BMJ Open

Impact of preconception vaginal microbiota on women's risk of spontaneous preterm birth: Protocol for a prospective case-cohort study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2019-035186
Article Type:	Protocol
Date Submitted by the Author:	23-Oct-2019
Complete List of Authors:	Lokken, Erica; University of Washington, Mandaliya, Kishorchandra ; University of Nairobi Srinivasan, Sujatha ; Fred Hutchinson Cancer Research Center, Vaccine and Infectious Diseases Division Richardson, BA; University of Washington, Department of Biostatistics; University of Washington, Department of Global Health Kinuthia, John; Kenyatta National Hospital, Research and Programs; Kenyatta National Hospital, Obstetrics and Gynaecology Lannon, Sophia; Northwest Perinatal Jaoko, Walter; University of Nairobi, Medical Microbiology Alumera, Hudson; University of Nairobi School of Dental Sciences Kemoli, Arthur; University of Nairobi School of Dental Sciences Fay, Emily; University of Washington, Obstetrics and Gynecology John-Stewart, G; University of Washington, Departments of Epidemiology, Global Health, and Medicine Fredricks, DN; Fred Hutchinson Cancer Research Center, Vaccine and Infectious Diseases Division; University of Washington, Medicine McClelland, Scott; University of Washington, Departments of Epidemiology, Global Health, and Medicine
Keywords:	Epidemiology < INFECTIOUS DISEASES, OBSTETRICS, MICROBIOLOGY

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Impact of preconception vaginal microbiota on women's risk of spontaneous preterm birth: Protocol for a prospective case-cohort study

Authors:
Erica M Lokken[1]
Kishorchandra Mandaliya[2]
Sujatha Srinivasan[3]
Barbra A Richardson[4,5]
John Kinuthia[6,7]
Sophia Lannon[8]
Walter Jaoko[9]
Hudson Alumera[10]
Arthur Kemoli[10]
Emily Fay[11]
Grace John-Stewart[1,5,12]
David N Fredricks[3,12]
R Scott McClelland[1,5,12]

Affiliations: Department of Epidemiology, University of Washington, Seattle, WA[1]; University of Nairobi Institute of Tropical & Infectious Diseases (UNITID), Nairobi, Kenya[2]; Vaccine and Infectious Diseases Division, Fred Hutchinson Cancer Research Center, Seattle, WA[3]; Department of Biostatistics, University of Washington, Seattle, WA[4]; Department of Global Health, University of Washington, Seattle, WA[5]; Research and Programs, Kenyatta National Hospital, Nairobi, Kenya[6]; Obstetrics and Gynaecology, Kenyatta National Hospital, Nairobi, Kenya[7]; Northwest Perinatal, Portland, OR[8]; Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya[9]; School of Dental Sciences, University of Nairobi, Nairobi, Kenya[10]; Department of Obstetrics and Gynecology, University of Washington, Seattle, WA[11]; Department of Medicine, University of Washington, Seattle, WA[12]

Corresponding Author:
Scott McClelland
University of Washington
Box 359909
325 9th Avenue
Seattle, WA 98102
Tel: 206-543-4278
Email: mcclell@uw.edu

Word Count-Abstract: 293/300
Word Count-Main Text: 3994/4000

ABSTRACT

INTRODUCTION

Bacterial vaginosis (BV) and vaginal microbiota disruption during pregnancy are associated with increased risk of spontaneous preterm birth (SPTB), but clinical trials of BV treatment during pregnancy have shown little or no benefit. An alternative hypothesis is that vaginal bacteria present around conception may lead to SPTB by compromising the protective effects of cervical mucus, colonizing the endometrial surface before fetal membrane development, and causing low-level inflammation in the decidua, placenta, and fetal membranes. This protocol describes a prospective case-cohort study addressing this hypothesis.

METHODS AND ANALYSIS

HIV-seronegative Kenyan women with fertility intent are followed from preconception through pregnancy, delivery, and early postpartum. Participants provide monthly vaginal specimens during the preconception period for vaginal microbiota assessment. Estimated date of delivery is determined by last menstrual period and first trimester obstetrical ultrasound. After delivery, a swab is collected from between the fetal membranes. Placenta and umbilical cord samples are collected for histopathology. Broad-range 16S rRNA gene PCR and deep sequencing of preconception vaginal specimens will assess species richness and diversity in women with SPTB versus term delivery. Concentrations of key bacterial species will be compared using quantitative PCR (qPCR). Taxon-directed qPCR will also be used to quantify bacteria from fetal membrane samples and evaluate the association between bacterial concentrations and histopathological evidence of inflammation in the fetal membranes, placenta, and umbilical cord.

ETHICS AND DISSEMINATION

This study was approved by ethics committees at Kenyatta National Hospital and the University of Washington. Results will be disseminated to clinicians at study sites and partner institutions,

presented at conferences, and published in peer-reviewed journals. The findings of this study could shift the paradigm for thinking about the mechanisms linking vaginal microbiota and prematurity by focusing attention on the preconception vaginal microbiota as a mediator of SPTB.

ARTICLE SUMMARY

STRENGTHS AND LIMITATIONS OF THIS STUDY

- This prospective case-cohort study enrolls Kenyan women with fertility intent, enabling follow-up and exposure measurement from preconception through pregnancy and the early postpartum period.
- Monthly specimen collection during the preconception period allows for examination of the vaginal microbiota close to the time of conception.
- Fetal membrane swabs and placental samples are collected at delivery, enabling assessment of the association between detection and concentrations of bacteria in the fetal membranes, inflammation, and preterm birth.
- A combination of broad-range 16S rRNA gene PCR with next generation sequencing and quantitative PCR assays provide both the relative and absolute quantities of bacteria in vaginal secretions and in the fetal membranes.
- Because this is an observational study, it cannot definitively establish a causal relationship between vaginal bacteria and preterm birth.

