[156]) will be recorded in a laboratory-based data system (REDCap) and linked to a participant's metadata via their unique study ID.

4.4.8.9 Molecular Methods/Interpretation of Sequence Data

Vaginal swabs will be subjected to sequencing of the V4 hypervariable region of the 16S rRNA gene using the well characterized 515F/805R primers; Illumina sequencing primers typically produces amplicons of ~290-292 base pairs. Paired end sequencing using an Illumina V2 sequencing kit 2x250bp produces reads with significant overlap, which will be processed through the DADA2 pipeline to assign high quality sequence variants. Taxonomic classification will be performed using the RDP classifier and silva version 128 database for precise assignment of taxonomy. Phyloseq[157] and QIIME[151] analysis packages will be used to assess taxonomic composition, and alpha and beta diversity of vaginal microbiome communities. Vaginal CSTs will be formed using the Phyloseq package based on hierarchical clustering of samples using Bray-Curtis distance.[158]

5 METHODOLOGY: AIM 5

5.1 DESCRIPTION OF THE STUDY DESIGN

This study will use a prospective cohort study design to address the main sub-study aim:

Aim 5) Determine the prevalence of *T. vaginalis* and the origins of repeat infection in pregnant women and its relation to the prevalence of drug-resistant *T. vaginalis.*

5.2 HYPOTHESES

Hypothesis 1: The prevalence of repeat T. vaginalis infections in South African pregnant women will be high at 10% (either due to re-infection or drug resistance).

Hypothesis 2: The prevalence of *T. vaginalis* drug resistance to 5-nitroimidazoles (MTZ, TDZ, and SEC) will be between 5-10% based on studies conducted in the U.S. [130]

5.3 METHODS AND PROCEDURES

5.3.1 DETERMINING TRICHOMONAS VAGINALIS PREVELANCE IN PREGNANT WOMEN IN SOUTH AFRICA

As outlined in the methodology for Aim 1 and 2, women designated in Arm 1 and Arm 2 of the parent RCT will undergo testing for *C. trachomatis, N. gonorrhoeae,* and *T. vaginalis* via PoC diagnostic Xpert test. At the time of enrollment, potential risk factors and baseline demographics including socio-economic status, obstetrical/gynecological history, sexual history, sexual behaviors, substance use history, partner characteristics, and previous history of STIs will be obtained from the parent study database organized by REDCap. Women in both arms are tested for *T. vaginalis* at study enrollment using the GeneXpert *T. vaginalis* NAAT and treated per standard-of-care (metronidazole 400 mg orally twice daily for 7 days) if positive. For women with positive GeneXpert *T. vaginalis* NAAT on enrollment, *T. vaginalis* InPouch cultures will be obtained. *T. vaginalis*-infected women in Arm 1 of this study receive a test-of-cure (TOC) for this STI three weeks after enrollment and if positive, are retreated. Women in Arm 2 of the study are re-tested for *T. vaginalis* between weeks 30-34 gestation. During the test-of-cure (TOC) visit (Arm 1) or 30-34 week follow-up visit (Arm 2), a follow-up study questionnaire will be administered re-examining signs & symptoms of STI, adherence to previously administered treatment, and interim partner history including if their prior sexual partners were tested for STIs and received treatment as well as history of sexual activity with new partners. Women are re-tested for *T. vaginalis* at each of the above-mentioned follow-up time points to assess for clearance of infection or repeat infection. The prevalence of women with repeat *T. vaginalis* infection at these two follow-up visits will be determined, and predictors of repeat infection will be examined to include interval sex with an untreated sexual partner(s), sex with a new sexual partner, treatment non-adherence, sexually-associated transmission events, or other potential etiologies.

Based on these questionnaires and *T. vaginalis* NAAT results, women will be categorized into three subgroups: 1) Probable re-infection from same partner (who was presumably not treated), 2) Probable re-infection from a new sexual partner or 3) Treatment failure. Baseline *T. vaginalis* prevalence will be calculated by dividing the number of positive *T. vaginalis* tests by the total number of test results. The proportion of *T. vaginalis* repeat infections will also be calculated.

5.3.2 TRICHOMONAS VAGINALIS DRUG SUSCEPTIBILITY TESTING

Women who were positive for *T. vaginalis* on enrollment and those with repeat *T. vaginalis* infection at their TOC visit (Arm 1) or their 32-34 weeks gestation follow-up visit (Arm 2) will have a vaginal specimen collected to inoculate a *T. vaginalis* InPouch™ culture. This culture will subsequently be incubated at 37°C and read 5 times over a 7-day period for the presence of motile trichomonads. If found to be positive, freeze-backs of the isolate will be prepared and stored at -80ºC until drug susceptibility testing is performed. Positive *T. vaginalis* InPouch cultures with low parasite loads at the end of the observation window (7 days), will have a portion of the media frozen back and a portion of the media passed into a fresh culture which would allow parasites to attempt to increase their numbers. *T. vaginalis* cultures and susceptibility testing will be performed.

5.3.2.1 *T. vaginalis* isolate selection and growth

T. vaginalis isolates from study subjects participating in the parent clinical trial [159] will be frozen at -80ºC and stored at the East London laboratory site. They will later be batch shipped on dry ice to Pretoria for drug susceptibility testing. At Pretoria, frozen isolates (2mL) will be thawed and added to 9 mL of warm Diamond's Trypticase-Yeast-Maltose (TYM) media contained in 15 mL polypropylene conical tubes. Cultures will then be placed in anerobic chambers with two AnaeroPack-Anaero pouches and placed in an incubator at 37°C for a minimum of 3 days to reach optimal cell density. Approximately 1 mL of the cultures will then be passed into new 15-mL conical tubes with fresh TYM media every other day. An antibiotic cocktail of 100X Penicillin/Streptomycin-Amphotericin B (MP Biomedicals, Solon, OH) will be added to each culture tube to prevent growth of bacteria and fungi. Once optimal growth conditions have been met (106 cells per culture), a 5 mL aliquot of the culture will be used for 5-nitromidazole susceptibility testing.

5.3.2.2 5-Nitromidazole susceptibility testing and analysis

MTZ, TDZ, and SEC resistance testing will be performed using a modified CDC susceptibility testing protocol [160,161]. Cultures will be transported to East Pretoria for susceptibility testing. Each antimicrobial agent will be solubilized in dimethyl sulfoxide (DMSO) and used to prepare 2-fold serial dilutions (400 µg/mL to 0.1 µg/mL) in Diamond's TYM media in a 96-well microtiter plate. Drug concentrations will be tested in triplicate and duplicate serial dilutions of equivalent final concentrations of DMSO will be included to control for parasite viability. Trichomonads (10^4/well) will be added to each well and plates incubated at 37°C for 46-50 hours under aerobic

conditions. The plates will then be examined using an inverted microscope at 100X magnification to evaluate cell motility with the lowest concentration of MTZ at which no viable parasites are observed will be recorded as the minimal lethal concentration (MLC). Data from the isolates will be analyzed to determine the median and 95th percentile MLC for each drug. Resistance to MTZ will be defined as an MLC ≥50 µg/mL, while resistance to TDZ will be defined as an MLC ≥ 6.3 µg/mL [160-162]. The resistance breakpoint for SEC has not been previously determined however our ongoing study suggests that a SEC MLC >12.5 µg/mL correlates with resistance (unpublished data) and this breakpoint will be used.

6 PARTICIPANT FOLLOW-UP AND RETENTION

To ensure retention, those providing informed consent will be asked to provide detailed contact information (e.g. phone numbers and home address for self, family, friend/neighbour). To develop and maintain a strong relationship with participants, study staff will conduct welcome phone calls within 3 days of enrolment and check in with participants during regular ANC clinic visits or monthly ART pickup for those with HIV. Appointment reminders will be sent via sms', and a telephonic reminder will be done prior to the visit. Women will also be given an appointment card as a reminder for future visits which will also include the research nurses' clinic details.

We will flag participant charts so that clinic staff will notify study staff on date of delivery. Seven days post-delivery, study staff will contact participants not yet attending a first postnatal clinic visit to schedule an outcomes interview. We will make up to 7 attempts to follow up with participants via text/phone call/home visits.

6.1 Participant reimbursement

When the participants return to report their pregnancy outcome, we will provide a reimbursement of a R150 gift voucher to use at local stores.

To increase the study retention rate of the RCT, participants will be additionally provided the following reimbursement amounts in form of gift voucher to use at local stores at their follow-up visit:

Visit	Reimbursement amount
Test-of-cure (Arm 1 only)	R 100
3rd trimester visit (all RCT participants)	R 100

To increase the study retention rate of the nested chlamydia case-control study, participants in that study will be additionally provided the following reimbursement amounts paid at their test of cure visit: R 200 per collection

Number of successfully collected specimens	Reimbursement amount
21 days Test of Cure (5 collections)	R 1000
42 days Test of Cure (10 collections)	R 2000

7 POTENTIAL CHALLENGES AND QUALITY ASSURANCE

7.1 Aim 1

Loss-to-follow up, postnatal specimen collection and interviews, and adequate 6-week infant follow-up visits may be the dominant Aim 1 challenges. In our current R21 study, optimized retention strategies resulted in >85% retention. Strategies included enhanced participant tracking, welcome phone calls, employing a community-based roving nurse that visited women in their homes for follow-up visits, and telephonic interviews to collect self-reported outcomes data. We will also hire a midwife research assistant with full access to MOUs to abstract medical records and discharge summaries. Based on current experiences, we believe that we are well prepared to overcome typical retention challenges. Given that syndromic screening/management is performed at all ANC visits, we will abstract medical records of all participants to determine if syndromic management was conducted outside research study events. We will take such events into consideration when analysing and interpreting our results. Finally, all research study personnel will meet weekly to review study enrolment, specimen collection, processing, test turn-around-time, data management, and treatment outcomes. Meetings will discuss descriptive study results to date, problems encountered and remedial actions to be taken.

7.2 Aim 2

The main challenge of Aim 2 involves accurate data collection of new-born hospital care costs, particularly those costs incurred by any higher-level neonatal care. If necessary, we will extend our follow-up of these infants beyond 6 weeks postpartum and will collect new-born cost data until discharge or death, whichever comes first; this will likely be a few months of hospital care for babies born very pre-term.[163-167]

7.3 Aim 3

Some participants might be uncomfortable with the number of swabs being taken. We will ensure that the participant is comfortable during specimen collection by training the staff in communication and specimen

collection, providing privacy and monitor the participant's well-being. The participant is at any time able to stop the specimen collection procedure. The study staff will take the specimens in a sequence that even if the participant is only tolerating less specimens to be taken, the major analyses of the study are still possible.

7.4 Aim 4

It may be challenging to collect specimens from women on a daily basis. Therefore, we propose an internal pilot phase to collect specimens from 20 women. After this phase, the study team will assess the acceptability and feasibility of the approach and adjust the specimen collection strategy if necessary. The UCT Human Research Ethics Community will be notified if any changes are made in specimen collection procedures. Another limitation is our inability to exclude re-infection as the cause for a positive test result at ToC. Consequently, we will exclude or adjust our analysis based on self-reported high-risk sexual behaviour between first ANC and ToC visits. To assess for re-infection, *Chlamydia* genotyping will be performed at the University of Pretoria on paired specimens of persistently positive participants using other existing funds.

7.5 Aim 5

It may be difficult for *T. vaginalis* isolates to grow in the culture medium if the burden of infection is low. However, the InPouch cultures will be read multiple times over a period of 5-7 days to assess for a positive result. The results of this Aim will be used for research purposes only and will not be used in clinical care.

7 ANALYTIC PLAN

7.1 PRIMARY OUTCOME DEFINITION

The primary objective of this study is to evaluate whether two approaches to antenatal screening and treatment of curable STIs in pregnancy leads to a reduction in preterm birth and low birth weight compared with standard antenatal care that involves syndromic management.

