SPECIFIC AIMS

In 2012, WHO estimated that over 350 million cases of *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG) and *Trichomonas vaginalis* (TV) occurred globally.[1] Sexually transmitted infections (STIs) during pregnancy are associated with premature rupture of membranes, preterm labor and delivery, low birth weight, congenital infections, perinatal death, and mother-to-child transmission of HIV infection.[2–13] STIs are common in pregnant women globally, but often go undiagnosed;[14–18] to address those concerns, we conducted a pilot study integrating molecular diagnostic testing for CT, NG and TV into **antenatal care (ANC)** services for HIV-infected pregnant women in South Africa. We found that diagnostic screening and **targeted treatment (TT)** during ANC was highly acceptable and feasible;[19] 97.8% of all eligible women agreed to be tested, and >93% with an STI received same-day treatment. We found a 41% STI prevalence, of which 65% of infections were asymptomatic.[19] Furthermore, our intervention decreased the prevalence of STIs at time-of-delivery by >50% compared to women receiving syndromic management (standard of care).

Though acceptable, feasible and effective, our current intervention design may have limitations. First, we found a 9.1% cumulative incidence of STIs between first ANC and delivery, suggesting a single point-in-time diagnostic screening + TT with **test of cure (ToC)** follow-up may not optimally decrease STIs at time of delivery. Consequently, *evaluating the impact and cost effectiveness of different diagnostic screening strategies that decrease the burden of STIs during pregnancy and at time-of-delivery is urgently needed.* Second, we found a 37% STI positivity at ToC. Participant interviews suggest that poor medication adherence and re-infection cannot explain the high post-treatment persistent positivity. Consequently, *biological factors that increase the risk for STI persistence and/or treatment failures must be further investigated.*

Research suggests the vaginal microbiome plays a critical role in STI acquisition, persistence, and treatment outcomes. Vaginal **community state types (CST)** with different concentrations of *Lactobacillus (L.)* spp. are associated with increased risk of acquiring STIs.[20–24] *In vitro* studies revealed that certain vaginal bacteria can inactivate metronidazole,[25–27] standard TV treatment, and bacterial vaginosis (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.[22,29,30]

In response to the need to 1) identify cost-effective screening strategies that optimally decrease the burden of STIs during pregnancy, and reduce adverse pregnancy and infant outcomes, 2) elucidate the role of the vaginal microbiome in treatment outcomes, and 3) inform STI screening and treatment guidelines in other low-middle income countries, we propose a novel, highly innovative study with the following three Specific Aims:

Aim 1: Evaluate different diagnostic screening interventions to decrease the burden of CT/NG/TV, and reduce adverse pregnancy and birth outcomes among pregnant women. Hypothesis 1 (H1): Compared to syndromic management, diagnostic screening with TT will significantly reduce adverse pregnancy/birth outcomes and decrease prevalent STIs at time of delivery. H2: Compared to single, point-in-time screening at 1st ANC with TT and ToC, periodic repeated diagnostic screening will decrease incident STIs at time of delivery. Approach: A hybrid type 1 effectiveness-implementation design three-arm randomized controlled trial will be conducted; Arm 1) diagnostic screening + TT at first ANC, with ToC follow-up; Arm 2) periodic diagnostic screening throughout ANC with TT, no ToC follow-up; Arm 3) syndromic management (standard of care). Prevalence and incidence of STIs at time of delivery, and frequency and type of adverse pregnancy/birth outcomes per intervention arm will be assessed.

Aim 2: Evaluate cost per pregnant woman diagnostically screened and treated, cost of adverse pregnancy and birth outcomes, and cost-effectiveness per STI and DALY averted. H1: Compared to syndromic management, diagnostic STI screening + TT will cost-effectively avert STIs at time of delivery, and reduce adverse pregnancy and birth outcomes. Approach: We will estimate the costs of alternative STI screening interventions relative to standard practice as well as the costs of managing adverse pregnancy/birth outcomes. Decision analytic modeling will estimate the cost-effectiveness per STI and DALY averted.

Aim 3. Investigate the relationship between the vaginal microbiome and CT treatment failure in pregnant women. H1: Chlamydia-infected pregnant women with BV-associated vaginal microbiota CSTs will be significantly more likely to have clinical treatment failure as identified at ToC. Approach: We will conduct a nested case-control (1:2) study using vaginal specimens collected from CT-infected women at first ANC, 1 and 2 weeks post-treatment and then at ToC (3 weeks post-treatment).

Our proposed 5-year study will enroll 1250 HIV-infected and 1250 HIV-uninfected pregnant women from three large ANC clinics in Tshwane District (ANC HIV positivity= 23.4%[31]), South Africa. Our research team, led by established researchers, has significant expertise and experience in all aspects of the proposed study. Our multi-institutional collaborations will allow us to leverage unique implementation platforms and resources, and allow for rapid dissemination of findings to South African and global stakeholders.

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,[31] 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.[32–34] Our current study using molecular diagnostic tests found 40.5% of HIV-infected pregnant women presenting for their first ANC clinic visit were infected with CT, NG and/or TV; >60% 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 WHOs global guidelines, the majority of STIs in HIV+ South African pregnant women go undiagnosed and untreated.

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)

	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%

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.[35–45] 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.[46] Yet the risks to infants born to HIV-infected mothers are greater than conjunctivitis. A study of HIV-infected women in Tanzania found that NG co-infection increased intrauterine HIV transmission by >450%.[2] Our recent analysis in a 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; [47–56] subsequent treatment of those STIs reduced the risk of HIV transmission.[57,58] Our own study in South Africa has recorded 34.8% (of 607) with adverse birth outcomes including 17.8% with preterm delivery, 14.8% low birth weight and 4.8% stillbirth.

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 unavailability of appropriate laboratory infrastructure.[59,60] Syndromic management involves the provision of treatment for STIs based on an algorithm of common signs and symptoms. Given that most STIs are asymptomatic, as shown by our current research (Table 1) and that of others, the majority of STIs go untreated in settings where syndromic management is used.[19,61,62] Two major limitations of the syndromic approach is the non-determination of infectious etiologies and the limited specificity, especially during pregnancy, of the "symptoms" algorithm, both of which lead to inappropriate treatment or over-treatment.[62,63] Diagnosis of CT, NG, and TV has traditionally relied on culture and microscopy, and even when highly sensitive PCR assays became available, dedicated laboratory infrastructure and trained laboratory personnel were required.[64–66] However, with the advent of new PCR based 'near-patient' or **'point-of-care' (PoC)** technology for the diagnosis of STIs,[67,68] implementation of diagnostic screening in variety of clinical settings is now possible.[19,69–73] Despite this, optimal models for PoC testing, especially during pregnancy, have not been identified. This is highlighted by our recent work integrating PoC diagnostic screening for CT, NG and TV into ANC services for HIV-infected women in South Africa. Specifically, while single PoC diagnostic screening and targeted treatment with **Test of Cure (ToC)** follow-up 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*[74] includes recommendations for the detection and treatment of STIs, including through PoC testing. However, to date, the South African government has not undertaken any efforts to identify any diagnostic platforms or testing algorithm. 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 urgently needed.