INTRODUCTION

Globally, approximately 10% of births are preterm (<37 weeks of gestation), but the prevalence can be as high as 18% in low-resource countries [1]. Preterm birth and its sequalae are the leading causes of death among children under five [2]. The majority of preterm deliveries are spontaneous preterm births (SPTB), and occur with or without preterm premature rupture of membranes (PPROM) [3]. The cause of SPTB is often unknown, but up to 40% may be associated with intrauterine infections [3,4]. Other maternal and fetal risk factors include other infections (urinary tract, sexually transmitted, systemic), socio-demographic characteristics (age, race, education) [3], extremes of body mass index [5], periodontal disease [6], pregnancy history (inter-pregnancy interval <6 months, prior SPTB), stress, depression, and smoking [3]. Further elucidation of the causes of SPTB may provide insight into novel approaches for reducing preterm birth risk.

Bacterial vaginosis (BV) is a common vaginal condition characterized by a shift from an optimal *Lactobacillus*-predominant vaginal microbiota to one characterized by high concentrations of diverse anaerobic species [7]. Numerous studies indicate that BV during pregnancy is associated with increased risk of SPTB [8]. However, a meta-analysis of clinical trials concluded that while antibiotic treatment prior to 20 weeks gestation was efficacious for eradicating BV, the risk of SPTB was not substantially reduced [9].

Molecular microbiology has transformed the understanding of vaginal microbiota to a much broader spectrum of phenotypes ranging from low-diversity *Lactobacillus*-dominated bacterial communities to a heterogeneous group of high-diversity BV-associated communities [10,11]. Studies exploring the relationship between the vaginal microbiota and SPTB have yielded conflicting findings. Some found significant associations between increased species diversity and preterm delivery [12–16], while others have not [17–19]. In addition, some studies have found that women with a low relative abundance of *Lactobacillus* species and higher relative abundance of

BV-related taxa such as *Gardnerella vaginalis* may be at higher risk of preterm birth [16,19–22]. Others found no association between vaginal bacterial community type and preterm birth [12,14,17]. Detection and higher concentrations of specific vaginal bacteria have also been associated with preterm delivery including *Leptotrichia/Sneathia* [16,23], BV-associated bacterium 1 (BVAB1) [16,23], *Megasphaera* [23], *G. vaginalis* [24,25], *Atopobium vaginae* [24], TM7-H1 [16], and some *Prevotella* species [16].

No previous study has addressed the hypothesis that the preconception vaginal microbiota is associated with SPTB. Bacteria present around the time of conception could compromise the protective effects of cervical mucus [26], gaining access to the endometrium before fetal membrane development [27]. These bacteria could colonize and cause low-level inflammation in the decidua, placenta, fetal membranes, or amniotic cavity [28,29]. The Microbiota and Preterm Birth Study (MPTB) was established to enroll HIV-negative Kenyan women trying to become pregnant into a prospective case-cohort study with frequent vaginal fluid sampling to address the following aims:

1) Compare the species diversity and richness of the vaginal microbiota sampled close to the time of conception in women with SPTB versus term delivery using broad-range 16S rRNA gene PCR and next generation sequencing.
2) Compare the presence and concentrations of select bacterial genera/species (based on published data [23] and results of Aim 1) using targeted qPCR assays in vaginal specimens sampled close to the time of conception in women with SPTB versus term births.
3) Perform species-specific qPCR assays on samples collected from fetal membranes and histological examination of membranes and umbilical cord to determine if vaginal bacteria ascend to the upper genital tract and cause inflammation in the fetal membranes and umbilical cord.

METHODS AND ANALYSIS

STUDY DESIGN, SETTING, AND TIMELINE

The MPTB Study is a prospective case-cohort study. Eligible women enroll into a prospective cohort prior to conception and are followed through preconception, pregnancy, delivery, and until six-weeks postpartum; participants will be selected into the case-cohort sample. Study sites are located at Kenyatta National Hospital (KNH) in Nairobi and at Ganjoni Health Center and Coast Provincial General Hospital (CPGH) in Mombasa. Enrollment began in Nairobi in April 2017 and Mombasa in April 2018. Enrollment will continue through approximately June 2019, with final deliveries expected in 2021.

ELIGIBILITY CRITERIA AND RECRUITMENT STRATEGIES

The target population is HIV-negative women who are currently planning to become pregnant. Additional eligibility criteria include being ≤45 years old, having had a menstrual period in the prior three months or recently discontinued contraceptive methods that induce amenorrhea (implant, hormonal intrauterine device [IUD], depo medroxyprogesterone acetate injectable [DMPA]), willing to comply with study procedures, and able to provide informed consent. Minors aged 14-17 are eligible if emancipated under Kenyan law. Exclusion criteria include current pregnancy, continuing contraception other than condoms for HIV/STI prevention, having a DMPA injection in the last three months, history of cervical or uterine surgery other than colposcopy, cryotherapy, loop electrosurgical excision procedure, or cesarean section, known autoimmune disease, antibiotic use in the prior four weeks, and history of infertility care-seeking. For women with known HIV-positive male partners, their partner must have a documented undetectable HIV viral load, or the participant must be taking pre-exposure prophylaxis.

Participants are recruited by study staff or referred by healthcare providers at sites providing reproductive or maternal health services. Women discontinuing contraception for the purpose of becoming pregnant are a key recruitment population.

STUDY VISITS AND PROCEDURES

Study participation occurs in six phases including screening/enrollment, periodontal examination, preconception, pregnancy, delivery, and postpartum (Figure 1). Specimen collection, laboratory testing, and other clinical procedures are summarized in Table 1.