The definition of 'curable STIs' in this trial includes *C. trachomatis, N. gonorrhoeae*, T. vaginalis. The two screening interventions are: 1) one-time testing for curable STIs at first antenatal visit before 27 weeks' gestation with a test of cure three weeks after for participants who tested positive at enrollment, and 2) twice testing — at the initial antenatal visit, and at a routine visit in the third trimester to coincide with gestational weeks 30 to 34.

The primary outcome comprises a composite endpoint of preterm birth (born alive before $37^{0/7}$ weeks' gestation) and/or low birth weight (less than 2500g) among all live births.

A dating ultrasound is conducted for all women at the time of enrollment. The gestational age at delivery will be calculated based on ultrasound-estimated gestation age. Birth weight data will be extracted from maternal case records at birth facilities and/or obtained from the participant's labor discharge summary during her postnatal study visit.

.

7.2 SECONDARY OUTCOME DEFINITIONS

Several secondary outcomes will also be assessed according to treatment arm.

The primary analytic models will utilize the most current endpoints, as defined by the World Health Organization (WHO). To ensure comprehensive comparability of our findings across global cohorts, additional models will consider other standardized endpoint definitions that may differ from the WHO endpoints.

Among all live births, we will assess the relationship between treatment arm and each of the following secondary outcomes, using the most updated endpoints defined by the World Health Organization (WHO):

A. Preterm birth, defined as <37 (model 1) and <34 (model 2) weeks gestational age (GA)
B. Low birthweight, defined as <2500g (model 1) and very low birth weight, defined as <1500g (model 2)
C. GA at delivery (continuous outcome)
D. Birth weight at delivery (continuous outcome)
E. Small for GA (SGA), defined as < 10^{th} percentile for gestational age, using the birthweight distribution in the control group for each gestational age. Model 2 will analyze Very SGA births, defined as birthweight below the 3^{rd} percentile for babies of the same GA.
F. Neonatal mortality, defined as death within 28 days of birth
G. Early neonatal mortality, defined as death within 7 days of birth.

Among all births, we will evaluate the relationship between treatment arm and each of the following secondary outcomes:

A. Stillbirth, defined as birth without sign of life at ≥28 weeks' gestation or clinically diagnosed as stillbirth in the maternity records between 20-28-weeks' gestation.
B. Perinatal mortality, defined as stillbirth and/or early neonatal mortality < 7 days of life.

Among all pregnancies, we will determine the effect of treatment arm on each of the following secondary outcomes:

A. Miscarriage/spontaneous abortion, defined as fetal loss <28 weeks (model 1) and early miscarriage <20 weeks (model 2).
    a. For non-WHO miscarriage endpoints, we will consider <22 weeks and <24 weeks
B. Any adverse birth outcome, defined in this trial to include preterm birth, low birth weight, small for gestational age, stillbirth and early neonatal death.
C. Hypertensive disorders in pregnancy:
    a. Chronic/Essential Hypertension: Maternal systolic blood pressure (SBP) ≥ 140 mm Hg and/or diastolic blood pressure (DBP) ≥90 mmHg before pregnancy or before 20 completed weeks of gestation. Where pre-pregnancy blood pressure is unknown, two measurements taken 4 hours apart can be used.
    b. Gestational Hypertension: Elevated maternal systolic blood pressure (SBP) ≥ 140 mm Hg and/or diastolic blood pressure (DBP) ≥90 mmHg, first detected after 20 weeks' gestation.
    c. Preeclampsia superimposed on existing hypertension: In a pregnant woman with chronic hypertension who develops worsening hypertension after 20 weeks of gestation, with new onset of proteinuria or other signs of organ dysfunction. *
    d. Preeclampsia-Eclampsia: As indicated in maternal case records, maternal SBP ≥140 mm Hg and/or DBP ≥90 mm Hg in a previously normotensive participant accompanied by proteinuria or one or more signs of organ failure, after 20 weeks of gestation.* Eclampsia, when seizures or coma occur, without another probable cause, in the same timeframe.

**Organ dysfunction is defined as thrombocytopenia (platelets <100K), elevated blood levels of liver transaminases (ALT and/or AST twice the normal concentration), and new onset renal insufficiency (creatine >1.1 mg/dL or doubling of creatine in the absence of other renal disease), pulmonary edema, or new cerebral or visual disturbances.*

7.3 **SECONDARY STI-RELATED OUTCOME DEFINITIONS**

Several secondary STI-related outcomes will also be assessed according to treatment arm. We will assess each of the following:

A. STI burden — Prevalence of *C. trachomatis*, N*. gonorrhoeae*, and/or *T. vaginalis* infection:
    a. Compare the change in prevalence between enrollment and 2 weeks post-delivery when the postnatal test is conducted. We will compare the % change between intervention arm 1 and control arm 3, and intervention arm 2 and control arm 3. We hypothesize that each intervention arm (1 or 2) will have a greater change in prevalence than the control arm (Arm 3)
    b. Compare the post-delivery prevalence only between intervention Arm 1 and control Arm 3, and intervention arm 2 and control arm 3. We hypothesize that both Arms 1 and 2 will have a lower post-delivery STI prevalence than the control arm (Arm 3)
    c. Compare a single diagnostic screening and test of cure (Arm 1) to repeat screening and treatment without a test of cure (Arm 2). We hypothesize that Arm 2 will have lower STI burden than Arm 1.
    d. Compare the combined treatment arms (1 & 2) to control with respect to (I) change in antenatal to post-delivery STI prevalence, and (II) post-delivery prevalence only. We hypothesize that either treatment arm is associated with a lower STI prevalence compared to control.

B. Vertical transmission of STI (from mother to child)
    a. Overall frequency of vertical transmission. This analysis is restricted to mothers testing positive for *C. trachomatis*, N*. gonorrhoeae*, or *T. vaginalis* infection within 14 days of delivery. We will assess the proportion of newborns testing positive for *C. trachomatis*, N*. gonorrhoeae*, or *T. vaginalis* infection (using ocular and nasopharyngeal swab specimens collected in the first 14 days of life) among STI-exposed newborns.
    b. Newborn STI Positivity. We will evaluate the incidence of newborn infection with *C. trachomatis*, N*. gonorrhoeae*, or *T. vaginalis* infection among all live deliveries, comparing each intervention arm (model 1) and composite intervention arms (model 2) to control.

7.4 **EXPLORATORY OUTCOMES/ASSESSMENT DEFINTIONS**

We will also conduct several exploratory, hypothesis-generating analyses.

A. Antenatal STI Epidemiology — We will calculate prevalence, incidence, and assess the distribution and correlates of *C. trachomatis*, N*. gonorrhoeae*, *T. vaginalis,* or *T. pallidum* infection as well as Bacterial Vaginosis.
B. Epidemiology of adverse birth outcomes — We will calculate the incidence and assess the distribution and correlates/risk factors of preterm birth, low birth weight, small for gestational age, and perinatal mortality.
C. Impact of untreated asymptomatic STIs in pregnancy— Analysis of enrollment specimens from participants in the control arm to compare individuals who presented with any STI exposure (*C. trachomatis*, *N. gonorrhoeae*, or *T. vaginalis*) but were asymptomatic and untreated at presentation. We will compare the incidence of any adverse birth outcome in asymptomatic participants untreated for infection vs. uninfected asymptomatic participants, treated asymptomatic participants, and treated symptomatic participants.
D. Microbial-induced inflammatory markers of preterm birth analyzing banked endocervical swab specimens.
    - Using a case-control design, with cases defined as pregnant women who had an STI in pregnancy and experienced a singleton spontaneous preterm birth and controls defined as women with STI who did not experience preterm birth. We will compare baseline inflammatory cytokine profile of cases vs. controls.
    - Using a case-control design, with cases defined as pregnant women who experienced a spontaneous preterm birth and had STI during pregnancy, and controls defined as pregnant women who experienced preterm birth but did not have STI in pregnancy. We will compare baseline inflammatory cytokine profile of cases vs. controls.
E. Inflammatory markers of preterm birth — Independent of STI status during pregnancy, we will explore the baseline proteomic markers among participants who experienced a singleton spontaneous preterm birth compared to participants who did not.
F. Bacterial Vaginosis (BV) and treatment outcomes for Chlamydia — We will explore the prevalence of BV based on Nugent scores of 7-10 at each visit. For the case control cohort to assess association of chlamydial treatment outcomes and BV, Nugent scores will be compared in cases vs. controls at the enrollment visit and at weekly or serial tests of cure. Associations between Nugent scores, chlamydia treatment outcomes, vaginal pH, and other clinical data will be analysed. Variations by symptomatic or asymptomatic chlamydial infection will be assessed. Vaginal pH will be considered as a proxy to estimate presence of dysbiosis. Key covariates that may influence these associations, HIV infection status, HIV (viral) load, CD4 count, anti-retroviral therapy exposure, and demographic characteristics will be considered for inclusion into the models to assess the effect of these factors on chlamydial treatment success rate.
G. Process evaluation measures as described in Table 4.

To adequately inform the selection of exposure variables in the exploratory analyses, relevant sociodemographic, clinical, behavioral, and obstetric factors will be identified through an extensive review of the literature. Final covariates and confounders will be chosen using a structured framework, such as directed acyclic graphs (DAGs), to support the analytic decisions.

7.5 **PLANNED SUBGROUP ANALYSES**

Additionally, we will perform several subgroup analyses to evaluate incidence of preterm birth / Low birth weight, modeling the relationship with treatment arm using logistic regression, as described below. We acknowledge that statistical power will be limited for these subgroup analyses.

Specifically, we will evaluate the subset of participants with:

1. Singleton pregnancies
2. Participants with an STI at baseline (C. trachomatis, N. gonorrhoeae, T. vaginalis). Additional covariates may include antibiotic treatment in pregnancy (yes/no) and timing of antibiotic treatment (<20 weeks vs. ≥20 weeks).
3. Nulliparous women. Nulliparous will be defined as the first pregnancy (nulliparous primigravida) or, if prior pregnancy, it did not result in a live birth (nulliparous, multigravida).
4. HIV positive. Additional covariates may include viral suppression (e.g., viral loads), immunological profile (CD4 count), and timing of antiretroviral therapy (pre vs. post conception).
5. Participants with anemia at baseline. Anemia will be defined as Hgb<11 g/dL (model 1) and <8 g/dL (model 2).
6. Alcohol use. Among participants reporting any alcohol using during pregnancy, we will assess the impact of amount of alcohol consumed (comparing those reporting harmful use/binge drinking to those reporting lower levels of consumption) as well as timing of alcohol consumption.
7. Genitourinary symptoms. Among those with genitourinary symptoms at baseline, we will assess differences in outcome according to whether the participant reported vaginal discharge. We will investigate the impact of antibiotic treatment in pregnancy, regardless of STI positivity.

7.6 **STATISTICAL CONSIDERATIONS**

7.6.1 Sample Size for Primary Research Question

We calculated the effective sample size required to compare each treatment arm (1 and 2) to the control arm (3) among live births. Using a test for multiple proportions with three arms, we considered a baseline frequency range of 20% to 26% for the primary endpoint (preterm birth and/or low birth weight), based on formative work in the population. We also looked at a range of incidence reductions, from 20% to 35%. Assuming an average baseline frequency of 23% for the primary outcome and anticipating an average clinically meaningful reduction of at least 25% in incidence between either intervention arm compared to control, we would require a total of 2079 participants with a live birth (693 per arm) to detect a difference of that magnitude. Considering a conservative 20% attrition rate due to pregnancy losses, protocol violations, or loss to follow-up, recruitment would necessitate a total of 2495 participants (832 per arm).

Although we do not expect a significant difference in treatment efficacy between the two intervention arms, we will compare them to obtain an estimate of the effect size for enhanced inference or to inform future studies. The study is not powered to detect equivalency between treatment arms, as this is not a primary aim. We assumed a two-sided p-value of 0.05 and 80% power, using PASS 2023 (NCSS, LLC, Kayesville, UT).