Risk factors associated with STI treatment failure 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. As part of our recent STI study aimed at integrating molecular diagnostic screening for CT, NG and TV into ANC services, we performed repeat ToCs until a participant cleared their infection, or had a document birth outcome. At ToC1, we identified a 37% persistent positivity; a number of women required multiple rounds of ToC and treatment before clearing their infection (Table 2). Interviews with women at ToC visits suggest that behaviors associated with poor treatment adherence or re-exposure from untreated partners cannot explain the high persistent positivity with CT or TV. 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.[28,75,76] Gatski *et al.*[28] revealed that in HIV+/TV+ women, BV was significantly associated with metronidazole treatment failure, suggesting that a 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 bacteria present in the vaginal microbiome.[25–27] Repeat CT positivity following treatment are not well understood; organism-related resistance is infrequently documented.[77] Reports have suggested that heterotypic resistance associated with high organism loads may factor in treatment failures, however, no solid evidence has been reported.[77–80] Given that multiple rounds of ToC and treatment are not a financially viable intervention to identify treatment failures, especially in resource constrained settings, understanding the biological mechanisms that contribute to treatment failures is an urgent priority.

Table 2. High frequency of persistent STI positivity following standard treatment at Test-of-Cure (ToC)

	ToC 1	ToC 2	ToC 3
Any STI	31.2%	12%	7%
CT	26.5%	7.9%	2.4%
NG	6.3%	0%	--
TV	19.1%	5.8%	4.7%

Vaginal microbiota may play an important role in STI treatment outcomes. Epidemiological studies have demonstrated that BV is associated with an increased risk of acquiring and transmitting HIV and STIs.[81–89] 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*,[20,90–92] and that these CSTs are associated with STIs such as CT and TV.[22,23,29] Furthermore, vaginal CSTs deficient in *Lactobacillus* spp. have been associated with increased risk of HIV acquisition,[21,24,93] while *L. crispatus*, specifically, is protective against HIV.[94] A recent study found that vaginal microbiota play an important role in modulating Tenofovir microbicide efficacy.[24] In that study, metabolism by *G. vaginalis* and other BV-associated bacteria led to Tenofovir depletion, decreasing the protection of pre-exposure prophylaxis (PrEP) and increasing the risk of acquiring HIV, regardless of medication adherence. Other work has shown Metronidazole treatment for TV to be inactivated by certain bacteria in the vaginal microbiome.[25–27] However, there are little data on the role of the vaginal microbiota on CT treatment outcomes in women.

Vaginal microbiota may play an important role in genital CT infections.[95–97] 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.[98] 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.[96] Indole-producing bacteria (e.g., *Prevotella* spp,[96] *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.

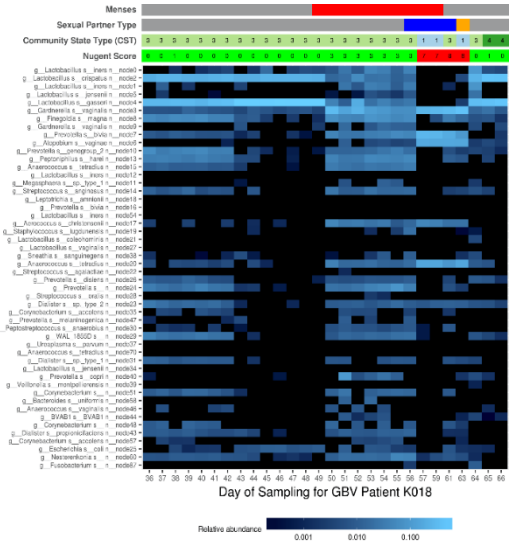
INNOVATION

1) Use of a hybrid type 1 effectiveness-implementation study design to decrease STIs and adverse birth outcomes at time of delivery: A major issue in health care is the relatively slow speed at which promising interventions, supported by rigorous research evidence, move into clinical practice. One way to 'speed up' the traditional step-wise progression from a clinical effectiveness trial to implementation science trial is to simultaneously combine the collection of effectiveness and implementation relevant data. Towards this end, we will conduct a hybrid type 1 effectiveness-implementation design study.[103] Our hybrid type 1 design allows for

the primary focus to be on collecting data on the effectiveness of our intervention, while also allowing us to incorporate process evaluation methods into our effectiveness randomized controlled trial. This will help us to explain our effectiveness results and efficiently inform future implementation efforts. Ultimately, should our intervention(s) effectively reduce the burden of STIs during pregnancy and adverse pregnancy and birth outcomes, we will be able to better inform the development of future intervention strategies.

2) Investigating clinical- and cost-effectiveness of routine CT/NG/TV testing of pregnant women: Our study will enhance knowledge of STIs during pregnancy, especially among high HIV prevalence populations, and the effectiveness of routinizing same-day PCR screening and treatment for these STIs in reducing adverse pregnancy and birth outcomes. Furthermore, until now there have been no studies in low and middle-income countries that have evaluated the costs and benefits of CT/NG/TV screening and treatment during pregnancy as it relates to pregnancy, neonatal and infant outcomes. Our cost/cost-effectiveness study has the potential to influence health policy in South Africa and globally, especially as it compares to syndromic management of STIs during pregnancy. If successful, this study would also provide reproducible cost-effectiveness analysis models for countries to better plan for and implement routine CT/NG screening and treatment in pregnancy.

Figure 1: Heatmap of incident BV case K018. Meta-data includes presence of menses (red), partner gender (gray=female, blue=male, orange= unknown), Nugent score, and CST.



3) Prospectively investigating associations between the vaginal microbiome and antibiotic treatment outcomes for STIs: CT and TV treatment failures not associated with poor medication adherence or drug resistance have been reported. [28,75,78,99–101] Studies have hinted at a role for the vagina microbiome in STI treatment failures, yet to our knowledge, none have investigated the vaginal microbiome and treatment failures in a prospective manner. As part of our study, we will longitudinally collect vaginal specimens from both HIV-infected and un-infected participants 1) before, during and after antibiotic treatment for STIs, and 2) from those with successfully treatment outcomes and treatment failures. These specimens will allow us to investigate the potential impact that the vaginal microbiome may play in STI persistence, with a focus on treatment failures.