Screening and enrollment

Following written informed consent for screening, study staff perform urine pregnancy testing and rapid HIV testing according to Kenyan guidelines [30]. An eligibility interview is conducted in English or Kiswahili. Eligible women can enroll immediately upon providing consent. If enrollment does not occur on the screening day, the participant provides another urine sample for pregnancy testing to re-confirm eligibility prior to enrollment. Enrollees complete a structured face-to-face interview regarding demographics, sexual behavior, substance use, depression symptoms (Patient Health Questionnaire-9), and reproductive, contraceptive, and medical history. A study clinician performs a general physical examination and speculum-assisted pelvic examination. If a woman is menstruating, the examination is deferred to avoid sampling during this interval when the vaginal microbiota undergoes rapid changes [31,32]. Two vaginal fluid specimens are collected by rolling push-off Dacron swabs (FitzCo, Inc) three rotations against the lateral vaginal wall; these are stored for vaginal microbiota and inflammatory response evaluation. Additional genital specimens are collected for STI diagnosis [*Neisseria gonorrhoeae, Chlamydia trachomatis,* and *Trichomonas vaginalis* by nucleic acid amplification testing (NAAT)], vaginal and cervical Gram stains, detection of prostate specific antigen (PSA) and elevated sialidase using a

point-of-care diagnostic test for BV (Diagnosit BVBLUE, Gryphus Diagnostics). A vaginal specimen is inoculated onto Rogosa agar for detection of *Lactobacillus*.

Syndromic management for STIs is provided following WHO and Kenyan guidelines [33]. Additional therapy is provided at the first preconception visit based on STI NAAT results. Symptomatic BV is treated according to standard of care [33].

Women receive counseling on healthy behaviors during preconception and pregnancy including smoking cessation, refraining from vaginal washing, and a healthy diet. Study clinicians discuss participants' menstrual cycle and identify the probable fertile window using calendar-based methods. Ovulation is estimated to occur 14 days prior to the first day of the next predicted menses, with the most fertile days emphasized as the five days before and day of ovulation [34]. All participants receive prenatal vitamins.

Periodontal Exam

Periodontitis has been associated with SPTB [6] and oral bacteria have been detected in the placenta of women with SPTB [35]. In this study, periodontal disease is a potential confounding factor, so each participant receives a periodontal examination. Since pregnancy increases gingival inflammation [36], examinations occur within four weeks of enrollment. Participants complete a modified version of the WHO's oral health questionnaire [37] and undergo an oral examination, including assessment for periodontitis using periodontal pocket depth and clinical attachment measurements, and Decay-Missing-Filled Index and Gingival Index assessment [37]. Presence and severity of periodontitis are defined using the 2007 Centers for Disease Control and Prevention/American Academy of Periodontology case definitions [38].

Monthly Preconception Visits

Participants return at one-month intervals while trying to become pregnant. A structured interview is conducted to update sexual behavior and medical history. Women self-collect vaginal swabs (with assistance if needed) for vaginal microbiota and inflammatory response analysis, vaginal Gram stain, PSA detection, *Lactobacillus* culture, and sialidase detection. Participants with genital symptoms are treated for STIs using syndromic management [33]. Counseling is provided to reinforce messages about healthy preconception behaviors. A urine pregnancy test is performed. Women whose pregnancy test is positive are scheduled for a first trimester visit between nine and twelve weeks of gestation. Most women who remain non-pregnant after six months exit the study; those who discontinued DMPA less than six months prior to enrollment are eligible for nine months of preconception 'trying time' due to delayed return to fertility after DMPA discontinuation [39,40].

First Trimester Visit (9-12 weeks of gestation)

At the first trimester visit, a structured interview is conducted to update information on sexual behavior and medical history. Women self-collect vaginal swabs for vaginal microbiota and inflammatory response analysis, vaginal Gram stain, PSA detection, and *Lactobacillus* culture. Women undergo an obstetrical ultrasound to confirm gestational age. The ultrasound is conducted by sonographers at KNH in Nairobi and at a private radiology facility in Mombasa (training and quality control described in Table 2). The American College of Obstetrics & Gynecology's 2014 guidelines are used to estimate gestational age if the ultrasound derived estimate differs from that calculated using the last menstrual period (LMP) by more than 7 days before 16 weeks [41]. If a later ultrasound is obtained, the sonographic dates are used if they differ from LMP dates by more than 10 days between 16 and 22 weeks, more than 14 days between 22 and 28 weeks, and more than 21 days after 28 weeks.

Pregnant participants are referred to routine antenatal care (ANC) and enrolled into a short message service (SMS) program to support retention in the study (see '*Retention During Pregnancy'* section). Routinely collected antenatal data (e.g., syphilis test results, blood pressure, fundal height) are abstracted from participants' ANC facility records and from the Mother and Child Health Booklet provided to all pregnant Kenyan women.

If a participant suffers a miscarriage, management of the pregnancy loss is conducted by non-study clinicians according to standard of care. Women who miscarry prior to 20 weeks of gestation may remain in the study if they would like to try for another pregnancy. Women who choose to re-enter for a second pregnancy attempt are eligible for six additional monthly preconception visits. Women who miscarry twice are not eligible to return for a third preconception attempt.

Retention During Pregnancy

Participants are offered enrollment into an adaptation of an automated two-way SMS program that was initially designed to support HIV-positive Kenyan women during pregnancy and postpartum [42]. Messages are sent at 16, 20, and 24 weeks gestation, bi-weekly beginning at 28 weeks, and weekly from 38 weeks through six weeks postpartum. In addition, study nurses call participants weekly starting at week 35 to confirm pregnancy status and planned delivery location. Participants are instructed to call at the onset of labor so study staff can coordinate collection of delivery samples for deliveries at KNH or CPGH, and to assist with identification of the delivery date for births occurring elsewhere.

