SPECIFIC AIMS

Sexually transmitted infections (STIs) during pregnancy cause 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 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] To study the benefit, acceptability and feasibility of STI diagnostic screening, we integrated point-of-care molecular testing for *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG) and *Trichomonas vaginalis* (TV) 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 delivery by >50% compared to women who received standard-of-care syndromic management.

Though acceptable, feasible and effective, our previous study had 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 enrollment may not optimally decrease STIs at time of delivery. Consequently, *evaluating the impact and cost effectiveness of different screening strategies to decrease STIs during pregnancy is urgently needed.* Second, our study was underpowered to detect 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. 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.*

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.)* 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.[21,29,30]

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 outcomes, we propose three Specific Aims:

Aim 1: Evaluate three different screening strategies to decrease the burden of CT/NG/TV among pregnant women, and reduce adverse birth outcomes. Hypothesis 1 (H1): Compared to a one-time diagnostic test for STIs at a woman's first ANC visit, repeat testing algorithms will significantly reduce adverse birth outcomes. H2: Compared to diagnostic screening with follow-up test-of-cure (ToC), repeat screening and treatment without any ToC will significantly decrease STIs at delivery. Approach: A three-arm randomized controlled hybrid-effectiveness trial will be conducted; **Arm 1**) diagnostic screening and treatment at first ANC + ToC follow-up; **Arm 2**) repeat screening and treatment throughout ANC (no ToC); **Arm 3**) one-time diagnostic screening and treatment at first ANC, no ToC (control). Prevalence and incidence of CT, NG and TV at delivery and frequency of adverse birth outcomes by study arm will be assessed.

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. H1: Compared to one-time diagnostic screening and treatment at first ANC, diagnostic screening with follow-up ToC and repeated screening with treatment (no ToC) will be more cost-effective to avert STIs at delivery, and reduce adverse birth outcomes. Approach: We will estimate and compare the costs of different STI screening strategies relative to control, and the costs of managing adverse 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 persistent Chlamydial infections in pregnant women. H1: CT-infected pregnant women with BV-associated vaginal microbiota CSTs will be significantly more likely to have persistent infections at test-of-cure compared to women with non-BV associated CSTs. Approach: A nested case-control (1:2) study using vaginal specimens collected from CT-infected women at first ANC, 1, 2 and 3 weeks post-treatment.

We will enroll 2500 pregnant women (50% HIV-infected/ 50% HIV-uninfected) from ANC clinics in Tshwane District (ANC HIV positivity= 23.4%[31]), South Africa. Our research team has expertise and experience in all aspects of the proposed study including prior work at study sites. Multi-institutional collaborations allow us to leverage unique implementation platforms and resources, and allow for rapid dissemination of findings.

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,[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 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.[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 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;[47-56] treatment of those STIs reduced HIV transmission.[57,58] 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%

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.[59,60] 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.[18,61,62] Major limitations of syndromic management include: 1) non-determination of infectious etiologies, 2) limited specificity, especially during pregnancy, of "symptoms" algorithms, and 3) inappropriate treatment or over-treatment.[62,63] 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.[64-66] 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,[67,68] 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.[19,69-73] 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*[74] 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. 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.[75,76] At the first test-of-cure, 26.5% were persistently positive; a number of women required multiple rounds of treatment before clearing their infection (Table 2). Interviews with women suggest that behaviors associated with poor treatment adherence or re-exposure from untreated partners cannot fully explain the high persistent positivity with CT or TV.[77] 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.[28,77-79] Gatski *et al.*[28] 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 exceedingly rare.[80] Reports have suggested that heterotypic resistance associated with high organism loads may factor in persistent infections; however, the evidence is limited.[80-83] 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.

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%)

Vaginal microbiota may play an important role in STI treatment outcomes and an important role in genital CT infections.[84-86] Epidemiological studies have demonstrated that BV is associated with an increased risk of acquiring and transmitting HIV and other STIs.[87-95] 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*,[21,96-98] and that these CSTs are associated with STIs such as CT and TV.[22,23,30] 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.[99] 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.[85] Indole-producing bacteria (e.g., *Prevotella* spp,[85] *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 hybrid type 1 effectiveness-implementation study design: 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 an implementation science trial is to **simultaneously combine the collection of effectiveness and implementation-relevant data**. Toward this end, we will conduct a hybrid type 1 effectiveness-implementation design study,[100] which allows the primary focus to be on collecting data on the effectiveness of our intervention, while also incorporating process evaluation methods into our effectiveness trial. This will help us to explain our effectiveness results and efficiently inform future implementation.