Table 6: Primary Analytic sample size and Power Calculations

Power/Alpha (2-sided)	Baseline Endpoint	Expected Reduction	Effective n by arm	Effective N (Total)	Effective N (Total) with 20% attrition
80/0.05	20%	20%	1318	3954	4745

80/0.05	20%	25%	822	2466	2959
80/0.05	20%	30%	555	1665	1998
80/0.05	20%	35%	373	1118	1342
80/0.05	23%	20%	1109	3327	3992
80/0.05	23%	25%	693	2079	2495
80/0.05	23%	30%	469	1407	1688
80/0.05	23%	35%	335	1005	1206
80/0.05	25%	20%	998	2994	3593
80/0.05	25%	25%	624	1872	2246
80/0.05	25%	30%	422	1266	1519
80/0.05	25%	35%	302	906	1087
80/0.05	26%	20%	949	2847	3416
80/0.05	26%	25%	593	1779	2135
80/0.05	26%	30%	402	1206	1447
80/0.05	26%	35%	288	864	1037

7.6.2 Analyses of Demographic and Clinical Characteristics

We will describe demographics and clinical characteristics of the study population according to treatment arm using mean $\pm$ standard deviation for numeric variables and counts with frequency for categorical variables. We expect that there will be a small amount of missing data. For variables with ≤20% missing data, we will use Markov-Chain Monte Carlo (MCMC) methods to impute missing data for important variables of interest. To assess the impact of imputation on our results, we will conduct a sensitivity analysis by comparing the results of the imputed dataset with results from models without imputation. Overlap in confidence intervals will be assessed to determine differences between the estimated parameters. Assuming overlapping 95% confidence intervals between models, we will present the primary analysis with imputed covariate data. Any variable with >20% missing data will not be used for analysis.

7.6.3 Study hypotheses

A. One-time diagnostic STI screening and treatment (for any of *C. trachomatis, N. gonorrhoeae, or T. vaginalis*) at a woman's first antenatal care visit with a subsequent test of cure (arm 1) or a twice screening algorithm without test of cure (arm 2), will significantly reduce preterm birth and/or low birthweight compared to the syndromic management (arm 3).

B. Either diagnostic STI screening intervention — both treatment arms assessed as a composite — will significantly reduce preterm birth and/or low birthweight compared to syndromic management.

C. One-time diagnostic STI screening and treatment with a test of cure (arm 1) or a twice screening algorithm with no test of cure (arm 2), will significantly decrease STIs by the time of delivery compared to the syndromic management (arm 3).

D. Compared to one-time diagnostic STI screening and treatment with a follow-up test of cure (arm 1), two-time screening and treatment without any test of cure (arm 2) will significantly decrease STIs at delivery.

The assessment of STIs at the time of delivery will employ testing conducted within 2 weeks after delivery.

The analysis of both primary and secondary hypothesis will be by intent-to-treat, and thus include all randomized participants regardless of fidelity to study intervention (screening and treatment). Additionally, a modified intent-to-treat analysis will be conducted including only randomized participants who completed screening and treatment interventions and have follow-up. Last, we will conduct a per-protocol analysis, including only participants who received screening and treatment according to protocol and have follow-up measures.

We will use generalized linear models, specifying a Gaussian distribution for birthweight and gestational age at delivery; and a binary distribution for preterm birth, low birthweight, diagnosis of any STI, occurrence of maternal child transmission, and other adverse birth outcomes. STI prevalence will be reported by intervention arm as proportion of individuals testing positive divided by number of individuals tested. Exact binomial 95% confidence intervals for prevalence will be estimated. Incident infections will be identified when a participant tests STI-negative during the first antenatal care visit and positive in the third trimester antenatal care visit. Time at risk will be the length of time between the first test date and the positive test date.

Incidence of the preterm birth/low birth weight will be compared between study arms using multivariable logistic regression. The dependent variable is a composite of preterm birth and low birth weight, and the independent variable is study arm. Covariates of interest include presence of STI at enrollment, parity/nulliparity, HIV infection, genitourinary symptoms, anemia, alcohol use, and any variable that appears unbalanced between groups at baseline. Potential predictors (socio-demographic, behavioral, medical, obstetric, pregnancy, partner, and perinatal characteristics) will also be considered for inclusion in all models.

Within Arm 2, a logistic regression model will be developed utilizing incident STIs (negative at first ANC visit, positive at 30-34 week ANC) to determine if there is an optimum gestational age at which a second STI screening would be most beneficial or if the data indicates a steady probability across gestational ages.

Variables with $p<0.25$ will be selected for further evaluation; only variables with $p\leq0.05$ or those that are confounders will be included in the final model. While we do not anticipate confounding due to the randomization procedure, any variable that alters the effect size (OR) between study arm and outcome by >15% will be included in the model. Model fit will be evaluated by the Hosmer-Lemeshow goodness-of-fit test (logistic regression) and via inspection of residuals, deviance, and leverage. Normal probability plots will be used to access the normality assumption for multiple linear regression models. If the normality assumption appears violated, non-parametric procedures will be utilized (e.g., nonparametric kernel regression). If model fit is not adequate, we will consider the inclusion of interaction terms between predictors. To assess the impact of imputation, a sensitivity analyses will be conducted as described previously. All models will include a test for effect modification by HIV infection status; a significant Likelihood Ratio test ($p<0.05$) will indicate significant interactions and trigger stratification by the effect modifier. Overall Type I error rate will be set at 0.05 and all analyses will be conducted with Stata 18.0 or higher (Stata Corporation, College Station, TX).

7.6.4 Analytic Plan for Process Evaluation Qualitative Data

We will employ aspects of deductive analysis that consider the RE-AIM framework through the creation of initial a priori codes. Data coding and analysis will be an iterative and interactive process. Interview transcripts will be

read to increase familiarity with data. A priori and emergent codes will be assigned. Transcripts will be re-read to create pattern codes that connect subsequent concepts under larger headings. Consistent patterns in meaning, concepts, and themes across interviews will be identified, and data matrices created as visual representations of findings.[145,146,150] We will also examine any differences based on stakeholder type (i.e., study staff, non-study clinic staff, NHLS and Health Department) to identify unique viewpoints. Coding and analytic activities will be discussed during qualitative data analysis meetings; discrepancies in coding and interpretation will be resolved through consensus.

7.6.5 Decision Analytic Modelling

We will build a decision analytic model to estimate costs and outcomes for each study arm and perspective (provider/patient). Box 1 (see Statistical Design and Power) summarizes formulae for calculating costs and DALYs for the provider perspective (arguably the more complex calculation). For DALY calculations, years of life lost are the difference between age at death and average South African life-expectancy for that age; years of life with disability and disability weights will be estimated from the Global Burden of Disease studies.[152,169] Deterministic sensitivity analyses will assess the impact of key parameter uncertainty (e.g. cost of GeneXpert machines within a scale-up scenario). Probabilistic sensitivity analysis will assess uncertainty around each utilization estimate from the trial.[170] However, if we find that the costs of Arms 1 and/or 2 exceed the costs of Arm 3, we will compute incremental costs per STI and Disability-Adjusted Life Year (DALY) averted. For the patient perspective, catastrophic expenditure will be computed by comparing patient costs to household expenditure using 10% and 20% thresholds per other South African and low and middle-income country studies.[171]

HUMAN SUBJECTS CONSIDERATIONS

7.7 Ethical Review

This protocol and the informed consent forms (ICF) contained in the Appendices will be reviewed and approved by the University of Cape Town, Research Ethics Committee with respect to scientific content and compliance with applicable research and human subjects' regulations. The protocol, ICF and all translations, participant education and recruitment materials, and amendments thereof will be reviewed and approved by the ethical review bodies responsible for oversight of research.

Subsequent to initial review and approval, the responsible IRBs/ECs will review the protocol for renewal once every 12 months. The Investigators will submit safety and progress reports to the IRBs/ECs at least annually, and within three months of study termination or completion. These reports will include the total number of participants enrolled in the study, the number of participants who completed the study, all changes in the research activity, and all unanticipated problems involving risks to human subjects or others.

7.8 Informed Consent

All interested candidates will be approached and those whom meet the eligibility criteria will undergo informed consent. It should be emphasized that participation is voluntary and that the care the participant will receive in the facility will not be any different from the care they would receive if they had opted not to participate. Before any specimens or data is collected, written informed consent will be obtained from all study participants. All staff will receive training on Protecting Human Research Participants and Good Clinical Practice.

7.8.1 Informed Consent Procedures

STI Counsellors responsible for enrolment of participants will be equipped with Informed Consent Forms (ICF). ICFs describing the purpose of the study, procedures to be followed, the risks and benefits of participation, in accordance with all applicable regulations will be translated into the most relevant local languages of each study site. All translated ICFs will be back translated into English to verify the accuracy of the translation. Furthermore,

all ICFs will be piloted before the commencement of enrolment to determine the level of understanding of the target population. All interested eligible candidates deemed to be literate will document their provision of informed consent by signing their informed consent forms. Illiterate participants will be asked to document their informed consent by marking their ICFs (e.g., with an X, thumbprint, or other mark) in the presence of a literate third-party witness. Any other local IRB/EC requirements for obtaining informed consent from non-literate persons will be adhered to.

For candidates that are eligible for the study, a private informed consent session will be held with each individual potential participant, during which the study will be explained. Potential candidates are free to withdraw at any given time without having to give a reason, they will be told that the care they receive from the facility will not be affected in any way if they do decide to withdraw.

7.8.2 Post-refusal Questionnaire

Participants who are not interested in partaking in the research study after the informed consent process, may decline consent. Study staff will ask whether the participant would like to give their reasons for not participating. This is however voluntary; participants do not have to answer any additional questions if they refuse to participate in the study. The responses will be captured on RedCap. This will provide information on the acceptability and feasibility of each intervention and depict whether there is a difference between those who consent or not.

7.9 Risks

7.9.1 Social Harms

7.9.1.1 Intimate Partner Violence

There are a number of potential unintended consequences to participating in an STI screening study. Partners are an important component in STI (re)infection and treatment. Participants whom test positive for an STI will be encouraged to disclose to their partner(s). As a result of gender and relationship power dynamics, women are often blamed for bringing the infection into the relationship. High rates of intimate partner violence in South Africa, may cause women to be scared to disclose their STI-status. Disclosure may also have unintended consequences of a social, emotional, physical and/or financial nature.

To mitigate this risk, we have formulated the following:

1) All participants will receive counselling as per clinic standards once STI results are available. All participants will be counselled on the unintended social risks with STI disclosure. Women testing positive for an STI will be counselled on safe disclosure to their partners.
2) Women will be given the referral for their partner(s) to present to the clinic.
3) All participants will be assessed for potential intimate partner violence related to disclosure.
4) Participants will be asked to report any social harms. Appropriate counselling, care and referral will be offered depending on the nature of the social harm reported. Any social harms that arise during the study will be recorded and reported as per NIH and local IRB guidelines.

5) In the case where issues relating to IPV or mental health is detected, participants will be offered to see the social worker or clinic psychologist. Additional information on organizations that provide IPV-support services (e.g. such as hotlines, local trauma centres), can be extended to participants where needed.

7.9.2 Confidentiality

Confidentiality of information collected is of fundamental importance. The research team will be trained to adhere to strict confidentiality guidelines and will protect the confidentiality of participants to the fullest extent possible. The following measures will be used to protect the confidentiality of the information collected:

1) Research staff will be trained on maintaining confidentiality.
2) Interviews will be conducted in a private setting.
3) Signed paper informed consent forms will be stored in a locked file cabinet separately from other study data.
4) Participants' contact information will be securely stored on password protected computers separate from other study data.
5) Names will not be written on any data collection forms; unique PIN numbers will be assigned to each participant. The master list linking the PIN numbers to the participants' identifiable information will be securely stored.
6) Identifiable information unintentionally collected in audio recordings will be redacted.
7) Data will be entered directly into password protected devices.
8) Data will be uploaded regularly onto a secure server at FPD Head Office in Pretoria.
9) Only a limited number of study staff whose role requires access to information with personal identifiers (i.e., signed informed consent forms, contact information, master list linking participant names with study PIN number and contact information) will have access to these types of data.
10) Participant personal identifiers will not appear in any data or documentation sent to collaborators.
11) Only authorized study staff will have access to study data.
12) Participants will not be identified by name in any report or publication resulting from study data. In reports and publications, data will be not be presented in a way where responses can be linked back to individual participants.