Delivery Procedures

Deliveries follow standard obstetrical procedures and are not conducted by study staff. Delivery samples are collected only for births at the Labor and Delivery (L&D) Wards at KNH and CPGH, where delivery samples are collected by trained L&D nurses (training and quality control

described in Table 2). Upon delivery of the placenta by vaginal or caesarean delivery, it is placed in a sterile container. Samples are collected as soon as feasible and within two hours of delivery. Using sterile technique, a pair of nurses collects samples from between the amnion and chorion [43]. A sterile push-off swab (FitzCo, Inc) is collected from between the membranes, placed in a cryovial, and temporarily stored in a -4°C (Nairobi) or -20°C (Mombasa) freezer in the L&D ward. The placenta is placed in 10% neutral-buffered formalin. Laboratory staff transport the fetal membrane swab to a -80°C freezer in study laboratories at KNH and CPGH within one working day. Laboratory staff also collect placental samples for histopathology. These include a four-centimeter fetal membrane roll from the ruptured edge of the membranes to the edge of the placental disc, a one-centimeter section of umbilical cord starting three centimeters from the placental disk, and a one-centimeter block of placenta with overlying membranes adjacent to the site of cord insertion. All pathological specimens are stored in 10% neutral-buffered formalin.

Study staff abstract data from L&D records, including type of delivery (i.e., vaginal or caesarean; labored or did not labor; spontaneous or induced labor), complications, live birth or stillbirth, and birthweight.

Postpartum Visit

At the six-week postpartum visit, clinicians abstract any ANC and delivery details not previously captured from the participant's delivery discharge report and Mother & Child Health Booklet. Participants complete an interview about the infant's heath and immunization status.

Incentives

Participants receive KSh 300 (about $3.00) at each study visit. Women who become pregnant receive a free obstetrical ultrasound. An incentive of 1000 KSH is provided for deliveries at KNH or CPGH.

LABORATORY METHODS

Microscopy, *Lactobacillus* culture, STI testing, and detection of sialidase and PSA in vaginal fluids

Vaginal Gram stained slides are evaluated for BV using the criteria developed by Nugent and Hillier [44]. Saline and potassium hydroxide wet mounts are examined for the presence of motile trichomonads, clue cells, yeast, and sperm. Endocervical Gram stained slides are scanned at low power, and polymorphonuclear leukocytes in three nonadjacent oil immersion fields are counted and averaged to evaluate cervical inflammation. Clinicians inoculate vaginal specimens directly on Rogosa agar for detection of cultivable *Lactobacillus* and store the plate in a candle jar until transportation to the laboratory within four hours [45]. Hydrogen peroxide (H_2O_2) production is evaluated by subculture of *Lactobacillus* isolates on tetramethylbenzidine (TMB) agar containing horseradish peroxidase [46]. A vaginal specimen is tested for *N. gonorrhoeae, C. trachomatis,* and *T. vaginalis* by NAAT (Aptima Combo-2 CT/NG Detection System, Aptima *Trichomonas vaginalis* assay; Hologic Corporation). One vaginal swab is used for detection of sialidase using a commercially available BV diagnostic test (Diagnosit BVBLUE; Gryphus Diagnostics). The minimum detectable level of sialidase is 0.25 μg, and samples above this concentration are considered positive [47]. Testing for PSA in vaginal samples is performed with a commercially available assay (ABAcard, Abacus Diagnostics), which can detect semen for 24-48 hours after condomless sex [48].

Molecular methods for identification of bacteria in vaginal and fetal membrane samples

Stored vaginal and fetal membrane swabs for bacterial PCR will be transported on dry ice to the Fredricks Laboratory at the Fred Hutchinson Cancer Research Center in Seattle, WA. Qiagen QIAamp BiOstic Bacteremia DNA Isolation Kits (Qiagen, Germantown, MD) will be used to extract DNA from vaginal swabs. This protocol uses bead beating and chaotropic lysis to break apart bacterial cells and recover DNA that is free of PCR inhibitors. Swabs that have not contacted a human surface will be processed in parallel to serve as sham DNA extraction (negative) controls. Extracted DNA will be subjected to broad-range 16S rRNA gene PCR using primers that anneal with highly conserved regions of the small subunit rDNA gene, amplifying a 470 base pair segment that contains a highly variable sequence useful for species identification. Bar coded primers will be used to multiplex samples [49]. Libraries of 16S rDNA gene amplicons will be mixed for sequencing on the Illumina MiSeq platform using 300 bp paired-end reads. The assembled reads will be binned into individual study samples using the nucleic acid bar codes. Approximately 10,000-30,000 sequence reads will be generated per sample, providing robust detection of minority species. Raw sequence reads will be demultiplexed using Illumina's on-board bclfastq conversion software v1.8.4 with zero mismatches in either forward or reverse indices. The DADA2 software package will be used to quality control, filter, pair and cluster the amplicon sequence reads [50]. Sequence variants will be assigned taxonomy using the phylogenetic placement tool *pplacer* [51] and a custom vaginal reference set [11]. Sham extraction controls will also be processed in parallel to exclude bacterial contamination of reagents. Bacterium-specific qPCR assays will also be performed. This study focuses on species hypothesized to be associated with increased risk of SPTB, such as BVAB1, *Megasphaera,* and *Sneathi*a. The final set of species/genera tested will be fine-tuned following analysis of the deep sequencing data from Aim 1, selecting additional bacteria based on a comparison of the relative abundance of individual taxa in women with and without SPTB. A standard exogenous jellyfish qPCR amplification control

will be used to assess for PCR inhibitors [52]. A broad-range bacterial 16S rDNA gene qPCR assay will be used to measure total bacterial load in each sample.

Histopathological examination of fetal membranes, placenta, and umbilical cord samples

Hematoxylin and eosin staining of fetal membrane rolls, placental samples, and umbilical cord sections will be graded according to published guidelines by an experienced pathologist (Dr. Mandaliya) [53]. Acute and subacute inflammatory lesions will be graded and staged for both maternal and fetal components. Chronic inflammatory lesions will be characterized including any observation of chronic deciduitis or the presence of decidual plasma cells.

STATISTICAL ANALYSIS

Sample size estimate and case-cohort population generating

Aim 2 requires the largest sample size and was used to guide sample size estimation for the study. The prospective case-cohort analysis set will include three women who delivered at term for each one with SPTB. A standard case-control method was employed to compute power [54]. Assuming three primary species/genera of interest, Simes' methodology was utilized to fix a type-1 error rate adjusted for three tests [55]. A sample of women with 80 SPTB and 240 term births would provide ≥80% power to detect a statistically significant 2.8-fold or greater difference in the odds of detecting a preconception vaginal bacterial species/genus in women with SPTB versus term delivery, assuming ≥10% prevalence of the organism at preconception visits in those delivering at term.