2) Investigating clinical- and cost-effectiveness of routine CT/NG/TV testing of pregnant women: Our study will inform global health practices regarding STI screening during pregnancy, especially among high HIV prevalence populations. We will also assess the effectiveness of routinizing diagnostic testing, with same-day test results and treatment, for in reducing adverse birth outcomes due to these STIs. There have been no RCTs in low and middle-income countries that have evaluated the costs and benefits of diagnostic CT/NG/TV testing and treatment during pregnancy as it relates to birth outcomes. Our cost/cost-effectiveness study has the potential to influence global health policy. If successful, this study would provide reproducible cost-effectiveness analysis models for countries to better plan for and implement routine STI testing and treatment in pregnancy.

3) Prospectively investigating associations between the vaginal microbiome and antibiotic treatment outcomes for STIs: Persistent CT and TV infections not associated with poor medication adherence, re-exposure/ re-infection or drug resistance have been reported.[28,79,81,101-103] Studies have suggested a role for the vagina microbiome in STI persistence, yet to our knowledge, none have prospectively investigated the role of the vaginal microbiome. Our study will longitudinally collect vaginal specimens from both HIV-infected and uninfected women 1) before, during and after antibiotic treatment for STIs, and 2) from those with successful treatment outcomes and treatment failures. This design will allow us to investigate the potential impact of the vaginal microbiome on STI persistence. If specific CSTs are found among pregnant women with persistent *C. trachomatis* infection, these data could be used to identify bacteria that interfere with azithromycin (i.e., CT treatment) and lead to possible alternatives to azithromycin (or co-treatment). Future studies may include trials of adjunctive treatment targeting specific bacteria or CSTs, designed to reduce cost and patient burden.

4) Vaginal microbiome data analysis: Numerous methods are use for sequencing and bioinformatics analysis of vaginal microbiome data.[104] Comparability studies of research methods for 16S rRNA gene sequencing and analysis have been performed by our group[105] and others.[106] Research by our group found that the bioinformatics pipelines to be used by the Taylor lab in Aim 3 (i.e., DADA2,[107] Ribosomal database project (RDP) classifier,[108] and Silva v132 database[109]) provide accurate classification of vaginal bacteria down to the species level. The Taylor lab has also developed methods to visualize changes in the vaginal microbiota over time, including graphic display of microbiome changes via longitudinal heat maps and analysis of CST changes.[110]

APPROACH

Study Setting: This study will take place in Tshwane District, Pretoria, South Africa. Study participants will be recruited from three large ANC clinics (Table 3) located in the referral zone of two **maternal obstetric units (MOUs)**; Kalafong Hospital and Laudium Community Health Centre. Our ANC study clinics and two hospital MOUs were selected due to their association with the South African Medical Research Council's (SA-MRC) Maternal and Infant Health Care Strategies Research Unit (MIHCSRU), directed by **co-I Pattinson**. Kalafong Hospital is co-located with the MIHCSRU and is one of the University of Pretoria's academic hospitals. The MIHCSRU and Kalafong Hospital are two of Africa's leadings centers for maternal-infant health research, with significant research funding and outputs (see Pattinson Letter of Support). The MIHCSRU regularly conducts studies within the two hospital MOUs and catchment area clinics; staff in the two study MOUs are well-trained to complete medical records and optimally collect factors related to birth outcomes consistent with high caliber research (see Dr. Pattinson's biosketch). Ultimately, the selected study sites are outstanding locations in which to conduct this study. Study clinics and MOUs are proximal to and provide care for persons living in informal settlements and lower SES communities. Key ANC indicators for our study clinics are shown in Table 3.

Facility Name	Annual ANC 1st visit headcount	Ave. Monthly 1st ANC Head Count	Annual ANC HIV Prevalence	New HIV diagnosis at 1st ANC (Annual)
Laudium Clinic	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, July 2016 – June 2017

Research Team: Details of the expert team may be found in the biosketches, and in the human subjects attachment highlighting the *Overall Structure of the Study Team*. 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 (2015-2018) from NICHD directly informs this new proposal and resulted in 25 scientific abstract presentations, six publications and additional three recently submitted articles in review.