All hard copies of data, electronic files, and databases will be stored securely at FPD's office in East London for up to five years after the completion of the study or as required by the institutional review boards and prevalent South African legislation. See data management section for additional details.

Although study sites will make every effort to protect participant privacy and confidentiality, it is possible that a participants' involvement in the study could become known to others, and that social harms may result. For

example, participants could be treated unfairly or discriminated against, or could have problems being accepted by their families and communities as a result of study participation. Qualitative responses will remain anonymous; names will not be used in IDIs or FGDs and any potentially identifying information will not be transcribed.

7.9.3 Sensitive Questions

Participants will be asked questions about their sexual behaviour and intimate partner violence that may make them feel uneasy. Participants do not have to answer any question that they do not want to and can stop answering the questions at any time. Additionally, skip patterns will be used to ensure to only ask more detailed questions to participants for whom these questions are relevant. Qualitative participants will not be pressured to disclose personal details they are not comfortable sharing.

7.9.4 Treatment Regimens

The study will make use of approved first-line syndromic treatment regimens for STIs as per CDC and South African guidelines:

Table 77: First-line treatment regimens for participant

Infection	**Treatment Regimen (Females)**
Chlamydia trachomatis	Oral Azithromycin (two 500mg tablets) single dose
Neisseria gonorrhoea	Ceftriaxone 500 mg IM single dose
Trichomonas vaginalis	Metronidazole 400 mg twice a day for 7 days

The treatment regimens have acceptable side-effects and comply with the standard treatment given.

Treatment given to women based on symptoms, will comply with the National Sexually Transmitted Infection Management Guidelines algorithms. Women presenting with STI-related symptoms but tested negative for all tested STIs on the GeneXpert, will be treated with Metronidazole 400 mg twice a day for 7 days.

7.9.5 Specimen Collection

Women may experience minor discomfort when specimens are obtained (e.g. inserting vulvovaginal swab). This is however normal diagnostic routine practice across the world and we do not foresee adverse events in this regard. Specimen collection will be done by a trained professional nurse and participants will receive adequate instructions and guidance should they have to self-collect a specimen. Women are also free to withdraw from specimen collection at any time.

7.10 BENEFITS

Women will benefit from better access to syndromic STI screening, treatment and diagnostic testing. Better STI management in turn allows for certain health benefits for both the participant and the neonate. Additionally, participants and others may benefit in the future from information learned from this study. Specifically, information learned in this study may help to reduce adverse birth outcomes and assist with PMTCT efforts.

Participants found to have persistent STI infections can be referred for specialized treatment and care. Clinical notes and/or referrals will assist the participant beyond the duration of the study.

7.10.1Treatment for genital infections

The standard of care (symptomatic treatment) is provided at every visit as per the South African guidelines for syndromic management of sexually transmitted infections. In addition, at base line (early in pregnancy) targeted treatment is provided as described in the previous paragraphs. For Aim 3, specimens taken at 30-34 weeks will be batch-tested after delivery and repeated target treatment will therefore not be provided because no clear evidence from RCTs is available to justify the risk of antibiotic treatment in late pregnancy versus the benefit of screening.

Mitigation strategies will be put in place to avoid the potential risks, as described above. Given the health benefit for both the participant and neonate, the benefits outweigh the potential risks. The protocol and procedures described above meet the four principles of: autonomy, justice, beneficence, and non-maleficence.

7.11 STUDY DISCONTINUATION

The study may be discontinued at any time by NIH other government or regulatory authorities, and/or IRBs/ECs.

7.12 DATA AND SAFETY MONITORING

Any unfavourable and unintended sign (including an abnormal laboratory finding), evidence of social harm (SH), or symptom or disease temporally associated with the use of a medical treatment or procedure, regardless of whether it is considered related to the intervention will be monitored. All research staff will be trained to identify, probe for and report AE and social harms. Occurrence of AEs and SHs will be collected at every visit. All AEs and SHs reported outside of research visits/activities will also be documented and reported. An AE list will be compiled and reviewed by the Data Safety and Monitoring Board (DSMB) which will be set up for this study. All DSMB members will have no direct association with this study.

8 ADMINISTRATIVE PROCEDURES

8.1 PROTOCOL REGISTRATION

The study protocol will be registered on the South African National Health Research Database (NHRED), South African National Clinical Trials Register (SANCTR) and the U.S. National Library of Medicine ClinicalTrial.gov portal. The protocol and protocol ICF(s) must be approved, as appropriate, all appropriate institutional review boards (IRB)/ethics committees (EC) and any other applicable regulatory entity (RE). Upon receiving final approval, protocol registration documents to the NIH. The NIH will review the submitted protocol registration packet to ensure that all of the required documents have been received.

8.2 STUDY ACTIVATION

Pending successful protocol registration and submission of all required documents, PI Andrew Medina-Marino (FPD), in full consultation with co-PI Dr. Jeffrey Klausner (UCLA), will “activate” the study. The study will not be implemented at identified sites, until permission letters are obtained from relevant local authorities including Provincial and District-level approvals. This includes submission of the protocol to the National Health Research Database. Study implementation (screening and enrolment) may not be initiated until a study activation notice is provided to a specific site by PI Medina-Marino, following full consultation with co-PI Klausner. Commencement of implementation activities at a specified site will be dependent on the readiness of the site. In addition, if study “activation” is determined to be necessary for any subsequent amendments, study implementation may not be initiated until a study activation notice is provided to the site by either PI Medina-Marino or PI Klausner.

8.3 STUDY COORDINATION

Study implementation will be directed by this protocol and the study manual of procedures. The manual of procedures will be based on the approved protocol and will outline procedures for conducting study visits; data and forms processing; AE assessment, management and reporting; dispensing study products and documenting product accountability; and other study operations. Study CRFs and other study instruments will be developed by the protocol team and FPD. Data will be transferred to FPD HQ for data entry, cleaning, reporting and analysis. Quality control reports and queries will be generated and distributed to the study sites on a routine schedule for verification and resolution.

Close coordination between protocol team members will be necessary to track study progress, respond to queries about proper study implementation, and address other issues in a timely manner. Rates of participant accrual, adherence, follow-up, and AE incidence will be monitored closely by the team as well as the FPD Study Monitoring Committee (SMC). The Protocol Chair, Protocol Biostatistician, FPD Project Manager, and FPD Study Team will address issues related to study eligibility and AE management and reporting as needed to assure consistent case management, documentation, and information-sharing across sites.

8.4 STUDY MONITORING

On-site study monitoring will be performed in accordance with GCP guidelines. Study monitors will visit the site to:

1) Verify compliance with human subjects and other research regulations and guidelines;
2) Assess adherence to the study protocol, study-specific procedures manual, and local counselling practices; and
3) Confirm the quality and accuracy of information collected at the study site and entered into the study database.

This study will allow study monitors to inspect study facilities and documentation (e.g., ICFs, clinic and laboratory records, other source documents, CRFs), and/or observe the performance of study procedures. Investigators also will allow inspection of all study-related documentation by authorized representatives of FPD, UCT, approving IRBs/ECs, and US regulatory authorities (OHRP). A site visit log will be maintained at each study site to document all visits.

8.5 PROTOCOL COMPLIANCE

The study will be conducted in full compliance with this protocol. The protocol will not be amended without prior written approval by the Protocol Chair. All protocol amendments must be submitted to and approved by the relevant IRB(s)/EC(s) prior to implementing the amendment.

8.6 INVESTIGATORS RECORDS

The Investigator will maintain, and store in a secure manner, complete, accurate, and current study records throughout the study. Under the US Department of Health and Human Services (DHHS) regulations, the Investigator is required to retain all study records relating to research for at least three (3) years after completion of the research, or longer if needed to comply with local regulations.

Completion of a clinical research study occurs when the following activities have been completed:

1) All research-related interventions or interactions with human subjects (e.g. when all subjects are off study);

2) All protocol-required data collection of identifiable private information described in the IRB/EC-approved research plan;
3) All analysis of identifiable private information described in the IRB/EC-approved research plan;
4) Primary analysis of either identifiable private or de-identified information.

Study records include administrative documentation — including protocol registration documents and all reports and correspondence relating to the study — as well as documentation related to each participant screened and/or enrolled in the study — including ICFs, locator forms, CRFs, notations of all contacts with the participant, and all other source documents.

8.7 USE OF INFORMATION AND PUBLICATIONS

All presentations, abstracts, or manuscripts must be submitted to the study PIs (Klausner and Medina-Marino) for review and approval prior to submission. Data sharing agreements will be governed per FPD and UCLA policy. If these policies are at odds, PIs Medina-Marino and Klausner will reach a mutually agreed upon outcome.

9 STUDY TIMELINE

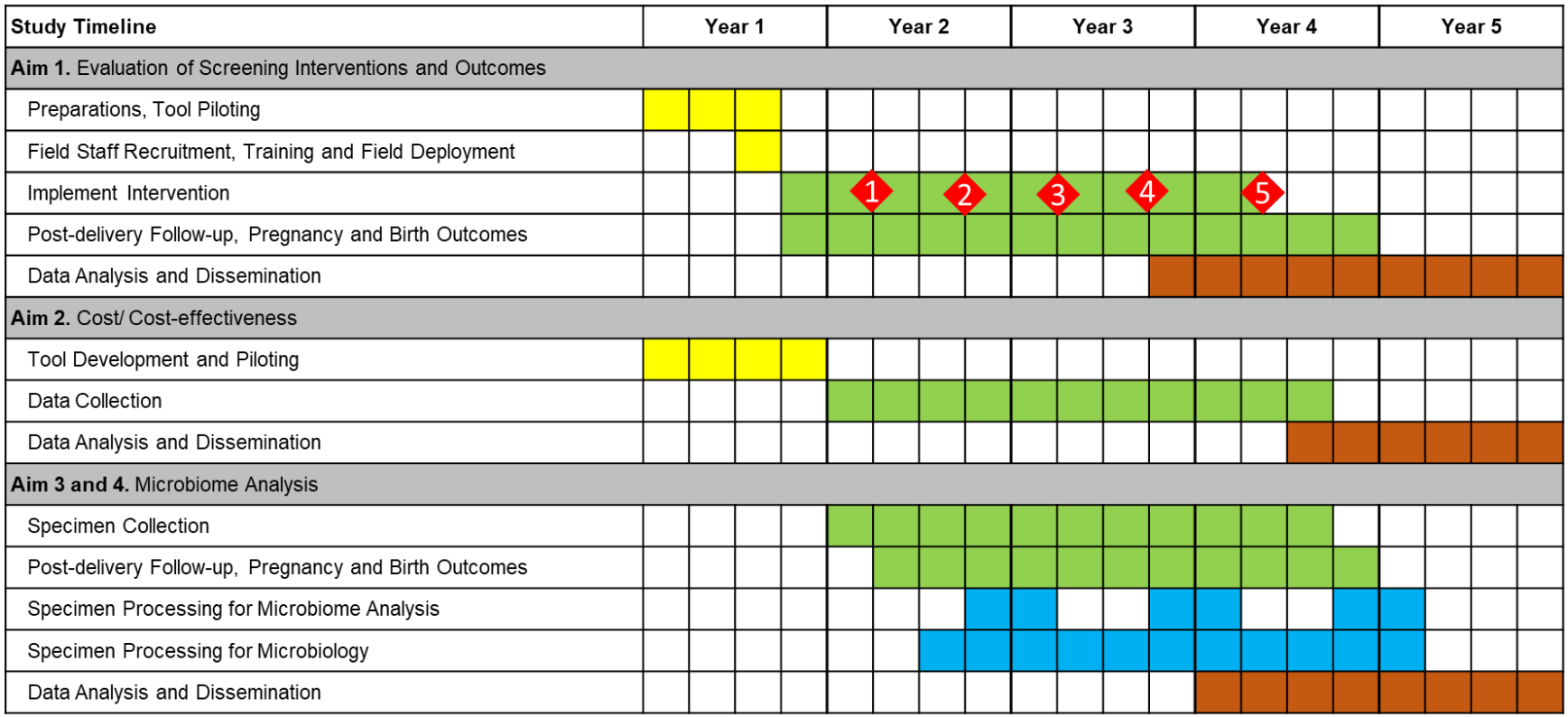
This study encompasses 3 major phases, as detailed in Table below.