To accrue 80 SPTB cases including women with spontaneous preterm labor or PPROM, a cohort of approximately 1100 women will be enrolled. Once 80 SPTB cases occur, the sample for the case-cohort will be defined. First, cases of spontaneous abortion (<20 weeks gestation) and preterm births without spontaneous labor or PPROM will be excluded. Next, a random sample of

the remaining pregnancies will be selected such that, when added to the remaining SPTB cases, the full case-cohort population will have a term birth to SPTB ratio of 3:1. A sampling fraction *f*, with *f* solved using the formula: *nf + (1-f)*80=320*, where *n* is the total number of women (cases and non-cases) with delivery data, will be used to select the random sample. Lastly, all of the remaining SPTB cases will be added to the random sample, creating the full case-cohort sample.

Statistical analysis plan

The goal of Aim 1 is to characterize and compare preconception vaginal species diversity and richness between women with SPTB versus term birth. All women with SPTB and a random sample of the same number of women with term birth from the case-cohort sample will be included. To describe the overall frequency and relative abundance of species, cumulative rank abundance plots will be generated for each group [56]. We will compare the cumulative distribution of preconception vaginal bacterial taxa between women with SPTB versus term birth using the Kolmogorov-Smirnov test. Rarefaction curves will be used to evaluate species richness (number of taxa at a 97% sequence similarity cutoff defining an operational taxonomic unit) in women with SPTB versus term birth. Finally, we will assess species diversity (Shannon Diversity Index [57]) and species richness (Chao1 richness estimator [58]) by comparing the mean values between women with SPTB versus term birth. We will perform logistic regression with SPTB status as the outcome and species rank abundance percentage for each species separately. We will first determine the score statistics (with SPTB status as the outcome) for each variable, then rank the variables from largest to smallest score statistic. Next, we will perform the logistic modeling on each of these in rank order of score statistic until we reach a p-value of 0.2 in univariate logistic regressions. These data will be examined to refine targets for the primary hypothesis test in Aim 2.

For Aim 2, based on the literature and informed by Aim 1 results, bacterial species/genera will be selected for evaluation using qPCR assays, comparing their presence and concentrations in vaginal specimens sampled before conception. These analyses will utilize data from the full case-cohort sample and will be weighted to account for the sampling scheme. For each bacterial taxon, we will first perform unadjusted logistic regression to examine the association between the presence of that taxon and the risk of SPTB. Multivariable logistic regression analysis will be used to determine the independent contributions of bacterial species to the risk of SPTB. Species associated with SPTB in univariate analyses ($p<0.10$) will be included in the multivariable model after addressing collinearity. Potential confounding factors are listed in Table 3. A manual forward stepwise model building approach will be used to address confounding. For the three species selected for the primary hypothesis test, $p<0.033$ will be considered statistically significant, using the Simes' correction for multiple comparisons. Other bacterial taxa may be evaluated as exploratory analyses. Additional exploratory analyses will be performed to investigate the relationship between preconception quantities of specific bacteria and risk of SPTB.

Aim 3 explores the association between bacteria detected in vaginal preconception samples and fetal membrane samples to further support the role of ascending vaginal bacteria in SPTB. This analysis will also evaluate the association between bacteria identified in the fetal membranes and histological evidence of deciduitis, chorioamnionitis, and funisitis. The analysis will use data from women in the case-cohort sample who contribute delivery samples. There are three different binary exposures, defined as preconception detection of each of the three vaginal species tested for the primary hypothesis in Aim 2. The set of binary outcomes includes detection of the same bacterial species in fetal membranes, deciduitis, chorioamnionitis and funisitis. Odds ratios will be estimated for each exposure using unadjusted logistic regression models, and multivariable logistic regression analyses will be utilized to adjust for potential confounding factors (Table 3).

PATIENT AND PUBLIC INVOLVEMENT

Neither patients nor the public were involved in the design of this research. Healthcare providers providing reproductive/maternal health services and study participants can refer potentially eligible women to the study.

ETHICS AND DISSEMINATION

This study was approved by the ethics committees at KNH and University of Washington. There are inherent risks to women and fetuses during pregnancy and delivery, but participation in this observational study does not increase these risks. Study participation risks include discomfort associated with the fingerstick for HIV testing, sensitive questions about sexual behavior, and the pelvic examination; stress associated with HIV or STI diagnosis; and breach of confidentiality. To minimize these risks, experienced study staff counsel participants on potential risks and explain that they can withdraw from the study at any time. The risk of breach of confidentiality is minimized by following Good Clinical Practice procedures for data security. Results will be disseminated to clinicians at study sites and partner institutions, presented at conferences, and published in peer-reviewed journals.

DISCUSSION

Most cases of SPTB occur without a known cause [3]. Bacterial vaginosis during pregnancy is associated with increased risk of SPTB, but clinical trials of BV treatment in pregnancy have shown minimal or no reduction in SPTB [9]. The MPTB Study addresses the hypothesis that the vaginal microbiota detected during the preconception period may be linked to SPTB. The findings could shift the paradigm for thinking about the mechanisms linking vaginal microbiota and preterm birth, and could be used to guide the development and evaluation of interventions aimed at lowering the risk of SPTB by identifying and eradicating high-risk vaginal bacteria prior to conception [59–61].