Preliminary Studies in Support of Aim 1 (All from Medina-Marino/ Klausner NIH R21HD084274):

1) 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 CT/NG/TV testing using self-collected vaginal swabs, 430 accepted screening (Acceptability= 97.3%).[111] 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 enrollment and implementation fidelity.*

2) 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 (CT= 26.5%; TV= 16.7%; NG= 6.3%).[112] 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

behaviors associated with re-infection or poor medication adherence cannot account for the high persistent positivity after treatment.[113] 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.*

3) 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%).[112] *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.*

4) 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.*

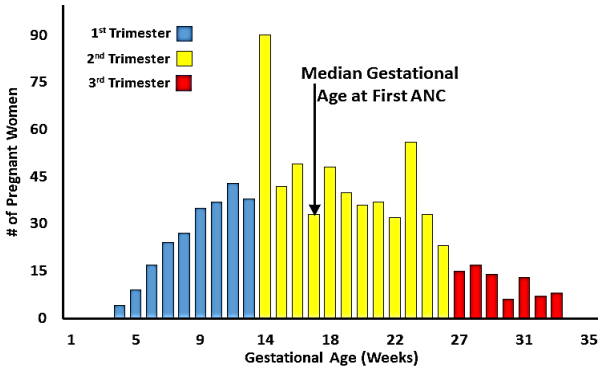

Figure 1: Gestational Age at First ANC Visit among HIV-infected Pregnant Women, Tshwane District, South Africa

5) Gestational age at first ANC and MTCT of STIs: Median gestational age was 17 weeks (IQR 12-22 weeks; Figure 1). In sub-analysis of 430 intervention arm women, enrolling in ANC during the 3rd trimester was associated with a higher prevalence of any STI compared to those who enrolled earlier.[19] Neonates born to mothers who enrolled for ANC during the 3rd trimester had significantly higher risk of nasopharyngeal colonization with maternal STI organisms compared to those whose mothers enrolled earlier (aPR=2.56; 95% CI: 1.22 – 5.38). *Those findings support our decision to not include gestational age as inclusion/ exclusion criteria.*

Preliminary Studies in Support of Aim 2:

6) Cost-effectiveness modeling 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.[114] 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.*

Preliminary Studies in Support of Aim 3:

7) Vaginal microbiome of HIV-negative South African women (Meiring; SA-NRF 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-negative 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; 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 PECAN[110] 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.[110] *Novel methodologies from this study will be incorporated into Aim 3.*

9) Consequences of the vaginal microbiota on IFNγ-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 CT clearance are 16S rRNA gene sequenced, targeting the V4 region, and DADA2 pipeline processed and taxonomy is assigned using the RDP classifier[115] and silva version 128 database.[116] Preliminary results show a prevalence of indole-producing microbiota in the vaginal microbiome of women with persistent CT 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 CT infections.*

10) Effect of BV on CT organism load and treatment outcomes (Muzny; U19AI113212). We are investigating the relationship of BV with 1) CT organism load, and 2) time to CT DNA clearance after treatment with 1g azithromycin in non-pregnant CT-infected women. To date, 17 CT-infected females have been assessed. We have found a general trend towards a longer median CT 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 CT load (p=0.0352), with a trend in higher Nugent score correlating with higher baseline log10 CT load (p=0.114). *Those preliminary data suggest that women with altered vaginal microbiota take longer to clear their CT infection, supporting our aim to investigate the role of the vaginal microbiome in persistent CT infection among pregnant women.*

METHODOLOGY AND STUDY AIMS

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

Aim 1 (Figure 2) will achieve three main sub-aims: **1(a)**: compare the effectiveness of multi-timed PoC diagnostic screening (Arms 1+2 Treatment Groups) to one-time diagnostic screening (Arm 3; Active Control) in reducing the frequency of adverse birth outcomes (e.g., preterm delivery, low birth weight, stillbirth/miscarriage); **1(b)**: compare the effectiveness of single point-in-time diagnostic screening with targeted treatment plus test-of-cure (Arm 1 Treatment Group) *vs* repeated diagnostic screening throughout ANC and treatment without test-of-cure (Arm 2 Treatment Group) in reducing prevalent and incident STIs at time of delivery; **1(c)** collect process measures to inform future implementation and scale-up.