Phase I: Develop and pilot final tools and standard operating procedures for recruitment, enrolment, and specimen and data collection. Adapt, optimize and finalize the different screening strategies. Hire and train staff to conduct STI screening at the identified facilities.

Phase II: Recruit and enrol study participants, and follow-up (pregnancy and birth outcomes) for 13 months.

Phase III: Analyse data and disseminate findings.

Table 8: Study Timeline



10 LITERATURE CITED

1. Newman L, Rowley J, Vander Hoorn S, et al. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. PLoS One 2015;10(12):e0143304. DOI: 10.1371/journal.pone.0143304.

2. Fawzi W, Msamanga G, Renjifo B, et al. Predictors of intrauterine and intrapartum transmission of HIV-1 among Tanzanian women. AIDS 2001;15(9):1157-65. DOI: 10.1097/00002030-200106150-00011.

3. Fichorova RN. Impact of T. vaginalis infection on innate immune responses and reproductive outcome. J Reprod Immunol 2009;83(1-2):185-9. DOI: 10.1016/j.jri.2009.08.007.

4. Silver BJ, Guy RJ, Kaldor JM, Jamil MS, Rumbold AR. Trichomonas vaginalis as a cause of perinatal morbidity: a systematic review and meta-analysis. Sex Transm Dis 2014;41(6):369-76. DOI: 10.1097/OLQ.0000000000000134.

5. Mann JR, McDermott S, Gill T. Sexually transmitted infection is associated with increased risk of preterm birth in South Carolina women insured by Medicaid. J Matern Fetal Neonatal Med 2010;23(6):563-8. DOI: 10.3109/14767050903214574.

6. Griffin M, Pushpanathan C, Andrews W. Chlamydia trachomatis pneumonitis: a case study and literature review. Pediatr Pathol 1990;10(5):843-52. DOI: 10.3109/15513819009064719.

7. Mardh PA. Influence of infection with Chlamydia trachomatis on pregnancy outcome, infant health and life-long sequelae in infected offspring. Best Pract Res Clin Obstet Gynaecol 2002;16(6):847-64. DOI: 10.1053/beog.2002.0329.

8. Rastogi S, Das B, Salhan S, Mittal A. Effect of treatment for Chlamydia trachomatis during pregnancy. Int J Gynaecol Obstet 2003;80(2):129-37. DOI: 10.1016/s0020-7292(02)00371-5.

9. Adachi K, Klausner JD, Bristow CC, et al. Chlamydia and Gonorrhea in HIV-Infected Pregnant Women and Infant HIV Transmission. Sex Transm Dis 2015;42(10):554-65. DOI: 10.1097/OLQ.0000000000000340.

10. Mullick S. Sexually transmitted infections in pregnancy: prevalence, impact on pregnancy outcomes, and approach to treatment in developing countries. Sexually Transmitted Infections 2005;81(4):294-302. (In en). DOI: 10.1136/sti.2002.004077.

11. Liu L, Johnson HL, Cousens S, et al. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. Lancet 2012;379(9832):2151-61. DOI: 10.1016/S0140-6736(12)60560-1.

12. Yeganeh N, Watts HD, Camarca M, et al. Syphilis in HIV-infected Mothers and Infants: Results from the NICHD/HPTN 040 Study. The Pediatric Infectious Disease Journal 2015;34(3):e52-e57. (In en). DOI: 10.1097/INF.0000000000000578.

13. Vermund SH. Screening for Sexually Transmitted Infections in Antenatal Care Is Especially Important Among HIV-Infected Women. Sex Transm Dis 2015;42(10):566-8. DOI: 10.1097/OLQ.0000000000000342.

14. Masha SC, Wahome E, Vaneechoutte M, Cools P, Crucitti T, Sanders EJ. High prevalence of curable sexually transmitted infections among pregnant women in a rural county hospital in Kilifi, Kenya. PLOS ONE 2017;12(3):e0175166. (In en). DOI: 10.1371/journal.pone.0175166.

15. Badman SG, Vallely LM, Toliman P, et al. A novel point-of-care testing strategy for sexually transmitted infections among pregnant women in high-burden settings: results of a feasibility study in Papua New Guinea. BMC Infectious Diseases 2016;16(1) (In en). DOI: 10.1186/s12879-016-1573-4.

16. Joseph Davey DL, Shull HI, Billings JD, Wang D, Adachi K, Klausner JD. Prevalence of Curable Sexually Transmitted Infections in Pregnant Women in Low- and Middle-Income Countries From 2010 to 2015: A Systematic Review. Sex Transm Dis 2016;43(7):450-8. DOI: 10.1097/OLQ.0000000000000460.

17. Vallely LM, Toliman P, Ryan C, et al. Prevalence and risk factors of Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis and other sexually transmissible infections among women attending antenatal clinics in three provinces in Papua New Guinea: a cross-sectional survey. Sexual Health 2016 (In en). DOI: 10.1071/SH15227.

18. Chico RM, Mayaud P, Ariti C, Mabey D, Ronsmans C, Chandramohan D. Prevalence of Malaria and Sexually Transmitted and Reproductive Tract Infections in Pregnancy in Sub-Saharan Africa: A Systematic Review. JAMA 2012;307(19) (In en). DOI: 10.1001/jama.2012.3428.

19. Mudau M, Peters RP, De Vos L, et al. High prevalence of asymptomatic sexually transmitted infections among human immunodeficiency virus-infected pregnant women in a low-income South African community. Int J STD AIDS 2018;29(4):324-333. DOI: 10.1177/0956462417724908.

20. Ravel J, Gajer P, Abdo Z, et al. Vaginal microbiome of reproductive-age women. Proceedings of the National Academy of Sciences 2011;108(Supplement_1):4680-4687. (In en). DOI: 10.1073/pnas.1002611107.

21. Gosmann C, Anahtar MN, Handley SA, et al. Lactobacillus-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased HIV Acquisition in Young South African Women. Immunity 2017;46(1):29-37. (In en). DOI: 10.1016/j.immuni.2016.12.013.

22. van der Veer C, Bruisten SM, van der Helm JJ, de Vries HJC, van Houdt R. The Cervicovaginal Microbiota in Women Notified for Chlamydia trachomatis Infection: A Case-Control Study at the Sexually Transmitted Infection Outpatient Clinic in Amsterdam, The Netherlands. Clinical Infectious Diseases 2017;64(1):24-31. (In en). DOI: 10.1093/cid/ciw586.

23. Brotman RM, Bradford LL, Conrad M, et al. Association Between Trichomonas vaginalis and Vaginal Bacterial Community Composition Among Reproductive-Age Women. Sexually Transmitted Diseases 2012;39(10):807-812. (In en). DOI: 10.1097/OLQ.0b013e3182631c79.

24. Klatt NR, Cheu R, Birse K, et al. Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. Science 2017;356(6341):938-945. (In en). DOI: 10.1126/science.aai9383.

25. Ralph ED, Clarke DA. Inactivation of metronidazole by anaerobic and aerobic bacteria. Antimicrob Agents Chemother 1978;14(3):377-383. (In eng).

26. Nagy E, Földes J. Inactivation of metronidazole by Enterococcus faecalis. J Antimicrob Chemother 1991;27(1):63-70. (In eng).

27. McFadzean JA, Pugh IM, Squires SL, Whelan JP. Further observations on strain sensitivity of Trichomonas vaginalis to metronidazole. Br J Vener Dis 1969;45(2):161-162. (In eng).

28. Gatski M, Mena L, Levison J, et al. Patient-Delivered Partner Treatment and Trichomonas vaginalis Repeat Infection Among HIV-Infected Women. Sexually Transmitted Diseases 2010:1. (In en). DOI: 10.1097/OLQ.0b013e3181d891fc.

29. National Department of Health SA. The 2013 National Antenatal Sentinel HIV Prevalence Survey South Africa. Pretoria, South Africa2015.

30. Menezes LJ, Pokharel U, Sudenga SL, et al. Patterns of prevalent HPV and STI co-infections and associated factors among HIV-negative young Western Cape, South African women: the EVRI trial. Sexually Transmitted Infections 2017:sextrans-2016-053046. (In en). DOI: 10.1136/sextrans-2016-053046.

31. De Jongh M, Lekalakala MR, Le Roux M, Hoosen AA. Risk of having a sexually transmitted infection in women presenting at a termination of pregnancy clinic in Pretoria, South Africa. Journal of Obstetrics and Gynaecology 2010;30(5):480-483. (In en). DOI: 10.3109/01443611003797687.

32. Moodley D, Moodley P, Sebitloane M, et al. High prevalence and incidence of asymptomatic sexually transmitted infections during pregnancy and postdelivery in KwaZulu Natal, South Africa. Sex Transm Dis 2015;42(1):43-7. DOI: 10.1097/OLQ.0000000000000219.

33. Adachi K, Nielsen-Saines K, Klausner JD. Chlamydia trachomatis Infection in Pregnancy: The Global Challenge of Preventing Adverse Pregnancy and Infant Outcomes in Sub-Saharan Africa and Asia. BioMed Research International 2016;2016:1-21. (In en). DOI: 10.1155/2016/9315757.

34. Rours GIJG, Duijts L, Moll HA, et al. Chlamydia trachomatis infection during pregnancy associated with preterm delivery: a population-based prospective cohort study. European Journal of Epidemiology 2011;26(6):493-502. (In en). DOI: 10.1007/s10654-011-9586-1.

35. Gravett MG, Nelson HP, DeRouen T, Critchlow C, Eschenbach DA, Holmes KK. Independent associations of bacterial vaginosis and Chlamydia trachomatis infection with adverse pregnancy outcome. JAMA 1986;256(14):1899-1903. (In eng).

36. Association of Chlamydia trachomatis and Mycoplasma hominis with intrauterine growth retardation and preterm delivery. The John Hopkins Study of Cervicitis and Adverse Pregnancy Outcome. Am J Epidemiol 1989;129(6):1247-1257. (In eng).

37. Heumann CL, Quilter LAS, Eastment MC, Heffron R, Hawes SE. Adverse Birth Outcomes and Maternal Neisseria gonorrhoeae Infection: A Population-Based Cohort Study in Washington State. Sexually Transmitted Diseases 2017;44(5):266-271. (In en). DOI: 10.1097/OLQ.0000000000000592.

38. Donders GG, Desmyter J, De Wet DH, Van Assche FA. The association of gonorrhoea and syphilis with premature birth and low birthweight. Genitourin Med 1993;69(2):98-101. (In eng).

39. Elliott B, Brunham RC, Laga M, et al. Maternal gonococcal infection as a preventable risk factor for low birth weight. The Journal of Infectious Diseases 1990;161(3):531-536. (In eng).

40. Cotch MF, Pastorek JG, Nugent RP, et al. Trichomonas vaginalis associated with low birth weight and preterm delivery. The Vaginal Infections and Prematurity Study Group. Sexually Transmitted Diseases 1997;24(6):353-360. (In eng).

41. Sutton MY, Sternberg M, Nsuami M, Behets F, Nelson AM, St Louis ME. Trichomoniasis in pregnant human immunodeficiency virus-infected and human immunodeficiency virus-uninfected congolese women: prevalence, risk factors, and association with low birth weight. Am J Obstet Gynecol 1999;181(3):656-662. (In eng).