REFERENCES

1 Beck S, Wojdyla D, Say L, *et al.* The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bull World Health Organ* 2010;**88**:31–8. doi:10.2471/BLT.08.062554

2 Liu L, Oza S, Hogan D, *et al.* Global, regional, and national causes of under-5 mortality in 2000–15: an updated systematic analysis with implications for the Sustainable Development Goals. *Lancet* 2016;**388**:3027–35. doi:10.1016/S0140-6736(16)31593-8

3 Goldenberg RL, Culhane JF, Iams JD, *et al.* Epidemiology and causes of preterm birth. *Lancet* 2008;**371**:75–84. doi:10.1016/S0140-6736(08)60074-4

4 Goldenberg RL, Culhane JF, Johnson DC. Maternal infection and adverse fetal and neonatal outcomes. *Clin Perinatol* 2005;**32**:523–59. doi:10.1016/j.clp.2005.04.006

5 Hendler I, Goldenberg RL, Mercer BM, *et al.* The Preterm Prediction study: Association between maternal body mass index and spontaneous and indicated preterm birth. *Am J Obstet Gynecol* 2005;**192**:882–6. doi:10.1016/j.ajog.2004.09.021

6 Daalderop LA, Wieland B V., Tomsin K, *et al.* Periodontal disease and pregnancy outcomes: Overview of systematic reviews. *JDR Clin Transl Res* 2018;**3**:10–27. doi:10.1177/2380084417731097

7 Hillier S, Marrazzo J, Holmes KK. Bacterial Vaginosis. In: Holmes KK, Sparling P, Stamm W, *et al.*, eds. *Sexually Transmitted Infections*. New York, NY: : McGraw Hill Companies 2007. 737–68.

8 Leitich H, Kiss H. Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. *Best Pract Res Clin Obstet Gynaecol* 2007;**21**:375–90. doi:10.1016/j.bpobgyn.2006.12.005

9 Brocklehurst P, Gordon A, Heatley E, *et al.* Antibiotics for treating bacterial vaginosis in pregnancy. *Cochrane Collab* 2013;:1–123. doi:10.1002/14651858.CD000262.pub4.

10 Ravel J, Gajer P, Abdo Z, *et al.* Vaginal microbiome of reproductive-age women. *Proc*

Natl Acad Sci 2011;**108**:4680–7. doi:10.1073/pnas.1002611107

11 Srinivasan S, Hoffman NG, Morgan MT, *et al.* Bacterial communities in women with bacterial vaginosis: High resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PLoS One* 2012;**7**:e37818. doi:10.1371/journal.pone.0037818

12 Hyman RW, Fukushima M, Jiang H, *et al.* Diversity of the vaginal microbiome correlates with preterm birth. *Reprod Sci* 2014;**21**:32–40. doi:10.1177/1933719113488838

13 Stout MJ, Zhou Y, Wylie KM, *et al.* Early pregnancy vaginal microbiome trends and preterm birth. *Am J Obstet Gynecol* 2017;**217**:356.e1-356.e18. doi:10.1016/j.ajog.2017.05.030

14 Freitas AC, Bocking A, Hill JE, *et al.* Increased richness and diversity of the vaginal microbiota and spontaneous preterm birth. *Microbiome* 2018;**6**:117. doi:10.1186/s40168-018-0502-8

15 Brown RG, Al-Memar M, Marchesi JR, *et al.* Establishment of vaginal microbiota composition in early pregnancy and its association with subsequent preterm prelabor rupture of the fetal membranes. *Transl Res* 2019;**207**:30–43. doi:10.1016/j.trsl.2018.12.005

16 Fettweis JM, Serrano MG, Brooks JP, *et al.* The vaginal microbiome and preterm birth. *Nat Med* 2019;**25**:1012–21. doi:10.1038/s41591-019-0450-2

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

18 Nelson DB, Shin H, Wu J, *et al.* The gestational vaginal microbiome and spontaneous preterm birth among nulliparous African American women. *Am J Perinatol* 2016;**33**:887–93. doi:10.1055/s-0036-1581057

19 Elovitz MA, Gajer P, Riis V, *et al.* Cervicovaginal microbiota and local immune response

modulate the risk of spontaneous preterm delivery. *Nat Commun* 2019;**10**:1305. doi:10.1038/s41467-019-09285-9

20 DiGiulio DB, Callahan BJ, McMurdie PJ, *et al.* Temporal and spatial variation of the human microbiota during pregnancy. *Proc Natl Acad Sci* 2015;**112**:11060–5. doi:10.1073/pnas.1502875112

21 Callahan BJ, DiGiulio DB, Goltsman DSA, *et al.* Replication and refinement of a vaginal microbial signature of preterm birth in two racially distinct cohorts of US women. *Proc Natl Acad Sci* 2017;**114**:9966–9971. doi:10.1073/pnas.1705899114

22 Stafford GP, Parker JL, Amabebe E, *et al.* Spontaneous preterm birth is associated with differential expression of vaginal metabolites by lactobacilli-dominated microflora. *Front Physiol* 2017;**8**:615. doi:10.3389/fphys.2017.00615

23 Nelson DB, Hanlon A, Nachamkin I, *et al.* Early pregnancy changes in bacterial vaginosis-associated bacteria and preterm delivery. *Paediatr Perinat Epidemiol* 2014;**28**:88–96. doi:10.1111/ppe.12106

24 Menard JP, Mazouni C, Salem-Cherif I, *et al.* High vaginal concentrations of Atopobium vaginae and Gardnerella vaginalis in women undergoing preterm labor. *Obstet Gynecol* 2010;**115**:134–40. doi:10.1097/AOG.0b013e3181c391d7

25 Nelson DB, Hanlon A, Hassan S, *et al.* Preterm labor and bacterial vaginosis-associated bacteria among urban women. *J Perinat Med* 2009;**37**:130–4. doi:10.1515/JPM.2009.026

26 Rahkonen L, Rutanen EM, Unkila-Kallio L, *et al.* Factors affecting matrix metalloproteinase-8 levels in the vaginal and cervical fluids in the first and second trimester of pregnancy. *Hum Reprod* 2009;**24**:2693–702. doi:10.1093/humrep/dep284

27 Andrews WW, Hauth JC, Cliver SP, *et al.* Association of asymptomatic bacterial vaginosis with endometrial microbial colonization and plasma cell endometritis in nonpregnant women. *Am J Obstet Gynecol* 2006;**195**:1611–6. doi:10.1016/j.ajog.2006.04.010

28 de Andrade Ramos B, Kanninen TT, Sisti G, *et al.* Microorganisms in the female genital tract during pregnancy: Tolerance versus pathogenesis. *Am J Reprod Immunol* 2015;**73**:383–9. doi:10.1111/aji.12326

29 Prince AL, Antony KM, Chu DM, *et al.* The microbiome, parturition, and timing of birth: More questions than answers. *J Reprod Immunol* 2014;**104**–**105**:12–9. doi:10.1016/j.jri.2014.03.006

30 National AIDS & STD Control Programme. Guidelines on Use of Antiretroviral Drugs for Treating and Preventing HIV in Kenya. Nairobi, Kenya: 2018.