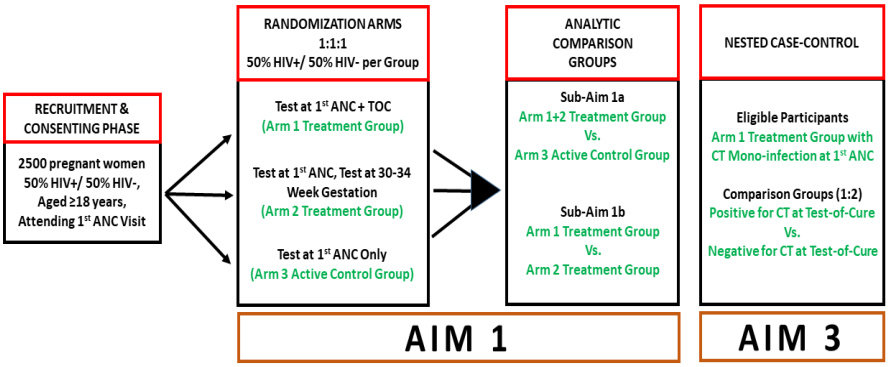

Figure 2: Study Diagram for Aim 1 Randomization Arms and Analytic Comparison Groups, and Aim 3 Nested Case-Control Study

To achieve Aim 1, we will conduct an effectiveness-implementation hybrid type 1 three-arm RCT, with **individual participants randomized (1:1:1)** from within each clinic to one of the following arms: **Arm 1 Treatment Group:** single point-in-time molecular PoC diagnostic screening and treatment for CT, NG and TV 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 and requested to return every 3 weeks for follow-up visits until a negative test-of-cure result or birth outcome is documented. **Arm 2 Treatment Group:** repeated molecular PoC diagnostic screening and treatment for CT, NG and TV at first ANC visit and week 30–34 gestation. No test-of-cure will be conducted for women with positive test results. **Arm 3 Active Control Group:** one-time diagnostic screening at first ANC visit, with targeted treatment but no follow-up ToC or repeat testing. Arms 1 and 2 are the intervention arm, Arm 3 is the comparison arm.

Of particular note, syndromic management is the standard of care in all low and middle-income countries. However, our previous work revealed that 64.9% of women were asymptomatic, thus leaving a large proportion of pregnancies and infants at risk for an adverse outcome from STIs. As such, the equipoise of retaining syndromic management standard care as the comparison arm necessitates an active control that includes once off diagnostic testing at first ANC visit (Arm 3).[117,118]

Recruitment and Eligibility: We will recruit 1250 HIV-infected and 1250 HIV-uninfected pregnant women presenting for ANC services at our 3 study clinics in Tshwane District, South Africa. Eligibility criteria: 1) Age ≥18 years, 2) Currently pregnant, 3) Attending first ANC visit for 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, as a substantial proportion (30%) of South African women enroll for ANC late in pregnancy (Fig 2). Further, inclusion of pregnant women across gestational ages will enable us to assess optimal timing for screening to prevent adverse birth outcomes.

All pregnant women will be screened for eligibility by study staff following standard HIV testing per South African National Guidelines.[119] Study staff will be trained in the study's methods, protocol, and human subjects research, and will receive training on South Africa's syndromic management algorithms for STIs. Staff will read all eligible women a brief study description. Interested women will then be read aloud, 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 12 with a 1:1:1 randomization into the 3 study arms. Prior to enrollment, each clinic will be provided two unique simple random allocation lists in Microsoft Excel, one for HIV-infected participants and one for HIV-uninfected participants; each study arm will be composed of 50% HIV-infected participants (purposive enrichment). While the impact of our intervention on prevalent STIs at time of delivery should be valid regardless of HIV-infection status, work by our group has shown maternal HIV infection is associated with increased adverse birth outcomes regardless of antiretroviral therapy (ART), CD4 count, or HIV viral load.[120] Given the complex interplay between HIV status and adverse birth outcomes, and the fact that approximately one-third of pregnant women in South Africa are HIV-infected, it is essential to demonstrate the impact and investigate the effect size of our proposed interventions on adverse birth outcomes among both HIV-infected and un-infected women.

Staff will record reasons for ineligibility/refusal. Basic de-identified information (i.e., age, gestational age, HIV/ART status) will be collected from clinic logs for descriptive analysis of the general ANC patient population.