42. Hardy PH, Hardy JB, Nell EE, Graham DA, Spence MR, Rosenbaum RC. Prevalence of six sexually transmitted disease agents among pregnant inner-city adolescents and pregnancy outcome. Lancet (London, England) 1984;2(8398):333-337. (In eng).

43. Minkoff H, Grunebaum AN, Schwarz RH, et al. Risk factors for prematurity and premature rupture of membranes: a prospective study of the vaginal flora in pregnancy. Am J Obstet Gynecol 1984;150(8):965-972. (In eng).

44. Hammerschlag MR. Chlamydial and gonococcal infections in infants and children. Clin Infect Dis 2011;53 Suppl 3:S99-102. (In eng). DOI: 10.1093/cid/cir699.

45. Johnson LF, Lewis DA. The Effect of Genital Tract Infections on HIV-1 Shedding in the Genital Tract: A Systematic Review and Meta-Analysis. Sexually Transmitted Diseases 2008;35(11):946-959. (In en). DOI: 10.1097/OLQ.0b013e3181812d15.

46. Kedzierska K, Crowe SM, Turville S, Cunningham AL. The influence of cytokines, chemokines and their receptors on HIV-1 replication in monocytes and macrophages. Rev Med Virol 2003;13(1):39-56. (In eng). DOI: 10.1002/rmv.369.

47. Mitchell CM, Balkus J, Agnew KJ, et al. Bacterial Vaginosis, Not HIV, Is Primarily Responsible for Increased Vaginal Concentrations of Proinflammatory Cytokines. AIDS Research and Human Retroviruses 2008;24(5):667-671. (In en). DOI: 10.1089/aid.2007.0268.

48. Anton G, Rid J, Mylonas I, Friese K, Weissenbacher ER. Evidence of a TH1-Shift of Local Vaginal Inflammatory Response During Bacterial Vaginosis. Infection 2008;36(2):147-152. (In en). DOI: 10.1007/s15010-007-7152-2.

49. Spear GT, Zariffard MR, Cohen MH, Sha BE. Vaginal IL-8 levels are positively associated with Candida albicans and inversely with lactobacilli in HIV-infected women. Journal of Reproductive Immunology 2008;78(1):76-79. (In en). DOI: 10.1016/j.jri.2007.11.001.

50. Hedges SR, Barrientes F, Desmond RA, Schwebke JR. Local and systemic cytokine levels in relation to changes in vaginal flora. The Journal of Infectious Diseases 2006;193(4):556-562. (In eng). DOI: 10.1086/499824.

51. Cummins JE, Christensen L, Lennox JL, et al. Mucosal Innate Immune Factors in the Female Genital Tract Are Associated with Vaginal HIV-1 Shedding Independent of Plasma Viral Load. AIDS Research and Human Retroviruses 2006;22(8):788-795. (In en). DOI: 10.1089/aid.2006.22.788.

52. Spear GT, Zariffard MR, Chen HY, et al. Positive Association between HIV RNA and IL-6 in the Genital Tract of Rwandan Women. AIDS Research and Human Retroviruses 2008;24(7):973-976. (In en). DOI: 10.1089/aid.2008.0004.

53. Cu-Uvin S, Hogan Joseph W, Caliendo Angela M, et al. Association between Bacterial Vaginosis and Expression of Human Immunodeficiency Virus Type 1 RNA in the Female Genital Tract. Clinical Infectious Diseases 2001;33(6):894-896. (In en). DOI: 10.1086/322613.

54. Sha BE, Zariffard MR, Wang QJ, et al. Female genital-tract HIV load correlates inversely with Lactobacillus species but positively with bacterial vaginosis and Mycoplasma hominis. The Journal of Infectious Diseases 2005;191(1):25-32. (In eng). DOI: 10.1086/426394.

55. Wang Chia C, McClelland RS, Reilly M, et al. The Effect of Treatment of Vaginal Infections on Shedding of Human Immunodeficiency Virus Type 1. The Journal of Infectious Diseases 2001;183(7):1017-1022. (In en). DOI: 10.1086/319287.

56. McClelland RS, Wang CC, Mandaliya K, et al. Treatment of cervicitis is associated with decreased cervical shedding of HIV-1. AIDS (London, England) 2001;15(1):105-110. (In eng).

57. Saigal S, Doyle LW. An overview of mortality and sequelae of preterm birth from infancy to adulthood. Lancet 2008;371(9608):261-9. DOI: 10.1016/S0140-6736(08)60136-1.

58. Romero R, Espinoza J, Kusanovic JP, et al. The preterm parturition syndrome. BJOG 2006;113 Suppl 3:17-42. DOI: 10.1111/j.1471-0528.2006.01120.x.

59. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet 2008;371(9606):75-84. DOI: 10.1016/S0140-6736(08)60074-4.

60. MacIntyre DA, Sykes L, Teoh TG, Bennett PR. Prevention of preterm labour via the modulation of inflammatory pathways. J Matern Fetal Neonatal Med 2012;25 Suppl 1:17-20. DOI: 10.3109/14767058.2012.666114.

61. Meis PJ, Goldenberg RL, Mercer B, et al. The preterm prediction study: significance of vaginal infections. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. AmJObstetGynecol 1995;173(4):1231-1235. (https://ac.els-cdn.com/0002937895913602/1-s2.0-0002937895913602-main.pdf?_tid=b6d5ae35-903f-44bc-a456-937abb5dc3f3&acdnat=1542331896_0b9d773f90710baac25f1b9f0d70b8f8).

62. Goldenberg RL, Andrews WW, Goepfert AR, et al. The Alabama Preterm Birth Study: umbilical cord blood Ureaplasma urealyticum and Mycoplasma hominis cultures in very preterm newborn infants. Am J Obstet Gynecol 2008;198(1):43 e1-5. DOI: 10.1016/j.ajog.2007.07.033.

63. Capoccia R, Greub G, Baud D. Ureaplasma urealyticum, Mycoplasma hominis and adverse pregnancy outcomes. Curr Opin Infect Dis 2013;26(3):231-40. DOI: 10.1097/QCO.0b013e328360db58.

64. Freitas AC, Bocking A, Hill JE, Money DM, Group VR. Increased richness and diversity of the vaginal microbiota and spontaneous preterm birth. Microbiome 2018;6(1):117. DOI: 10.1186/s40168-018-0502-8.

65. Donders GG, Van Calsteren K, Bellen G, et al. Predictive value for preterm birth of abnormal vaginal flora, bacterial vaginosis and aerobic vaginitis during the first trimester of pregnancy. BJOG 2009;116(10):1315-24. DOI: 10.1111/j.1471-0528.2009.02237.x.

66. Brown RG, Marchesi JR, Lee YS, et al. Vaginal dysbiosis increases risk of preterm fetal membrane rupture, neonatal sepsis and is exacerbated by erythromycin. BMC Med 2018;16(1):9. DOI: 10.1186/s12916-017-0999-x.

67. Andrews WW, Goldenberg RL, Mercer B, et al. The Preterm Prediction Study: association of second-trimester genitourinary chlamydia infection with subsequent spontaneous preterm birth. American Journal of Obstetrics & Gynecology 2000;183(3):662-668.

68. Andrews WW, Klebanoff MA, Thom EA, et al. Midpregnancy genitourinary tract infection with Chlamydia trachomatis: association with subsequent preterm delivery in women with bacterial vaginosis and Trichomonas vaginalis. Am J Obstet Gynecol 2006;194(2):493-500. DOI: 10.1016/j.ajog.2005.08.054.

69. Sweet RL, Landers DV, Walker C, Schachter J. Chlamydia trachomatis infection and pregnancy outcome. Am J Obstet Gynecol 1987;156(4):824-33. (https://www.ncbi.nlm.nih.gov/pubmed/3107388).

70. Romero R, Hassan SS, Gajer P, et al. The vaginal microbiota of pregnant women who subsequently have spontaneous preterm labor and delivery and those with a normal delivery at term. Microbiome 2014;2:18. DOI: 10.1186/2049-2618-2-18.

71. Fettweis JM, Serrano MG, Brooks JP, et al. The vaginal microbiome and preterm birth. Nat Med 2019;25(6):1012-1021. DOI: 10.1038/s41591-019-0450-2.

72. Vuylsteke B. Current status of syndromic management of sexually transmitted infections in developing countries. Sexually Transmitted Infections 2004;80(5):333-334. (In eng). DOI: 10.1136/sti.2004.009407.

73. Lewis DA, Latif AS, Ndowa F. WHO global strategy for the prevention and control of sexually transmitted infections: time for action. Sexually Transmitted Infections 2007;83(7):508-509. (In eng). DOI: 10.1136/sti.2007.028142.

74. Johnson LF, Dorrington RE, Bradshaw D, Coetzee DJ. The effect of syndromic management interventions on the prevalence of sexually transmitted infections in South Africa. Sexual & Reproductive Healthcare 2011;2(1):13-20. (In en). DOI: 10.1016/j.srhc.2010.08.006.

75. White RG, Moodley P, McGrath N, et al. Low effectiveness of syndromic treatment services for curable sexually transmitted infections in rural South Africa. Sexually Transmitted Infections 2008;84(7):528-534. (In en). DOI: 10.1136/sti.2008.032011.

76. van Gemert C, Hellard M, Bradshaw CS, et al. Syndromic management of sexually transmissible infections in resource-poor settings: a systematic review with meta-analysis of the abnormal vaginal discharge flowchart for Neisseria gonorrhoea and Chlamydia trachomatis. Sexual Health 2017 (In en). DOI: 10.1071/SH17070.

77. Papp J, Schachter J, Gaydos C, Van Der Pol B. Recommendations for the laboratory-based detection of Chlamydia trachomatis and Neisseria gonorrhoeae - 2014. MMWR Recomm Rep 2014;63(RR-02):1-19. (https://www.ncbi.nlm.nih.gov/pubmed/24622331).

78. Van Der Pol B, Kraft CS, Williams JA. Use of an Adaptation of a Commercially Available PCR Assay Aimed at Diagnosis of Chlamydia and Gonorrhea To Detect Trichomonas vaginalis in Urogenital Specimens. Journal of Clinical Microbiology 2006;44(2):366-373. (In en). DOI: 10.1128/JCM.44.2.366-373.2006.

79. Sonkar SC, Wasnik K, Kumar A, Mittal P, Saluja D. Comparative analysis of syndromic and PCR-based diagnostic assay reveals misdiagnosis/ overtreatment for trichomoniasis based on subjective judgment in symptomatic patients. Infectious Diseases of Poverty 2016;5(1) (In en). DOI: 10.1186/s40249-016-0133-x.

80. Peters RPH, de Vos L, Maduna L, et al. Laboratory Validation of Xpert Chlamydia trachomatis/Neisseria gonorrhoeae and Trichomonas vaginalis Testing as Performed by Nurses at Three Primary Health Care Facilities in South Africa. Journal of Clinical Microbiology 2017;55(12):3563-3565. (Comparative Study

Letter

Multicenter Study)
(http://ovidsp.ovid.com/ovidweb.cgi?T=JS&CSC=Y&NEWS=N&PAGE=fulltext&D=medc&AN=29021154

https://jcm.asm.org/content/jcm/55/12/3563.full.pdf).

81. Sanac. Let Our Actions Count: South Africa's National Strategic Plan for HIV,TB and STIs 2017-2022. The South African National AIDS Council 2017;1(March):1-132.

82. Huppert JS, Hesse E, Kim G, et al. Adolescent women can perform a point-of-care test for trichomoniasis as accurately as clinicians. Sexually Transmitted Infections 2010;86(7):514-519. (In en). DOI: 10.1136/sti.2009.042168.

83. Huppert JS, Hesse EA, Bernard MA, et al. Acceptability of self-testing for trichomoniasis increases with experience. Sexually Transmitted Infections 2011;87(6):494-500. (In en). DOI: 10.1136/sextrans-2011-050037.