4) Vaginal microbiome data analysis approach: We have developed methods to visualize changes in vaginal microbiota over time, including methods to display microbiome changes via heatmaps and analysis of changes in CSTs over time (manuscript in submission; Figure 1). Another innovative aspect of our analytic methods will be the use of the PECAN classifier, developed in Jacques Ravel's lab (U Maryland). PECAN uses a specialized vaginal microbiota database for accurate classification of vaginal microbiome components down to species level.[102] By using PECAN in tandem with the DADA2 processing pipeline, we are able to interrogate common bacteria sequence variants.

APPROACH

Study Setting: This study will take place in Tshwane District, South Africa. Study participant recruitment will be conducted in three large ANC clinics (Table 3; Figure 2 on next page). Clinics are located in the referral zone of two **maternal obstetric units (MOUs)**; Kalafong Hospital and Laudium Community Health Centre. Together, the three clinics see ~442 pregnant women each monthly attending a first ANC visit. Research staff embedded within the Kalafong and Laudium MOUs will collect birth outcomes. Study clinics are proximal to, and provide care for persons living in informal settlements and lower socio-economic status communities (Figure 2 pink shapes). Key ANC indicators for the three study clinics are shown in Table 3.

Facility Name	Annual ANC 1st visit headcount	Average Monthly 1st ANC Head Count	Annual ANC HIV Prevalence	HIV diagnosis at 1st ANC (Annual)
Laudium CHC	2853	238	23.3% (665)	403 (60.7%)
Olievenhout Clinic	1125	94	24.7% (278)	131 (47.1%)
Phomolong Clinic	1323	110	23.1% (306)	129 (42.2%)
Total	5301	442	23.6% (1249)	663 (53.1%)

Table 3: Key ANC indicators for selected study clinics in Tshwane District, South Africa extracted from the South African District Health Information Systems (July 2016 – June 2017)

Research Team: Details of the expert team may be found in the biosketches. Of note, Drs. Klausner and Medina-Marino have collaborated successfully on grants, publications, NIH-Fogarty training and infectious disease/ reproductive health projects since first meeting and working together at CDC-PEPFAR South Africa in 2010. Their current successful R21 (2015-2017) from NICHD directly informs this new proposal.

Preliminary Studies: The proposed study will build upon our study team's preliminary work and expertise.

1) Acceptability/Feasibility of STI testing among HIV-infected pregnant women, South Africa (Medina-Marino/Klausner, co-PIs; R21HD084274): We recently completed enrollment in a study of HIV-infected pregnant women attending their 1st ANC visit (N=845) and offered diagnostic testing for CT, NG and TV on self-collected vaginal swabs.[104] Of 442 eligible women approached in the screening arm, 430 accepted screening (Acceptability= 97.3%). 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 test results and treatment. *Those results show that integrating diagnostic STI screening into ANC services is acceptable and feasible, and that our study team has the capacity and experience to conduct the proposed study*.

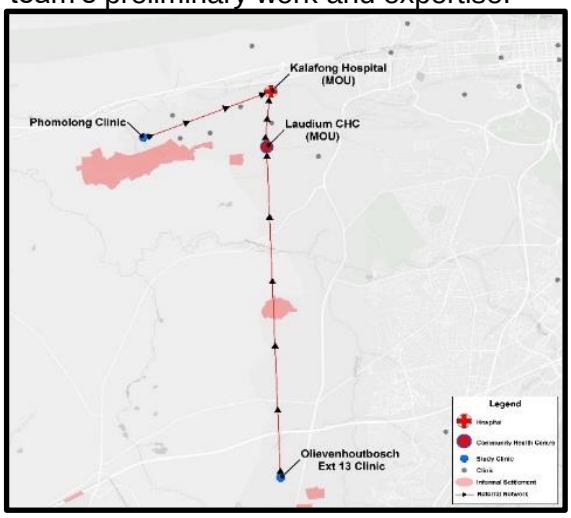


Figure 2. Study Clinics & Referral Network

2) Antenatal prevalence and behavioral risk factors of STIs (Medina-Marino/Klausner, co-PIs; R21HD084274): Our current work has identified an overall STI prevalence of 40.5% (CT= 29.5%; TV= 20.2%; NG= 5.6%) among HIV-infected pregnant women attending their first ANC visit.[105] Of those with STIs, 64.4% were asymptomatic. Factors associated with STIs at first ANC consultation were alcohol use during pregnancy (aOR=1.96, 95% CI=1.06-3.64) and having a non-cohabitating partner (aOR=1.42, 95% CI=0.97-2.03, p=0.07), controlling for maternal age and employment status.

3) Test of Cure (ToC) and treatment outcomes (Medina-Marino/Klausner, co-PIs; R21HD084274): Among 174 participants with a positive STI result at first ANC, 80% returned for a ToC 3 weeks later. Of these, 37% had any positive ToC results (CT= 26.5%; TV= 19.1%; NG= 6.3%).[106] ToC interviews revealed 91.2% of women disclosed their results to their partner(s), and 55.2% provided their partner(s) with a treatment packet. Interviews also suggested that behaviors associated with re-infection or poor medication adherence cannot account for the high persistent positivity after treatment. Those findings suggest that *a single point-in-time diagnostic screening with targeted treatment may not optimally decrease STIs at time of delivery*. Furthermore, *biological mechanisms that increase the risk for STI persistence and/or treatment failures must be further investigated.*

4) STI incidence during pregnancy and prevalence at time of delivery (Medina-Marino/Klausner, co-PIs; R21HD084274): Among 148 women negative for CT, NG, and TV at first ANC visit, we identified 11 incident infections immediately post-delivery. Moreover, in those women with a documented negative test result after treatment, an additional 10 had an incident infection immediately post-delivery, resulting in a 9.1% cumulative incidence of STIs between first ANC and delivery. Comparing the postnatal STI prevalence of intervention and control arm participants, we found that our diagnostic screening intervention decreased the prevalence of 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%).[106] Ultimately, *while diagnostic screening and targeted treatment significantly decreased the prevalence of STIs at time of delivery, a single point-in-time diagnostic test cannot identify incident infections, thus leaving women and neonates at risk of sequelae associated with STIs*.