Data Collection at Enrollment/First ANC: Trained study staff will administer an audio-computer assisted self-interview (ACASI)-based questionnaire to all participants. The ACASI 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: 1) 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,[121] 4) partner characteristics and HIV status,[122,123] 5) knowledge and previous history of STIs, and 6) screenings for depression,[124,125] substance abuse,[126] interpersonal violence and social support. Staff will translate questionnaires into the major local languages (i.e., Sepedi, Setswana, Zulu, and Ndebele). Participants may select their preferred language for 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 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, 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 South African NHLS 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: 2 swabs for STI testing, 1 swab for microbiome analysis (Aim 3), and 1 swab for bio-banking (NOTE: all pregnant women from our recently completed study found it acceptable and feasible to collect up to four vaginal swabs at a visit). 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.[127] If a participant is not comfortable with self-collecting a vulvo-vaginal swab 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*). 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 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 stored at 2-8°C and transported to **Dr. Peters** (co-investigator; Dept. of Microbiology, University of Pretoria) on a bi-weekly basis according to Good Laboratory Practice. Specimens will be flash frozen and stored at -80°C for bio-banking. Frozen specimens will be shipped quarterly for microbiome processing and analysis to University of Cape Town.

Diagnostic Testing: Vaginal specimens collected from participants will be tested for CT, NG and TV using the Xpert® CT/NG and Xpert® TV assays [Cepheid, Sunnyvale, CA]. Trained staff (STI Test Counselors 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 CT and NG, while Xpert® TV provides 60 min detection of TV; both test cartridges have high sensitivity and specificity[127] 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.

Testing, Reporting and Treatment: The GeneXpert systems consist of an instrument, computer, and preloaded software for running tests and displaying results. STI Test Counselors will report all test results to the ANC Research Nurse embedded within study clinics. Research nurses provide test results notification, treatment, partner treatment counseling and treatment packets to STI-infected participants per National STI treatment protocols.[128,129] Arm 1 and 2 participants will be provided same day results and immediate treatment. Arm 3 participants will be provided results and treatment at their routine follow up ANC visit; reporting of results and provision of treatment at a woman's 2nd routine ANC is in line with South African guidelines for syphilis test result reporting and treatment provision, thus better approximating a likely future scenario.[128,129]

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 the option to either request their partner(s) present to a 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; in lieu of the recommended intramuscular injection of ceftriaxone for NG infections, which would require a male partner to present to a clinic, WHO and South African National Guidelines recommend oral Cefixime 400mg tablet/ azithromycin 1gm oral to be administered for NG infection.[128,130] 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 R21 study. Several mechanisms will be used to ascertain that partners sought care or actually took medication provided via partner pill packets: 1) women will complete a questionnaire during the test-of-cure visit, with questions about whether their partner(s) sought care at a clinic or swallowed pills from the treatment packet, 2) partner referral letters will detail a fast track servicing by research nurses should they wish to receive STI treatment at one of the three study facilities, and 3) participants consent to study staff contacting their partner, and the partner verbally consented to a brief telephonic interview regarding STI treatment behaviour. Partner interviews will include: 1) assessment of disclosure dynamics; 2) determination of receipt and self-administration of partner treatment packet; 3) preference for partner treatment packet vs. attending clinic for care; 5) knowledge, attitudes, practices regarding STIs; and 6) STIs in their pregnant partner and their own health. Characteristics of contacted partners may be biased given that women who provide consent for contacting may have differential partnership dynamics indicative of particular health behaviors in these partners.

Arm 1 Specific Activities: Per Table 4, at first ANC visit, participants randomized to Arm 1 will collect four vaginal swab specimens as described above (*Specimen Collection*). Two specimens will be used for pH, CT/NG and TV testing, and two for bio-banking. 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

Table 4: STI Testing Schedule Per Randomization Arm

Clinic Visit	Participant	Specimen Collected	CT, NG and TV Testing
First ANC Visit	**All Pregnant Women**	**Vaginal Swabs**	**All Arms**
ToC 3-Weeks Post-treatment	**Arm 1 Only**	**Vaginal Swabs**	**Arm 1 Only**
30 – 34 Weeks Gestation	**Arm 2 Only**	**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 Swab**	**All Infants***

***Post-delivery maternal and infant swabs will be batch tested at the end of the study**

which they were treated). At the ToC visit, women will again self-collect vaginal specimens for STI ToC and bio-banking. Women with positive ToC will again be treated (and given partner treatment packet) and asked to return 3 weeks later for another ToC; ToC will be repeated until negative test result or documented birth outcome.