84. Hsieh Y-H, Hogan MT, Barnes M, et al. Perceptions of an Ideal Point-of-Care Test for Sexually Transmitted Infections – A Qualitative Study of Focus Group Discussions with Medical Providers. PLoS ONE 2010;5(11):e14144. (In en). DOI: 10.1371/journal.pone.0014144.

85. Dean D, Turingan RS, Thomann H-U, et al. A Multiplexed Microfluidic PCR Assay for Sensitive and Specific Point-of-Care Detection of Chlamydia trachomatis. PLoS ONE 2012;7(12):e51685. (In en). DOI: 10.1371/journal.pone.0051685.

86. Medina-Marino A. An Innovative Cluster of Studies on The Undiscussed (STIs), The Underfunded (TB) and The Continuing (HIV) Epidemics in South Africa. Lecture presented at: HIV Grand Rounds, University of Pennsylvania2018.

87. Medina-Marino A. Decreasing the Burden of South Africa's Trifecta of Infectious Diseases: Screening for STIs During Pregnancy, Estimating the Magnitude of Missed Cases of TB and Implementing PrEP for HIV Prevention. Lecture presented at: Center for Interdisciplinary Research on AIDS, Yale University, New Haven2019.

88. Keizur EM, Klausner JD. The need for new treatment recommendations for trichomoniasis among women. The Lancet Infectious diseases 2018;18(11):1168-1169. DOI: 10.1016/S1473-3099(18)30544-9.

89. Gatski M, Martin DH, Levison J, et al. The influence of bacterial vaginosis on the response to Trichomonas vaginalis treatment among HIV-infected women. Sexually Transmitted Infections 2011;87(3):205-208. (In en). DOI: 10.1136/sti.2010.046441.

90. Kissinger P, Secor WE, Leichliter Jami S, et al. Early Repeated Infections with Trichomonas vaginalis among HIV-Positive and HIV-Negative Women. Clinical Infectious Diseases 2008;46(7):994-999. (In en). DOI: 10.1086/529149.

91. Somani J, Bhullar Vinod B, Workowski Kimberly A, Farshy Carol E, Black Carolyn M. Multiple Drug–Resistant Chlamydia trachomatis Associated with Clinical Treatment Failure. The Journal of Infectious Diseases 2000;181(4):1421-1427. (In en). DOI: 10.1086/315372.

92. Smith KS, Guy R, Danielewski J, et al. Biological and Behavioral Factors Associated With Positive Chlamydia Retests. Sexually Transmitted Diseases 2017;44(7):417-422. (In en). DOI: 10.1097/OLQ.0000000000000616.

93. Jones RB, Van der Pol B, Martin DH, Shepard MK. Partial characterization of Chlamydia trachomatis isolates resistant to multiple antibiotics. The Journal of Infectious Diseases 1990;162(6):1309-1315. (In eng).

94. Suchland RJ, Geisler WM, Stamm WE. Methodologies and cell lines used for antimicrobial susceptibility testing of Chlamydia spp. Antimicrob Agents Chemother 2003;47(2):636-642. (In eng).

95. Masson L, Arnold KB, Little F, et al. Inflammatory cytokine biomarkers to identify women with asymptomatic sexually transmitted infections and bacterial vaginosis who are at high risk of HIV infection. Sex Transm Infect 2016;92(3):186-93. DOI: 10.1136/sextrans-2015-052072.

96. Masson L, Barnabas S, Deese J, et al. Inflammatory cytokine biomarkers of asymptomatic sexually transmitted infections and vaginal dysbiosis: a multicentre validation study. Sex Transm Infect 2019;95(1):5-12. DOI: 10.1136/sextrans-2017-053506.

97. Beigi RH, Yudin MH, Cosentino L, Meyn LA, Hillier SL. Cytokines, pregnancy, and bacterial vaginosis: comparison of levels of cervical cytokines in pregnant and nonpregnant women with bacterial vaginosis. J Infect Dis 2007;196(9):1355-60. DOI: 10.1086/521628.

98. Kutteh WH, Franklin RD. Quantification of immunoglobulins and cytokines in human cervical mucus during each trimester of pregnancy. Am J Obstet Gynecol 2001;184(5):865-72; discussion 872-4. DOI: 10.1067/mob.2001.113853.

99. Goldenberg RL, Culhane JF. Prepregnancy health status and the risk of preterm delivery. Arch Pediatr Adolesc Med 2005;159(1):89-90. DOI: 10.1001/archpedi.159.1.89.

100. van de Wijgert JH, Jespers V. The global health impact of vaginal dysbiosis. Res Microbiol 2017;168(9-10):859-864. DOI: 10.1016/j.resmic.2017.02.003.

101. Santiago GL, Tency I, Verstraelen H, et al. Longitudinal qPCR study of the dynamics of L. crispatus, L. iners, A. vaginae, (sialidase positive) G. vaginalis, and P. bivia in the vagina. PLoS One 2012;7(9):e45281. DOI: 10.1371/journal.pone.0045281.

102. Ziklo N, Huston WM, Taing K, Katouli M, Timms P. In vitro rescue of genital strains of Chlamydia trachomatis from interferon-γ and tryptophan depletion with indole-positive, but not indole-negative Prevotella spp. BMC microbiology 2016;16(1):286. (In eng). DOI: 10.1186/s12866-016-0903-4.

103. Aiyar A, Quayle AJ, Buckner LR, et al. Influence of the tryptophan-indole-IFNγ axis on human genital Chlamydia trachomatis infection: role of vaginal co-infections. Front Cell Infect Microbiol 2014;4:72. (In eng). DOI: 10.3389/fcimb.2014.00072.

104. Sherchand S, Ibana JA, Quayle AJ, Aiyar A. Cell Intrinsic Factors Modulate the Effects of IFNγ on the Development of Chlamydia trachomatis. J Bacteriol Parasitol 2016;7(4) (In eng). DOI: 10.4172/2155-9597.1000282.

105. Taha TE, Hoover DR, Dallabetta GA, et al. Bacterial vaginosis and disturbances of vaginal flora: association with increased acquisition of HIV. AIDS (London, England) 1998;12(13):1699-1706. (In eng).

106. Atashili J, Poole C, Ndumbe PM, Adimora AA, Smith JS. Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. AIDS (London, England) 2008;22(12):1493-1501. (In eng). DOI: 10.1097/QAD.0b013e3283021a37.

107. Wiesenfeld HC, Hillier SL, Krohn MA, Landers DV, Sweet RL. Bacterial vaginosis is a strong predictor of Neisseria gonorrhoeae and Chlamydia trachomatis infection. Clin Infect Dis 2003;36(5):663-668. (In eng). DOI: 10.1086/367658.

108. Martin HL, Richardson BA, Nyange PM, et al. Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. The Journal of Infectious Diseases 1999;180(6):1863-1868. (In eng). DOI: 10.1086/315127.

109. Peters SE, Beck-Sagué CM, Farshy CE, et al. Behaviors Associated with Neisseria gonorrhoeae and Chiamydia trachomatis: Cervical Infection Among Young Women Attending Adolescent Clinics. Clinical Pediatrics 2000;39(3):173-177. (In en). DOI: 10.1177/000992280003900307.

110. Cherpes TL, Meyn LA, Krohn MA, Lurie JG, Hillier SL. Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. Clin Infect Dis 2003;37(3):319-325. (In eng). DOI: 10.1086/375819.

111. Coleman JS, Hitti J, Bukusi EA, et al. Infectious correlates of HIV-1 shedding in the female upper and lower genital tracts. AIDS 2007;21(6):755-759. (In en). DOI: 10.1097/QAD.0b013e328012b838.

112. Cohn JA, Hashemi FB, Camarca M, et al. HIV-inducing factor in cervicovaginal secretions is associated with bacterial vaginosis in HIV-1-infected women. J Acquir Immune Defic Syndr 2005;39(3):340-346. (In eng).

113. Cherpes TL, Melan MA, Kant JA, Cosentino LA, Meyn LA, Hillier SL. Genital tract shedding of herpes simplex virus type 2 in women: effects of hormonal contraception, bacterial vaginosis, and vaginal group B Streptococcus colonization. Clin Infect Dis 2005;40(10):1422-1428. (In eng). DOI: 10.1086/429622.

114. Gajer P, Brotman RM, Bai G, et al. Temporal Dynamics of the Human Vaginal Microbiota. Science Translational Medicine 2012;4(132):132ra52-132ra52. (In en). DOI: 10.1126/scitranslmed.3003605.

115. Anahtar Melis N, Byrne Elizabeth H, Doherty Kathleen E, et al. Cervicovaginal Bacteria Are a Major Modulator of Host Inflammatory Responses in the Female Genital Tract. Immunity 2015;42(5):965-976. (In en). DOI: 10.1016/j.immuni.2015.04.019.

116. Fettweis JM, Brooks JP, Serrano MG, et al. Differences in vaginal microbiome in African American women versus women of European ancestry. Microbiology 2014;160(Pt_10):2272-2282. (In en). DOI: 10.1099/mic.0.081034-0.

117. van Houdt R, Ma B, Bruisten SM, Speksnijder AGCL, Ravel J, de Vries HJC. Lactobacillus iners-dominated vaginal microbiota is associated with increased susceptibility to Chlamydia trachomatis infection in Dutch women: a case–control study. Sexually Transmitted Infections 2017:sextrans-2017-053133. (In en). DOI: 10.1136/sextrans-2017-053133.

118. Brotman RM, Klebanoff MA, Nansel TR, et al. Bacterial vaginosis assessed by gram stain and diminished colonization resistance to incident gonococcal, chlamydial, and trichomonal genital infection. The Journal of Infectious Diseases 2010;202(12):1907-1915. (In eng). DOI: 10.1086/657320.

119. Kissinger P. Trichomonas vaginalis: a review of epidemiologic, clinical and treatment issues. BMC Infect Dis 2015;15:307. (In eng). DOI: 10.1186/s12879-015-1055-0.

120. Kissinger P, Secor WE, Leichliter JS, et al. Early repeated infections with Trichomonas vaginalis among HIV-positive and HIV-negative women. Clin Infect Dis 2008;46(7):994-9. (In eng). DOI: 10.1086/529149.

121. Krashin JW, Koumans EH, Bradshaw-Sydnor AC, et al. Trichomonas vaginalis prevalence, incidence, risk factors and antibiotic-resistance in an adolescent population. Sexually transmitted diseases 2010;37(7):440-4. (In eng). DOI: 10.1097/OLQ.0b013e3181cfcd8c.

122. Kissinger P, Schmidt N, Mohammed H, et al. Patient-delivered partner treatment for Trichomonas vaginalis infection: a randomized controlled trial. Sexually transmitted diseases 2006;33(7):445-50. (In eng). DOI: 10.1097/01.olq.0000204511.84485.4c.

123. Muzny CA, Rivers CA, Mena LA, Schwebke JR. Genotypic characterization of Trichomonas vaginalis isolates among women who have sex with women in sexual partnerships. Sexually transmitted diseases 2012;39(7):556-8. (In eng). DOI: 10.1097/OLQ.0b013e31824f1c49.

124. Burch TA, Rees CW, Reardon LV. Epidemiological studies on human trichomoniasis. Am J Trop Med Hyg 1959;8(3):312-8. (In eng). DOI: 10.4269/ajtmh.1959.8.312.

125. Adu-Sarkodie Y. Trichomonas vaginalis transmission in a family. Genitourin Med 1995;71(3):199-200. DOI: 10.1136/sti.71.3.199.

126. Crucitti T, Jespers V, Mulenga C, Khondowe S, Vandepitte J, Buve A. Non-sexual transmission of Trichomonas vaginalis in adolescent girls attending school in Ndola, Zambia. PLoS One 2011;6(1):e16310. DOI: 10.1371/journal.pone.0016310.

127. Low N, Chersich MF, Schmidlin K, et al. Intravaginal practices, bacterial vaginosis, and HIV infection in women: individual participant data meta-analysis. PLoS Med 2011;8(2):e1000416. DOI: 10.1371/journal.pmed.1000416.