31 Gajer P, Brotman RM, Bai G, *et al.* Temporal dynamics of the human vaginal microbiota. *Sci Transl Med* 2012;**4**:132ra52. doi:10.1126/scitranslmed.3003605

32 Srinivasan S, Liu C, Mitchell CM, *et al.* Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PLoS One* 2010;**5**:e10197. doi:10.1371/journal.pone.0010197

33 National AIDS & STI Control Programme of Kenya. Kenya National Guidelines for Prevention, Management and Control of Sexually Transmitted Infections. Nairobi, Kenya: 2018.

34 Lynch CD, Jackson LW, Buck Louis GM. Estimation of the day-specific probabilities of conception: current state of the knowledge and the relevance for epidemiological research. *Paediatr Perinat Epidemiol* 2006;**20**:3–12. doi:10.1111/j.1365-3016.2006.00765

35 Prince AL, Ma J, Kannan PS, *et al.* The placental membrane microbiome is altered among subjects with spontaneous preterm birth with and without chorioamnionitis. *Am J Obstet Gynecol* 2016;**214**:627.e1-627.e16. doi:10.1016/j.ajog.2016.01.193

36 Laine MA. Effect of pregnancy on periodontal and dental health. *Acta Odontol Scand* 2002;**60**:257–64. doi:10.1080/00016350260248210

37 World Health Organization. Oral Health Surveys - Basic Methods: Fifth Edition. Geneva, Switzerland: 2013.

38 Page RC, Eke PI. Case definitions for use in population-based surveillance of periodontitis. *J Periodontol* 2007;**78**:1387–99. doi:10.1902/jop.2007.060264

39 Pardthaisong T, Gray R, McDaniel E. Return of fertility after discontinuation of dept medroxyprogesterone acetate and intra-uterine devices in northern Thailand. *Lancet* 1980;**315**:509–12. doi:10.1016/s0140-6736(80)92765-8

40 Schwallie P, Assenzo J. The effect of depo-medroxyprogesterone acetate on pituitary and ovarian function, and the return of fertility following its discontinuation: a review. *Contraception* 1974;**10**:181–202. doi:10.1016/0010-7824(74)90073-0

41 Committee opinion no 611: Method for estimating due date. *Obs Gynecol* 2014;**124**:863–6. doi:10.1097/01.AOG.0000454932.15177.be

42 Odeny TA, Newman M, Bukusi EA, *et al.* Developing content for a mHealth intervention to promote postpartum retention in prevention of mother-to-child HIV transmission programs and early infant diagnosis of HIV: A qualitative study. *PLoS One* 2014;**9**:e106383. doi:10.1371/journal.pone.0106383

43 Lannon SMR, Adams Waldorf KM, Fiedler T, *et al.* Parallel detection of lactobacillus and bacterial vaginosis-associated bacterial DNA in the chorioamnion and vagina of pregnant women at term. *J Matern Neonatal Med* 2019;**32**:2702–10. doi:10.1080/14767058.2018.1446208

44 Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *J Clin Microbiol* 1991;**29**:297–301.

45 Martin HL, Richardson BA, Nyange PM, *et al.* Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. *J Infect Dis* 1999;**180**:1863–8. doi:10.1086/315127

46 Eschenbach DA, Davick PR, Williams BL, *et al.* Prevalence of hydrogen peroxide-producing lactobacillus species in normal women and women with bacterial vaginosis. *J Clin Microbiol* 1989;**27**:251–6.

47 Gryphus Diagnostics. Diagnosit BV Blue - Rapid Sialidase Test for Bacterial Vaginosis Package Insert.

48 Hobbs M, Steiner M, Rich K, *et al.* Good performance of rapid prostate-specific antigen test for detection of semen exposure in women: Implications for qualitative research. *Sex Transm Dis* 2009;**36**:501–6. doi:10.1097/OLQ.0b013e3181a2b4bf

49 Golob JL, Pergam SA, Srinivasan S, *et al.* Stool Microbiota at Neutrophil Recovery Is Predictive for Severe Acute Graft vs Host Disease after Hematopoietic Cell Transplantation. *Clin Infect Dis* 2017;**65**:1984–91. doi:10.1093/cid/cix699

50 Callahan BJ, McMurdie PJ, Rosen MJ, *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;**13**:581–3. doi:10.1038/nmeth.3869

51 Matsen F, Kodner R, Armbrust E. pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. *BMC Bioinformatics* 2010;**11**:538. doi:10.1186/1471-2105-11-538

52 Khot PD, Ko DL, Hackman RC, *et al.* Development and optimization of quantitative PCR for the diagnosis of invasive aspergillosis with bronchoalveolar lavage fluid. *BMC Infect Dis* 2008;**8**:73. doi:10.1186/1471-2334-8-73

53 Kraus F, Redline R, Gersell D, *et al. Atlas of Nontumor Pathology - Placental Pathology*. Washington, DC: : American Registry of Pathology 2004.