Arm 2 Specific Activities: Per Table 4, at BOTH at first ANC visit and during ANC visit occurring between 30-34 weeks gestation, participants randomized to Arm 2 will collect four vaginal swab specimens; two for pH, CT/NG and TV testing, and two for bio-banking. No ToC activities will be performed for Arm 2 participants.

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; two for pH, CT/NG and TV testing, and two for bio-banking. Reporting of test results and provision of treatment (self and partner) for those with a positive STI result will be provided at a women's next routine ANC visit.

Retention and Follow-up: 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/neighbor). 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 monthly ART pickup for those with HIV. 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.

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) Enrollment tracking sheets 2) Enrollment 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
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.*[121])

Post-partum and Infant Specimen Collection: During the first postnatal visit (typically 3-6 days post MOU discharge), four vaginal swab specimens will be collected from all post-partum women and two nasopharyngeal (NP) swabs specimens will be collected from all infants. Specimens will be labeled with random specimen IDs that link to participant IDs. Specimens will be transported to the Univ. of Pretoria and stored as previously described. Vaginal and NP swabs will be batch tested using Xpert® CT/NG and Xpert® TV assays at study end. Test results from all participants will be used specifically for study outcomes, not clinical management.

Data Collection at Postnatal Clinic Visit: We will collect data on pregnancy and birth outcomes from all study participants via abstraction of labor/postnatal ward clinical records and face-to-face interviews with participants during the first postnatal clinic visit. All clinical data relating to labor, 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 labor 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 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 NHLS database. At the routine 6-week immunization visit, we will assess for neonatal health outcomes and morbidities (or mortality) (i.e., 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 provide assistance to attend clinic. If neonatal mortality is identified, a verbal autopsy will be

performed, and death will be confirmed via medical records. A study supervisor will perform weekly reviews to ensure data completeness and validity; discrepancies will be resolved via interview with the birth attendant.

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**[131-133] 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).

Data Analysis: Data will be analyzed using R [R Foundation for Statistical Computing, Vienna, Austria] and SAS 9.4 [Cary, North Carolina]. Participant demographic and clinical characteristics will be described per study arm using proportions (categorical variables), as well as measures of central tendency (sample mean, sample median) and dispersion (sample variance, interquartile range) for continuous variables. Outcome difference among treatment arms will be assessed for statistical significance using Chi-square tests and logistic regression models for categorical/binary outcomes. Analysis of Variance (ANOVA) and multiple linear regression models will be used for continuous outcomes. Normal probability plots will be used to access the normality assumption for ANOVA and multiple linear regression models. If the normality assumption appears violated, non-parametric procedures will be utilized. Within Arm 1, we will use 95% confidence intervals for proportions to estimate the percent of women with a negative ToC, but with an STI at birth outcome. These confidence intervals, calculated by HIV status as well as pooled across HIV status, will allow an estimation of the percent of STI prevalence at birth outcome which is due to new infections between ANC visits. 4) 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.

All analyses will be conducted using intent-to-treat principles. Overall Type I error rate will be set at 0.05; for multiple comparisons among study arms Type I error will be set to a Bonferroni-corrected Type I error of 0.01667. We will use multiple imputation of missing data when missing values exceed 10%, and will conduct sensitivity analyses to determine how imputed data affects the study results.

Primary Outcomes to be compared among study arms, adjusted/controlling for HIV status include: 1) frequency of adverse birth outcomes (sub-Aim 1a) and 2) change in STI prevalence between baseline (1st ANC) and birth outcome). 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 generalized estimating equations to test for variation among study arms with regard to change in prevalence of CT/NG/TV between baseline and delivery, adjusting for potential effect modifiers and confounding variables. Secondary Outcomes: 1) prevalence and risk factors for CT, NG, and TV colonization in neonates controlling for HIV status; 2) among mothers, the prevalence and risk factors for STI infection at birth outcome, 4) factors associated with STIs at first ANC; and 5) process evaluation measures as described in Table 5. Exploratory Outcomes: 1) type and frequency of adverse birth outcomes as a function of STI and HIV status; 2) infant outcomes, including pneumonia and neonatal conjunctivitis, at 6 weeks.