5) Gestational age measurements (Medina-Marino/Klausner, co-PIs; R21HD084274): We assessed the reliability of last menstrual period (LMP) dating in estimating gestational age at first ANC with estimates obtained from vaginal ultrasound. Among 153 women, the median estimated gestational age was 19 weeks (IQR: 14 – 24 weeks) by LMP and 19 weeks (IQR: 15 – 24 weeks) by ultrasonography. Gestational age estimate obtained by LMP differed from the ultrasonography estimate by ≤2 weeks in 76.5% (n=117) of participants. The mean difference between gestational age estimated by last menstrual period vs. ultrasonography was 0.26 weeks (95% CI: -4.8 weeks to 5.3 weeks; manuscript in preparation).

6) Adverse birth outcomes (Medina-Marino/Klausner, co-PIs; R21HD084274): Among 607 women delivered to date, the median gestational age at delivery was 38 weeks. We have recorded 34.8% with adverse outcomes including preterm delivery 17.8% (4.9% <33 weeks), low birth weight 14.8% (1.3% very low or extremely) and miscarriage or stillbirth 4.8%. Two early neonatal deaths < 24 hours after birth have been recorded.

7) Vaginal microbiome of HIV-negative South African women (Meiring, PI; NRF RCA 13100150715/91478): We recently completed a study assessing the association between the vaginal microbiome and prevalent human papillomavirus (HPV) infection in 87 reproductive age HIV-uninfected Black South African women. A minority of the women (N=23, 26.4%) were found to have *Lactobacillus* spp. dominant vaginal microbiota; two (2.3%) were CST I, *L. crispatus* dominated; two (2.3%) were CST V, *L. jensenii* dominated; 19 (21.8%) were CST III, *L. iners* dominant; zero were *L. gasseri* dominant. The majority of women (n=64, 73.5%) had diverse vaginal microbiota

with low to no *Lactobacilli* spp. present and complex mixtures of BV-associated bacteria. *Gardnerella vaginalis*, *Atopobium vaginae* and *Sneathia* were identified as putative biomarkers for prevalent hrHPV (manuscript in preparation). *This work provides insight into the structure and composition of the vaginal microbiome of HIV-uninfected South African women, and can provide a useful comparison for our proposed study.*

8) Pathogenesis of BV in African American women who have sex with women (Muzny, PI; K23AI106957). Women with a baseline Nugent score of 0-3 were followed prospectively. Women with incident BV and controls were matched by age, race, and days of menstrual cycle; 16S rRNA sequencing targeting V4 was performed on specimens for 21 days prior to incident BV. DADA2 was used to process raw MiSeq reads. Species-level taxonomy was assigned to variants using PECAN[102] and merged with RDP assigned taxonomy using GreenGenes13_5. Longitudinal microbiome data for BV-candidate bacteria and lactobacilli of interest were analyzed using the phyloseq library. Of 31 participants that completed the study, 14 (45.2%) developed incident BV. Sequencing was performed on 448 specimens from 14 cases and 8 controls. Of controls, 75% were dominated by *L. crispatus* while 78.6% of cases were dominated by *L. iners* and/or *L. jensenii* and *L. gasseri* prior to incident BV. The relative abundance of *L. crispatus* became significantly lower in cases 14 days before incident BV. The relative abundance of *Gardnerella vaginalis, Prevotella bivia,* and *Atopobium vaginae* became significantly higher in cases 7-8 days before, 4 days before, and on the day of incident BV, respectively. There was no significant difference between cases and controls in the relative abundance of *Megasphaera* Type 1, *Sneathia sanguinegens*, BVAB1-BVAB3 or of *L. iners* leading up to incident BV (submitted *JID*). *Novel methodologies used in this study will be incorporated into Aim 3 of this proposal.*

9) Consequences of vaginal microbiota on IFNγ-mediated clearance of *Chlamydia trachomatis* (Taylor, MPI; 1R01AI118860-01A1). This study will assess the influence of vaginal microbiota on the incidence of CT clearance without treatment. Vaginal swabs from women with persistent or spontaneous CT clearance are subjected to 16S rRNA gene sequencing targeting the V4 region and processed through the DADA2 pipeline. Species-level taxonomy is assigned using the PECAN classifier and vaginal-specific database. Preliminary results have shown a prevalence of indole-producing microbiota in the vaginal microbiome of women who had persistent CT infection, and a lack of indole-producing microbiota in the vaginal microbiome of women who did clear this infection without treatment. *These results help elucidate the role of the vaginal microbiome in CT testing outcomes and further support our rationale for studying the vaginal microbiome in pregnant women with CT treatment failure.*

METHODOLOGY AND STUDY AIMS

Specific Aim 1: Evaluate different diagnostic screening interventions to decrease the burden of CT/NG/TV, and reduce adverse pregnancy and birth outcomes among pregnant women.

Methods and Procedures: To achieve Aim 1, we will conduct an effectiveness-implementation hybrid type 1 3-arm randomized controlled trial, with participants randomized from within each clinic (1:1:1) to one of the following arms: **Arm 1)** single point-in-time molecular diagnostic screening for CT, NG and TV with targeted treatment at first ANC visit and infection-specific ToC 3 weeks post-treatment. Women with a positive ToC will be re-treated and requested to return every 3 weeks for follow-up ToC visits until a negative ToC or birth outcome is documented. **Arm 2)** periodic molecular diagnostic screening for CT, NG and TV at first ANC visit and week 30–34 gestation with targeted treatment. No ToC will be conducted for women with positive test results. **Arm 3)** syndromic management (standard of care) at every ANC visit per South African National Guidelines.[128,129]

Through these methods we plan to achieve four main sub-aims: **1(a)**: compare the effectiveness of diagnostic screening (Arms 1/2) to syndromic management (Arm 3; standard of care) in reducing the prevalence of STIs during pregnancy and at time of delivery; **1(b)**: compare the effectiveness of (i) single point-in-time diagnostic screening and targeted treatment plus ToC follow-up (Arm 1) *versus* (ii) periodic diagnostic screening throughout ANC and targeted treatment without ToC follow-up (Arm 2) in reducing prevalent and incident STIs at time of delivery; **1(c)**: estimate the frequency of adverse pregnancy and birth outcomes (e.g., premature rupture of membranes, preterm labor or delivery, low birth weight/small for gestational age) and their association with screening interventions; **1(d):** collect process measures to inform future implementation and scale-up activities.