128. Bartley JB, Ferris DG, Allmond LM, Dickman ED, Dias JK, Lambert J. Personal digital assistants used to document compliance of bacterial vaginosis treatment. Sexually transmitted diseases 2004;31(8):488-91. (In eng). DOI: 10.1097/01.olq.0000135990.21755.51.

129. Kissinger PJ, Gaydos CA, Seña AC, et al. Diagnosis and Management of Trichomonas vaginalis: Summary of Evidence Reviewed for the 2021 Centers for Disease Control and Prevention Sexually Transmitted

Infections Treatment Guidelines. Clin Infect Dis 2022;74(Suppl_2):S152-s161. (In eng). DOI: 10.1093/cid/ciac030.

130. Kirkcaldy RD, Augostini P, Asbel LE, et al. Trichomonas vaginalis antimicrobial drug resistance in 6 US cities, STD Surveillance Network, 2009-2010. Emerging infectious diseases 2012;18(6):939-43. (In eng). DOI: 10.3201/eid1806.111590.

131. Schwebke JR, Barrientes FJ. Prevalence of Trichomonas vaginalis isolates with resistance to metronidazole and tinidazole. Antimicrob Agents Chemother 2006;50(12):4209-10. (In eng). DOI: 10.1128/aac.00814-06.

132. Sariego Ramos I, Rojas Rivero L, Fraga Nodarse J. Estudio de la susceptibilidad al metronidazol en aislamientos cubanos de Trichomonas vaginalis. Revista Cubana de Obstetricia y Ginecología 2011;37(2):271-276.

133. Marques-Silva M, Lisboa C, Gomes N, Rodrigues AG. Trichomonas vaginalis and growing concern over drug resistance: a systematic review. Journal of the European Academy of Dermatology and Venereology 2021;35(10):2007-2021. DOI: https://doi.org/10.1111/jdv.17461.

134. Mtshali A, Ngcapu S, Govender K, Sturm AW, Moodley P, Joubert Bronwyn C. In Vitro Effect of 5-Nitroimidazole Drugs against Trichomonas vaginalis Clinical Isolates. Microbiology Spectrum 2022;10(4):e00912-22. DOI: 10.1128/spectrum.00912-22.

135. Kawamura N. Metronidazole and tinidazole in a single large dose for treating urogenital infections with Trichomonas vaginalis in men. The British Journal of Venereal Diseases 1978;54(2):81-83. DOI: 10.1136/sti.54.2.81.

136. Mabaso N, Tinarwo P, Abbai N. Lack of association between Mycoplasma hominis and Trichomonas vaginalis symbiosis in relation to metronidazole resistance. Parasitol Res 2020;119(12):4197-4204. (In eng). DOI: 10.1007/s00436-020-06930-x.

137. Medina-Marino A, Mudau M, De Vos L, et al. Acceptability and Feasibility of Integrating Diagnostic STI Screening into Antenatal Care Services. Durban, South Africa2017.

138. Daniels J, De Vos L, Mogos W, et al. Factors influencing sexually transmissible infection disclosure to male partners by HIV-positive pregnant women in Pretoria townships, South Africa: a qualitative study. Sex Health 2019;16(3):274-281. DOI: 10.1071/SH18177.

139. Jones Davey D, Medina-Marino A, Mudau M, et al. Behavioral risk factors among HIV-infected pregnant women with a sexually transmitted infection in South Africa. 9th IAS Conference on HIV Science. Paris, France2017.

140. Wynn A, Moucheraud C, Klausner J, Leibowitz A. Assessing the costs and estimating scale-up of testing pregnant women for curable sexually transmitted infections in Botswana. Implementation Science 2018;13.

141. Muzny C, Blanchard E, Taylor C, et al. Identification of Key Bacteria Involved in the Induction of Incident Bacterial Vaginosis: A Prospective Study. The Journal of Infectious Diseases 2018(In Press). DOI: 10.1093/infdis/jiy243.

142. Muzny C. Factors influencing urogenital Chlamydia Trachomatis DNA clearance after treatment. Woudschoten in Zeist, The Netherlands2018:14th International Symposium on Human Chlamydial Infections (ISHCI).

143. Holm JB, Gajer P, Ravel J. PECAN: A fast, novel 16S rRNA gene sequence non-clustering based taxonomic assignment tool. Montreal, Canada2016:16th International Symposium on Microbial Ecology.

144. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research 2012;41(D1):D590-D596. (In en). DOI: 10.1093/nar/gks1219.

145. Malaba TR, Phillips T, Le Roux S, et al. Antiretroviral therapy use during pregnancy and adverse birth outcomes in South African women. International Journal of Epidemiology 2017;46(5):1678-1689. (In en). DOI: 10.1093/ije/dyx136.

146. Patton MQ. Qualitative research & evaluation methods. 3. ed., [Nachdr.] ed. Thousand Oaks, Calif.2009.

147. Vallely AJ, Pomat WS, Homer C, et al. Point-of-care testing and treatment of sexually transmitted infections to improve birth outcomes in high-burden, low-income settings: Study protocol for a cluster randomized crossover trial (the WANTAIM Trial, Papua New Guinea). Wellcome Open Res 2019;4:53. DOI: 10.12688/wellcomeopenres.15173.2.

148. Hagedorn HJ, Stetler CB, Bangerter A, Noorbaloochi S, Stitzer ML, Kivlahan D. An implementation-focused process evaluation of an incentive intervention effectiveness trial in substance use disorders clinics at two Veterans Health Administration medical centers. Addiction Science & Clinical Practice 2014;9(1):12. (In en). DOI: 10.1186/1940-0640-9-12.

149. Glasgow RE, Vogt TM, Boles SM. Evaluating the public health impact of health promotion interventions: the RE-AIM framework. Am J Public Health 1999;89(9):1322-1327. (In eng).

150. Kessler RS, Purcell EP, Glasgow RE, Klesges LM, Benkeser RM, Peek CJ. What Does It Mean to “Employ” the RE-AIM Model? Evaluation & the Health Professions 2013;36(1):44-66. (In en). DOI: 10.1177/0163278712446066.

151. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. Nature methods 2010;7(5):335-336. DOI: 10.1038/nmeth.f.303.

152. Blencowe H, Vos T, Lee AC, et al. Estimates of neonatal morbidities and disabilities at regional and global levels for 2010: introduction, methods overview, and relevant findings from the Global Burden of Disease study. Pediatr Res 2013;74 Suppl 1:4-16. DOI: 10.1038/pr.2013.203.

153. South African National Department of H. Sexually Transmitted Infections Management Guidelines 2015 Adapted from: Standard Treatment Guidelines and Essential Drugs List PHC. Pretoria, South Africa2015.

154. South African National Department of H. First line comprehensive management and control of sexually transmitted infections (STIs): Protocol for the management of a person with a Sexually Transmitted Infection. Pretoria, South Africa2008.

155. Jordan J. Bacterial Vaginosis in Swabs: Laboratory Procedure Manual. 1997:National Health and Nutrition Examination Survey.

156. Shafer WM. Does the Cervicovaginal Microbiome Facilitate Transmission of Neisseria gonorrhoeae From Women to Men? Implications for Understanding Transmission of Gonorrhea and Advancing Vaccine Development. The Journal of Infectious Diseases 2016;214(11):1615-1617. (In eng). DOI: 10.1093/infdis/jiw331.

157. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS One 2013;8(4):e61217. (In eng). DOI: 10.1371/journal.pone.0061217.

158. DiGiulio DB, Callahan BJ, McMurdie PJ, et al. Temporal and spatial variation of the human microbiota during pregnancy. Proceedings of the National Academy of Sciences 2015;112(35):11060-11065. (In en). DOI: 10.1073/pnas.1502875112.

159. Medina-Marino A, Cleary S, Muzny CA, et al. Sexually transmitted infection screening to prevent adverse birth and newborn outcomes: study protocol for a randomized-controlled hybrid-effectiveness trial. Trials 2022;23(1):441. DOI: 10.1186/s13063-022-06400-y.

160. Meingassner JG, Thurner J. Strain of Trichomonas vaginalis resistant to metronidazole and other 5-nitroimidazoles. Antimicrob Agents Chemother 1979;15(2):254-7. (In eng). DOI: 10.1128/aac.15.2.254.

161. Narcisi EM, Secor WE. In vitro effect of tinidazole and furazolidone on metronidazole-resistant Trichomonas vaginalis. Antimicrob Agents Chemother 1996;40(5):1121-5. (In eng). DOI: 10.1128/aac.40.5.1121.

162. Ghosh AP, Aycock C, Schwebke JR. In Vitro Study of the Susceptibility of Clinical Isolates of Trichomonas vaginalis to Metronidazole and Secnidazole. Antimicrob Agents Chemother 2018;62(4) (In eng). DOI: 10.1128/aac.02329-17.

163. Murray CJL, Lopez AD. Health Dimensions of Sex and Reproduction. Cambridge1998.

164. Mathers CD, Bernard C, Iburg KM, et al. Global burden of disease in 2002: data sources, methods and results. Global Programme on Evidence for Health Policy Discussion 2003(54):1-116.

165. Cleary SM, McIntyre D, Boulle AM. The cost-effectiveness of antiretroviral treatment in Khayelitsha, South Africa--a primary data analysis. Cost effectiveness and resource allocation : C/E 2006;4:20. DOI: 10.1186/1478-7547-4-20.

166. Cleary S, Boulle A, Castillo-Riquelme M, McIntyre D. The burden of HIV/AIDS in the public healthcare system. South African Journal of Economics 2008;76(May):3-14.

167. Pepper DJ, Burch VC, Levitt NS, Cleary S. Hyperglycaemic emergency admissions to a secondary-Level hospital — an unnecessary financial burden Hyperglycaemic emergency admissions to a secondary-level hospital – an unnecessary financial burden. S Afr Med J 2007;12(2):56-60. DOI: 10.1080/22201009.2007.10872157.

168. Creswell JW. Qualitative inquiry and research design: choosing among five approaches. third edition ed. Los Angeles, Calif. London New Dehli Singapore Washington DC2013.

169. Salomon JA, Haagsma JA, Davis A, et al. Disability weights for the Global Burden of Disease 2013 study. Lancet Glob Health 2015;3(11):e712-23. DOI: 10.1016/S2214-109X(15)00069-8.

170. Cleary SM, Birch S, Moshabela M, Schneider H. Unequal access to ART: exploratory results from rural and urban case studies of ART use. Sexually transmitted infections 2012;88(2):141-6. DOI: 10.1136/sextrans-2011-050136.

171. Butchart A, Garcia-Moreno C, Mikton C, World Health O, London School of H, Tropical M. Preventing intimate partner and sexual violence against women: global trends and determinants of prevalence, safety, and acceptability. Geneva2010.

172. van de Wijgert JH, Verwijs MC, Agaba SK, et al. Intermittent Lactobacilli-containing Vaginal Probiotic or Metronidazole Use to Prevent Bacterial Vaginosis Recurrence: A Pilot Study Incorporating Microscopy and Sequencing. Scientific reports 2020;10(1):3884. (In eng). DOI: 10.1038/s41598-020-60671-6.

173. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nature methods 2016;13(7):581-3. (In eng). DOI: 10.1038/nmeth.3869.

174. Pruesse E, Quast C, Knittel K, et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic acids research 2007;35(21):7188-96. (In eng). DOI: 10.1093/nar/gkm864.

175. Fettweis JM, Serrano MG, Sheth NU, et al. Species-level classification of the vaginal microbiome. BMC genomics 2012;13 Suppl 8(Suppl 8):S17. (In eng). DOI: 10.1186/1471-2164-13-s8-s17.

176. Breiman L, Friedman JH, Olshen RA, Stone CJ. Classification and regression trees. Monterey, CA: Wadsworth & Brooks/Cole Advanced Books & Software, 1984.

177. Breiman L. Random Forests. Machine Learning 2001;45:5-32. DOI: doi:10.1023/A:1010933404324.