Development of persistent STI risk score calculator: We will use a predictive modelling approach to develop a STI risk calculator.[134] To assure model utility, we will select variables that are readily available to clinicians *a priori*. Model building will utilize 10-fold cross validation where the data is randomly divided into 10 datasets. For each model fitting iteration, 9 of the datasets will be used to fit the model. This resulting model will then be used to predict outcome in the 10th dataset. The final model will be a weighted average of the models observed in each of the 10 cross-validation steps. Weights will be assigned based upon observed degree of fit with models exhibiting higher degree of fit (better prediction) receiving higher weights. To assess external validity of the model, the model will be applied to the dataset from our prior study (R21HD084274). Risk calculators will be developed for any STI as well as separately for CT, NG, and TV.

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 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.[120,133,135] 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, 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 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 cost per pregnant woman screened and treated, cost of adverse birth outcomes, and cost-effectiveness per STI and DALY averted*.*

Rationale: While Aim 1 will determine the efficacy of screening interventions in improving birth outcomes for pregnant women, Aim 2 will assess whether Arms 1 and/or 2 are cost-effective in comparison to Arm 3, from the 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 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.[120,135] 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.[120] 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.[136] 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: 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.[137,138] 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.[139] 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 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.[120]

Potential Challenges: 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. If necessary, we will extend our follow-up of these infants beyond 6 weeks postpartum and will collect newborn 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.[140-144]

Specific Aim 3. Investigate the relationship between the vaginal microbiome and persistent Chlamydial infections 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 (Figure 2). We will accomplish two main sub-aims: 3(a): determine the impact of vaginal microbiota on CT treatment outcomes; and 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 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.[145-147]

Specimen collection, handling and shipping: The Laboratory of co-I Peters will use the swab collected for bio-banking to smear a glass slide for Nugent score and determination of BV prior to its storage.[148] 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, provided a partner treatment packet 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.

Nugent scoring for BV: Air-dried slide smears will be heat-fixed and Gram stained per standard procedure.[149] Nugent scores (0-3: normal, 4-6: intermediate and 7-10: BV flora[148]) 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 along with weekly vaginal swab specimens from "cases" who remained persistently CT positive 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 RDP classifier and silva version 128 database for precise assignment of taxonomy. Phyloseq[150] and QIIME[136] 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.[151]

Estimated effective sample size: Based on 834 pregnant women randomized to Arm 1 (see *Sample Size Calculations*), and a 30% CT prevalence among pregnant women (Table 1), ~246 CT infected women will be included in Aim 3. Considering 26.5% of CT-infected women had a positive ToC (Table 2), we anticipate approximately 65 "cases" and 130 "controls" (1:2 match). Furthermore, given that 7.9% of CT-infected women may still be positive for CT at the second test of ToC (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, vaginal pH and other clinical data. We will compare the relative abundance of microorganisms between cases and controls to determine which organisms are associated with persistent CT infection in pregnant women. Several statistical methods have been proposed to evaluate differential abundance in microbiome data (DESeq, DESeq2, and Voom).[152-154] We propose to use the DESeq2 method, which is based on the negative binomial Wald test, as it provides increased sensitivity and several desirable characteristics compared to other competing methods.[155] Data will be analyzed at 4 time points, correlating to specimen collection (see above). Preliminary analysis at each time point will account for individual effects of different microbiota at different study stages, and to understand any time/environment-specific differences in microbiome composition over time. CSTs will be constructed using linkage clustering of microbiome species data. Given the repeated measurements for each participant and the longitudinal nature of this aim, the primary analytic method for continuous outcome measures will be linear mixed models. Normality assumptions will be accessed using normal probability plots. For binary outcomes, generalized estimating equations will be used. Covariates for all models will be HIV status, presence/absence of specific community states, vaginal PH, and demographic variables; covariates affecting the microbiome (e.g. CD4 count, ART exposure) will be included to assess their effect on treatment success rates. We will also use linear and generalized linear mixed models to detail the effects of individual microorganisms on CT treatment. 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.[156] 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 3 (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, E. 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.[157] 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.[143] 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, co-I 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 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%.

Regarding aim 3, we assume 65 cases and 130 controls will provide four vaginal swabs allowing us to study the longitudinal association of vaginal microbiome characteristics and changes with persistent CT infection. Given the repeated observations within an individual, the non-independence of observations within a subject must be accounted for in the 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, an odds ratio of 1.97.

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