Recruitment and Eligibility: We will recruit 1250 HIV-infected and 1250 HIV-uninfected pregnant women presenting for their first ANC visit at one of our 3 study clinics in Tshwane District (Figure 2), South Africa. Eligibility criteria: 1) Age ≥18 years, 2) Currently pregnant, 3) Attending first ANC visit for their current pregnancy, 4) Willingness to self-collect up to four vulvo-vaginal swabs, 5) Residence in Tshwane District, and 6) Intent to stay in Tshwane District through delivery. Gestational age will NOT be used as an inclusion/exclusion criterion.

All pregnant women attending their first ANC visit will be screened for eligibility by study staff following standard HIV testing per South African National Guidelines for the prevention of MTCT of HIV.[130] Study staff will be trained in the study's methods, protocol, and human subjects research. Study staff will also receive training

on South Africa's syndromic management algorithms for STIs. Staff will read all eligible women a brief description of the study. Interested women will then be read aloud, in their preferred language, the study consent form which will provide specific information about CT, NG and TV infections, the consequences and treatment of those infections, and study risks and benefits, and then will be invited to participate in the study. Those providing informed consent will be enrolled and within each clinic randomized (1:1:1) into one of the 3 study arms using a simple random allocation list created in Microsoft Excel before the initiation of recruitment activities; each study arm will be composed of 50% HIV-infected (purposive enrichment) and 50% HIV-uninfected women.

Those providing informed consent will be asked to provide detailed contact information (e.g., phone numbers and "home address" for self, family, friend/neighbor) to ensure follow-up. Staff will record reason for ineligibility or refusal. Staff will collect basic de-identified information from clinic logs (i.e., age, cultural group, gestational age, HIV status) to use for descriptive analysis of the general ANC patient population.

Data Collection at Enrollment/First ANC: Trained study staff will administer an ACASI-based questionnaire to all participants. The ACASI questionnaire, adapted in part from measures used by Drs. Medina-Marino, Klausner and Pattinson in previous and current STI screening and maternal-child health studies, or documented in the literature, will include: 1) participant demographics and socio-economic status, 2) obstetric, gynecological and sexual health history, 3) sexual behaviors, risk factors and self-perception of risk for HIV and STI acquisition before and during pregnancy,[131] 3) partner characteristics and HIV status,[132,133] 4) knowledge and previous history of STIs, and 8) screenings for depression,[134,135] substance abuse,[136] interpersonal violence and social support. Staff will translate ACASI questionnaires into the major local languages (i.e., English, Sepedi, Setswana, Zulu, and Ndebele). Participants may select their preferred language in which to take the ACASI questionnaire, but will also be able to toggle between languages during the questionnaire to ensure linguistic comprehension of all questions. Staff will abstract from clinical records additional clinical history, including HIV status, date of diagnosis, and immunological characteristics associated with HIV infection (e.g., CD4 T-cell level, HIV viral load, antiretroviral therapy (ART) use and 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 National Health Laboratory Service corporate data warehouse, both of which contain individual-level heath data.

Specimen Collection, Handling, Transport and Storage: Consenting participants will be instructed on how to self-collect a vulvo-vaginal swab specimen and asked to provide up to four swabs: 2x swabs for STI testing, 1x swab for microbiome analysis (Aim 3), and 1x swab for bio-banking (NOTE: pregnant women in our current study found it acceptable and feasible to collect up to four vaginal swabs at a visit). If a participant is not comfortable with self-collecting a vulvo-vaginal swab specimen they will be given the option to provide a urine specimen for testing and bio-banking (women that only provide urine specimens for testing will not be included in the cohort for microbiome analysis, Aim 3). 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 Data Collection section). For immediate GeneXpert testing, participants will use the GeneXpert Vaginal/ Endocervical Specimen Collection kit [Cepheid, Sunnyvale, CA] for vaginal swab specimen collection. For vaginal microbiome analysis, participants will use a Dacron swab [Qiagen, Digene] for self-collection, with subsequent storage in DNA AssayAssure® [Sierra Molecular, Incline Village, Nevada] at ambient, air-conditioned room temperature. For specimen bio-banking, participants will use a dry FLOQswab® [COPAN, Murrieta, CA] for specimen collection, with subsequent storage in a sterile tube. Collection of vaginal swabs for microbiome analysis and bio-banking (Aim 3) will occur before any STI treatment. Specimens will be securely stored at 2-8°C and transported to the Department of Medical Microbiology, University of Pretoria, on a bi-weekly basis according to Good Laboratory Practice. Specimens will then be flash frozen and stored at -80°C for long-term bio-banking. We will ship specimens for microbiome processing and analysis to the University of Cape Town on Dry Ice quarterly.

Diagnostic Testing: Vaginal specimens collected from participants will be PCR-tested for CT, NG and TV using the Xpert® CT/NG and Xpert® TV cartridges [Cepheid, Sunnyvale, CA]. Trained staff (STI Test Counselors and Research Nurses) will conduct the Point-of-care (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 CT and NG, while Xpert® TV provides 60 min detection of TV; both test cartridges have high sensitivity and specificity[137] and function well in resource-constrained environments and clinical settings such as those proposed here. 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.

Reporting and Treatment: The GeneXpert systems consist of an instrument, computer, and preloaded software for running tests and displaying results as either positive or negative. STI Test Counselors will report all test results to the ANC Research Nurse embedded within each study clinic. Research nurses will be responsible for providing same-day test results notification and immediate treatment (and partner treatment) to all STI-infected study participants per the South African Department of Health's STI treatment protocols.[48,49]

Partner Treatment: All women testing positive for an STI will be asked to notify their partners, and given the option to either request their partner(s) to present to the clinic for treatment, or be given an infection-specific partner treatment packet of oral medication to take to their partner(s). Targeted treatment for partners will be provided according to the South African STI National Guidelines; however, in lieu of the recommended intramuscular injection of ceftriaxone for NG infections, which would require a male partner to present to a clinic, South African National Guidelines allow for oral cefixime 400mg tablet/ azithromycin 1 gm oral to be administered for NG infection.[128,129] Partner treatment will be placed inside a small yellow envelope labelled with the medication name, dosage, instructions, expiration date, and lot number. This manner of providing partner treatment was found to be highly acceptable and feasible in our current study.

Arm 1 Specific Activities: Per Table 4, at first ANC visit, participants randomized to Arm 1 will be asked to collect four vaginal swab specimens as described above (Specimen Collection section). Two specimens will be used for CT/NG and TV testing (as described in Diagnostic Testing section), while specimens for microbiome analysis (Aim 3) and bio-banking will be handled as described in the *Specimen Handling, Transport and Storage* section. Test result reporting and the provision of treatment for those with a positive test result will be conducted as described in the *Reporting and Treatment* section. Test of Cure (ToC): Arm 1 study participants that were treated for a diagnostically detected CT, NG and/or TV infection at first ANC visit will be requested to return to the clinic 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, women will be asked to self-collect vaginal specimens, as described above, for STI ToC as well as for microbiome analysis and bio-banking. Women with a positive ToC will again be provided treatment (and partner treatment) and asked to return 3 weeks later for another ToC; ToC will be repeated until a participant has a negative test result or a documented birth outcome.

Arm 2 Specific Activities: Per Table 4, at first ANC visit, and an ANC visit occurring between 30-34 weeks gestation, participants randomized to Arm 2 will be asked to collect four vaginal swab specimens as described (Specimen Collection section). Two specimens will be used for CT/NG and TV testing (as described in Diagnostic Testing section), while specimens for microbiome analysis and bio-banking will be handled as described in the Specimen Handling, Transport and Storage section. Test result reporting and the provision of treatment for those with a positive test result will be conducted as described in the Reporting and Treatment section. No ToC activities will be performed for Arm 2 participants.

Clinic Visit	Specimen Collection	CT, NG and TV PoC Testing	Syndromic Management
First ANC Visit	All Participants	Arms 1 and 2	Arm 3 Only
ToC 3-Weeks Post-treatment	Arm 1 Only	Arm 1 Only	-----
30 – 34 Weeks Gestation	Arm 2 Only	Arm 2 Only	As needed
First Postnatal Clinic Visit	All Maternal-Infant Pairs	All Maternal-Infant Pairs	-----

Table 4: Specimen Collection and STI Testing Schedule

Arm 3 Specific Activities: Per Table 4, at first ANC visit participants randomized to Arm 3 will be asked to collect four vaginal swab specimens as described (Specimen Collection section). All specimens will be immediately bio-banked as described in the Specimen Handling, Transport and Storage section. Arm 3 participants will be provided standard of care syndromic screening and management for STIs per South African National Guidelines.[128,129] Specimens collected from Arm 3 participants will be tested for CT, NG and TV infections after a birth outcome is documented; specimens will be tested using the GeneXpert systems as described in the Diagnostic Testing section.

Retention and Follow-up: To ensure retention, we will collect multiple forms of contact information for all participants. To develop and maintain a strong relationship with participants, study staff will conduct welcome phone calls within 3 days of enrollment, and check in with participants during regular ANC clinic visits, or during monthly ART pickup for those with HIV infections. To further support active follow up, participants will also be encouraged to enroll in the UNICEF-funded MomConnect program, which sends reminder messages to pregnant women's mobile phones via text messages to remind them about their upcoming ANC visits, and automatically generates alerts for clinic staff when appointments are missed. Clinic and study staff will contact participants who do not return for scheduled ANC or ART visits and encourage return for care. We will flag participant charts so that clinic staff will notify study staff on the date of delivery. Seven days post-delivery, study staff will contact participants that have not yet presented for their 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, and home visits.

Post-partum and Infant Testing: Per Table 4, ALL STUDY PARTICIPANTS will be asked to provide four vaginal swab specimens (as described in *Specimen Collection* section) during their first postnatal clinic visit; the first postnatal clinic visit is typically 3-6 days after discharge from the MOU or at the earliest time possible after they give birth. Two specimens will be used for CT/NG and TV testing (as described in *Diagnostic Testing section*), while specimens for microbiome analysis and bio-banking will be handled as described in the *Specimen Handling, Transport and Storage* section. Reporting of test results and provision of treatment for those with a positive test result will be conducted as described in the *Reporting and Treatment* section. Two **nasopharyngeal (NP)** swab specimens will be collected from all infants during the first postnatal visit. For any woman with a post-partum prevalent STI, staff will test the infant's NP swab at that visit for that same infection (CT, NG and/or TV) to exclude mother-to-child transmission. If no maternal infections are identified, both NP swabs will be bio-banked. Any neonate who has an NP specimen positive for CT, NG and/or TV will be treated according to the South African Medical Formulary recommendations and guidelines.[128]

Data Collection at Postnatal Clinic Visit: We will collect data on adverse pregnancy events in all study participants via abstraction of MOU discharge records and face-to-face interviews with participants during the first postnatal clinic visit. Staff will collect information on fetal loss, preterm labor, 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 South African National Health Laboratory Service (NHLS)'s database. At the routine 6-week immunization visit, we will access neonatal morbidities (i.e., respiratory distress, conjunctivitis, sepsis) via maternal interviews and patient medical records. A study supervisor will perform weekly reviews to ensure completeness and validity of the data collected; discrepancies will be resolved via interview with the birth attendant (midwife or physician).

Data Collection for Process Evaluation: We will use the **R**each-**E**ffectiveness-**A**doption-**I**mplementation-**M**aintenance (RE-AIM) model as our **conceptual framework**[138–140] to guide the collection of valuable information during our effectiveness trial. Per Table 5, 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).

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? 3) What aspects of the intervention do patient like/dislike?	1) Recruitment rates 2) Socio-demographics of all eligible participants stratified by consent/refused 3) Perception of participants	1) Enrollment tracking sheets 2) Enrollment tracking sheets 3) Post-intervention participant survey
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
Implementation	1) What does the intervention cost? 2) What support and tools are needed for consistent delivery of intervention?	1) Cost/Cost-effectiveness data 2) Perceptions of study and clinic staff, NHLS and NDoH	1) Study datasets 2) Post-intervention interviews w/ clinic & national stakeholders
Maintenance	1) What resources will be needed for the intervention to be sustainable? 2) What adaptions are needed to integrate intervention into current practices?	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

Table 5: RE-AIM Conceptual Framework Guiding Process Evaluation (adapted from Hagedorn *et al.*[138])

Data Analysis: We will analyze data using R [R Foundation for Statistical Computing, Vienna, Austria]. Participant demographic and clinical characteristics will be described per study arm using proportions (categorical variables) and mean, median, and interquartile range (continuous variables). Statistical significance

will be assessed using chi-square and t-tests, or their non-parametric equivalents. Primary Outcomes, stratified by HIV status include: 1) change in STI prevalence between baseline (1st ANC) and delivery (1st postnatal clinic visit) per study arm, and 2) frequency of adverse pregnancy and birth outcomes per study arm. We will assess primary outcome measures based on intention-to-treat, modified intent-to-treat and a per-protocol analysis. Secondary Outcomes: 1) incident infections identified at time of delivery by study arm; 2) incidence and risk factors of CT, NG, and TV colonization in neonates, stratified by HIV status; 3) rates and risk factors for treatment failures, stratified by HIV status; 4) factors associated with STIs at first ANC and risk factors for incident STIs during pregnancy; and 5) process evaluation measures, as described in Table 5. Exploratory Outcomes: 1) type and frequency of adverse pregnancy/birth outcomes as a function of STI exposure time; 2) infant outcomes, including pneumonia and neonatal conjunctivitis, at 6-week immunization clinical visit. Significance level will be set at p-value <0.05 and all tests for significance will be two-tailed. We will calculate the change in CT, NG, and TV prevalence by subtracting the prevalence at delivery from the prevalence at baseline. We will use difference in differences analysis and average treatment effects estimation techniques to compare net changes in CT/NG/TV prevalence between baseline and delivery. We will assess differences in the frequency of adverse pregnancy and birth outcomes between study arms in both absolute and relative terms using risk (R) and relative risk (RR) estimates, respectively. We will use stratified analysis and multivariate multinomial logistic regression analysis to compute relative risks adjusted for potential effect modifiers, and confounding variables, such as exposure time to an STI. We will consider multiple imputation of missing data when missing values exceed 10% and not more than 30%, and satisfy the condition of "missing at random." We will conduct sensitivity analyses to determine how imputed data affect the study results. Analytic Plan for Process Evaluation Qualitative Data: We will employ aspects of deductive analysis that take into account the RE-AIM framework through the creation of initial *a priori* codes. Data coding and analysis will be iterative and interactive processes. We will first read all interview transcripts in order to increase familiarity with the data. Next, we will assign *a priori* codes and create emergent codes. Transcripts will then be re-read to create pattern codes that connect subsequent concepts under larger headings. Consistent patterns in meaning, concepts, and themes across all interviews will be identified, and data matrices created as visual representations of the findings.[141-143] 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.

Potential Challenges and Quality Assurance: Loss-to-follow up at ToC, postnatal specimen collection and interviews, and 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 maternal-obstetric units to collect maternal and neonatal specimens, and 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 analyzing and interpreting our results. Finally, all research study personnel will meet weekly to review study enrollment, 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.

Aim 2: Evaluate the cost per pregnant woman diagnostically screened, the costs of adverse pregnancy and birth outcomes, and the cost-effectiveness per STI and DALY averted.

Rationale: 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. This crucial 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.

Data Collection: *The Provider Perspective*: We will assess the full economic costs of each study arm and the full economic costs of adverse pregnancy and 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 modeling 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 combined bottom-up and step-down approach, 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.[122,123] 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.[122] 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.[144] 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.

Decision Analytic Modeling: Upon estimating unit costs and utilization, we will build a decision analytic model to estimate the cost per pregnant woman diagnostically screened, screened positive, treated, and cured at time of delivery for each study arm and each perspective (provider/patient). Deterministic sensitivity analyses will assess the impact of key parameter uncertainty (e.g. the cost of GeneXpert machines within a scale-up scenario). Probabilistic sensitivity analysis will assess uncertainty around each utilization estimate from the trial.[145] If Arm 3 costs (hypothesized to include higher costs for adverse pregnancy and birth outcomes) are greater than Arm 1 or Arm 2 costs, the intervention(s) are cost-saving and no further analysis would be required. 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. DALYs associated with preterm births will come from common reliable sources such as the WHO and the Institute for Health Metrics and Evaluation.[146–148] DALYs will be modeled in two steps: 1) we will model the proportion of preterm infants dying within the first year. For that group of preterm infants, the lost DALYs are the estimated life expectancy for South African neonates surviving at age one. Second, we will model the 1) proportion of preterm infants surviving past the first year, 2) average life expectancy of these children, and 3) average degree of disability of these children. That will enable an estimation of DALYs and DALYs averted within each study arm. 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.[122]

Potential Challenges: Assuming sample size, STI prevalence, and birth outcome data are sufficient, the main challenge of Aim 2 involves accurate data collection of newborn hospital care costs, particularly those costs incurred by any higher-level neonatal care. Building on previous experience of costing a variety of conditions within hospital settings, we plan to collect newborn cost data until discharge or death, whichever comes first, though this will likely be a few months of hospital care for babies born very pre-term.[109,149–152]

Specific Aim 3. Investigate the relationship between the vaginal microbiome and CT treatment failure in pregnant women.

Methods and Procedures: For Aim 3, we will conduct a nested case-control study (1:2) using selected bio-banked vaginal specimens collected from participants enrolled and randomized in Aim 1. We will accomplish two main sub-aims: 3(a): determine the impact of vaginal microbiota on CT treatment outcomes; 3(b): explore the natural history of the vaginal microbiome in the context of antibiotic treatment for CT infections.

Recruitment and follow-up visits: Participants randomized into Arm 1 of Aim 1, and who test positive for a CT mono-infection during their first ANC visit will be invited to participate in a weekly vaginal specimen collection

activity until a negative ToC result or a birth outcome is documented. Participants with multiple STIs will be excluded from this sub-study, as the presence of TV and NG may also alter vaginal microbiota.[153–155]

Specimen collection, handling and shipping: We will use the swab collected for bio-banking to smear a glass slide for Nugent score determination prior to its storage.[156] At week 1, 2 and 3 (i.e., ToC visit), vaginal specimen collection for microbiome analysis, glass slide smearing for Nugent scoring and specimen bio-banking will occur. At ToC, participants will be repeat CT-tested (Aim 1: Diagnostic Testing section). Those with positive CT test results at ToC will again be treated with azithromycin 1g, and asked to return for subsequent weekly specimen collection (weeks 4 and 5) and ToC2 (week 6). Specimens will be collected and stored as previously described. (Aim 1: Specimen Collection, Handling, Transport and Storage section).

Nugent scoring: Air-dried slide smears will be heat-fixed and Gram stained per standard procedure.[157] 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.

Selection of Stored Specimens for Nugent Scoring and Vaginal Microbiota Analysis: "Cases" will be defined as participants who test positive for CT by GeneXpert at first ANC visit (week 0) and at ToC visit (week 3; 'no clearance'). "Controls" will be participants who test positive for CT by GeneXpert at first ANC visit (week 0) but test negative at ToC (week 3; 'clearance'). The four stored vaginal swab specimens (weeks 0-3) from cases and controls will be selected for Nugent scoring and vaginal microbiota analysis. Additional weekly vaginal swab specimens from "cases" who remained persistently positive for CT by GeneXpert at first ToC will also be selected for vaginal microbiota analysis.

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 PECAN classifier (https://github.com/pgajer/MCclassifier) and vaginal_319_806_rc_MCo7p2 database for precise assignment of taxonomy. Phyloseq[158] and QIIME[159] 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.[160]

Estimated effective sample size: Based on 834 pregnant women randomized to Arm 1 of Aim 1 (see Sample Size Calculations below), and a 30% CT prevalence among pregnant women (Table 1), approximately 246 CT infected women will be included in Aim 3. Considering 26.5% of CT-infected women had a positive ToC (Table 2), approximately 65 women will be "cases" and 130 women will be "controls" (1:2 match). Furthermore, given that 7.9% of CT-infected women may still positive for CT at ToC2 (week 6), 5 women will continue to collect weekly vaginal specimens. Given that each participant will have 4 stored specimens, ~800 vaginal specimens will be sequenced.

Data analysis and statistical considerations: We will analyze associations between Nugent scores, vaginal CSTs, CT treatment outcomes, and other clinical data. We intend to compare the relative abundance of microorganisms between cases and controls to determine which organisms are associated with CT treatment failure in pregnant women. Several statistical methods have been proposed to evaluate differential abundance in microbiome data (DESeq, DESeq2, and Voom).[161–163] We propose to use the DESeq2 method, which is based on the negative binomial Wald test, as it provides increased sensitivity and has several desirable characteristics compared to other competing methods.[164] Data will be analyzed at 4 time points, correlating to specimen collection (see above). We will perform preliminary analysis at each time point to account for individual effects of different microbiota at different study stages, and to understand any time/environmental-specific differences in microbiome composition over time. We will then perform longitudinal data analysis to account for longitudinal trends, random effects and fixed effects of different factors. We will use the generalized linear mixed model framework to remove the confounding effects of these factors on microbiome composition, and detail the effects of individual microorganisms on CT treatment. CSTs will be constructed using linkage clustering of microbiome species data. We will use generalized linear mixed models again to determine if different CSTs are associated with successful CT treatment. Other covariates affecting the microbiome (e.g. HIV status, CD4 count, ART exposure) will be included in the models to assess the effect of these factors on the treatment success rate. Primary Outcomes: Association of CT treatment outcomes and BV-associated CSTs. Findings from this sub-study could be clinically significant, as they may suggest that all pregnant women who are persistently positive for CT should be screened and treated for BV, even if they are asymptomatic. Current evidence is insufficient to recommend routine screening for BV in asymptomatic pregnant women for the prevention of preterm birth.[165] Secondary Outcomes: 1) prevalence of BV in cases vs. controls based on Nugent score of 7-10 at first ANC

visit, week 1 and 2, and ToC visit, and 2) Association of composition and structure of the vaginal microbiome over time at first ANC visit, week 1 and 2, and ToC in cases vs. controls at as a function of HIV viral load, CD4 count, and ART exposure. Exploratory Objectives: 1) Change in average relative abundance of indole-producing bacteria (i.e. *Prevotella* spp, *Fusobacterium nucleatum*, *Propionibacterium acnes, Porphyromonas gingivalis, Escherichia coli*, and *Enterococcus faecalis)* over time in cases vs. controls, and 2) association of BV-associated CSTs with symptomatic or asymptomatic CT infection in cases vs. controls at first ANC visit.

Potential Limitations: Changes in the structure and composition of the vaginal microbiome can occur rapidly, at times within days.[166] As such, our currently proposed weekly sampling frame may limit our resolution to detect important changes. However, a recent prospective microbiome study in pregnant women found that vaginal community taxonomic composition and diversity remained remarkably stable during pregnancy.[160] 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 behavior between first ANC and ToC visits. To assess for re-infection, study consultant Dr. Remco Peters will perform CT genotyping on paired specimens of persistently positive participants using other existing funds.

Sample Size Calculations: Aim 1 analyses will explore intervention effects on reducing probabilities for two primary outcomes: adverse pregnancy / birth events, and prevalent STIs. Based on a total sample size of approximately 2500 participants (834 participants in each study arm), calculations show that we will have at least 80% power to detect study arm differences of approximately 10% or larger at birth. We conducted two sets of calculations. 1) Calculations for the probability of an adverse pregnancy / birth event were conducted in PASS 2008 software (https://www.ncss.com/) for differences in proportions at a single timepoint (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 timepoints (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); simulations used a range of values from 10% to 20%. Second, we fit random effects logistic regressions to each simulated data set and recorded the number of significant intervention effects for a given sample size. We assumed an attrition rate of 15%.

A sample size of 2500 is a reasonable target based on data contained within the South African District Health Information System (Table 3). The annual total head count of women presenting for first ANC services at the three participating clinics is 5301. Given the combined estimated HIV prevalence rates at the three study clinics (Table 3), we expect a total of 663 HIV-infected pregnant women to seek services each year. The number of HIV-infected pregnant women will support a 50:50 ratio of HIV-infected to HIV-uninfected pregnant women in each study arm. Based on our recent study, and the rate at which we identified eligible participants that provided consent, we expect to meet our proposed sample size within 27 months from the start of enrollment.

Timeline: This study encompasses four major phases, as color highlighted in Table 6.

- Phase 1 (yellow): Protocol development, IRB submission; Develop and pilot clinical and costing data collection tools; Develop participant, specimen and implementation tracking tools; Staff hiring and training
- Phase 2 (green): Participant recruitment, testing, treatment, ToC and follow-up; Microbiome specimen collection; Clinical and costing data collection; Postnatal follow-up, testing and outcomes data collection
- Phase 3 (blue): Specimen selection for Nugent scoring and vaginal microbiota analysis; Microbiome specimen processing and sequencing
- Phase 4 (brown): Data analysis, dissemination of findings, and preparation for future research.

Table 6: Study Timeline	Year 1				Year 2				Year 3				Year 4				Year 5			
Aim 1. Evaluation of Screening Interventions and Outcomes																				
Preparations, Tool Piloting, Training																				
Implement Intervention																				
Post-delivery Follow-up, Pregnancy and Birth Outcomes																				
Data Analysis and Dissemination																				
Aim 2. Cost/ Cost-effectiveness																				
Tool Development and Piloting																				
Data Collection																				
Data Analysis and Dissemination																				
Aim 3. Microbiome Analysis																				
Specimen Collection																				
Specimen Processing																				
Data Analysis and Dissemination																				

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