54 Kim M, Xue X, Du Y. Approaches for calculating power for case-cohort studies. *Biometrics* 2006;**62**:929–33. doi:10.1111/j.1541-0420.2006.00639_1.x

55 Simes R. An improved Bonferroni procedure for multiple tests of significance. *Biometrika* 1986;**73**:751–4.

56 Hughes JB, Hellman JJ, Ricketts TH, *et al.* Counting the Uncountable: Statistical Approaches to Estimating Microbial Diversity. *Appl Environ Microbiol* 2001;**67**:4399–406. doi:10.1128/AEM.67.10.4399–4406.2001

57 Krebs C. *Ecological Methodology*. New York: : Harper Collins 1989.

58 Chao A. Non-parametric estimation of the number of classes in a population. *Scand J Stat* 1984;**11**:265–70.

59 McClelland RS, Balkus JE, Lee J, *et al.* Randomized trial of periodic presumptive treatment with high-dose intravaginal metronidazole and miconazole to prevent vaginal infections in HIV-negative women. *J Infect Dis* 2015;**211**:1875–82. doi:10.1093/infdis/jiu818

60 McClelland RS, Richardson BA, Hassan WM, *et al.* Improvement of vaginal health for Kenyan women at risk for acquisition of human immunodeficiency virus rype 1: results of a randomized trial. *J Infect Dis* 2008;**197**:1361–8. doi:10.1086/587490

61 Sobel JD, Ferris D, Schwebke J, *et al.* Suppressive antibacterial therapy with 0.75% metronidazole vaginal gel to prevent recurrent bacterial vaginosis. *Am J Obstet Gynecol* 2006;**194**:1283–9. doi:10.1016/j.ajog.2005.11.041

62 Wanyonyi SZ, Napolitano R, Ohuma EO, *et al.* Image-scoring system for crown-rump length measurement. *Ultrasound Obstet Gynecol* 2014;**44**:649–54. doi:10.1002/uog.13376

AUTHORS' CONTRIBUTIONS: RSM is the principal investigator. RSM and DF generated the idea for this study and supervised study protocol development and implementation. GJS contributed to essential elements of the protocol related to recruitment and retention of pregnant and postpartum women in Kenya. JK and WJ served as site principal investigators, overseeing study staff and implementation at Kenyatta National Hospital and Ganjoni Health Center. EML, SL, EF, KM, AK, and HA participated in designing the study, protocol, data collection tools, and staff training. BAR, RSM, DF, and SS developed the statistical analysis plan for the study. DF, SS, and KM oversaw laboratory methods. EML coordinates the study and wrote the first draft of the manuscript. All authors reviewed and approved the final manuscript.

FUNDING STATEMENT: This work was supported by a National Institutes of Health grant (NICHD R01 HD087346-RSM). RSM received additional support for mentoring (NICHD K24 HD88229). EML was supported by a pre-doctoral fellowship (NIAID T32 AI07140-Lukehart). Data collection and management are made possible using REDCap electronic data capture tools hosted at the University of Washington's Institute of Translational Health Science supported by grants from NCATS/NIH (UL1 TR002319, KL2 TR002317, and TL1 TR002318).

COMPETING INTERESTS STATEMENT: RSM has received honoraria for consulting from Lupin Pharmaceuticals and receives research funding, paid to the University of Washington, from Hologic Corporation.

ACKNOWLEDGEMENTS: We would like to acknowledge the clinic, laboratory, and administrative study staff in Nairobi, Mombasa, and Seattle for their dedication and teamwork. We are also grateful to Kenyatta National Hospital, Coast Provincial General Hospital, and the Mombasa County Department of Health for supporting this research and providing clinical and laboratory space. We thank the study participants whose commitment has made the ongoing

implementation of this protocol possible. Development and implementation of the ultrasound quality assurance measures has been generously and skillfully supported by Dr. Robert Nathan (University of Washington) and Dr. Christine Mamai (Kenyatta National Hospital). Lastly, the authors thank Dr. Anne Pulei, Dr. Lydia Okutoyi, Raheli Mukhwana and Grace Wang'ombe (Kenyatta National Hospital), Charity Muthoni and Josephine Mwangi (Coast Provincial General Hospital), and the labor and delivery nurses at KNH and CPGH for their contributions to the successful training, oversight, and implementation of delivery sample collection.

Figure Title & Caption:

Figure 1. Microbiota and Preterm Birth Study Phases – Additional participant pathways: 1) Participants who discontinued DMPA injectable contraception within 6 months of study enrollment are eligible for 9 months of preconception follow-up. 2) Participants who miscarry are eligible to re-enter preconception follow-up for a second pregnancy attempt.

Table 1. Specimen collection, laboratory testing, and other procedures by study visit

Procedure Type	Screening	Enrollment	Periodontal Exam	Pre-conception	9-12 Week Gestation	Delivery[i]	Postpartum
Specimen Collection							
Urine pregnancy test	X	X		X			
Genital swabs							
APTIMA swab		X					
Push-off swabs[ii]		X		X	X		
Cotton & Dacron swabs		X		X	X		
Sialidase test swab		X		X			
Fetal membrane swab						X	
Placental punch						X	
Fetal membrane roll						X	
Umbilical cord						X	
Laboratory Testing							
HIV Rapid Test	X						
N. gonorrhoeae, C. trachomatis, & T. vaginalis		X					
Cervical Gram stain		X					
Vaginal pH		X					
Vaginal wet mount		X					
Vaginal Gram stain		X		X	X		
Lactobacillus culture[iii]		X		X	X		
Sialidase test		X		X			
PSA test		X		X	X		
Participant Questionnaires							
Eligibility screen	X						
Demographics		X					
Reproductive history		X					
Medical history & current symptoms		X		X	X		
Sexual behavior		X		X	X		
Substance use		X		X	X		
Dental health history			X				
Neonatal heath							X
Obstetric Ultrasound					X		

Abbreviations: PSA-prostate specific antigen

[i] Delivery follows standard obstetrical procedure and is not conducted by study staff
[ii] Storing for vaginal microbiota and vaginal inflammatory response testing
[iii] Rogosa agar, followed by sub-culture for hydrogen peroxide production on tetramethylbenzidine agar containing horseradish peroxidase

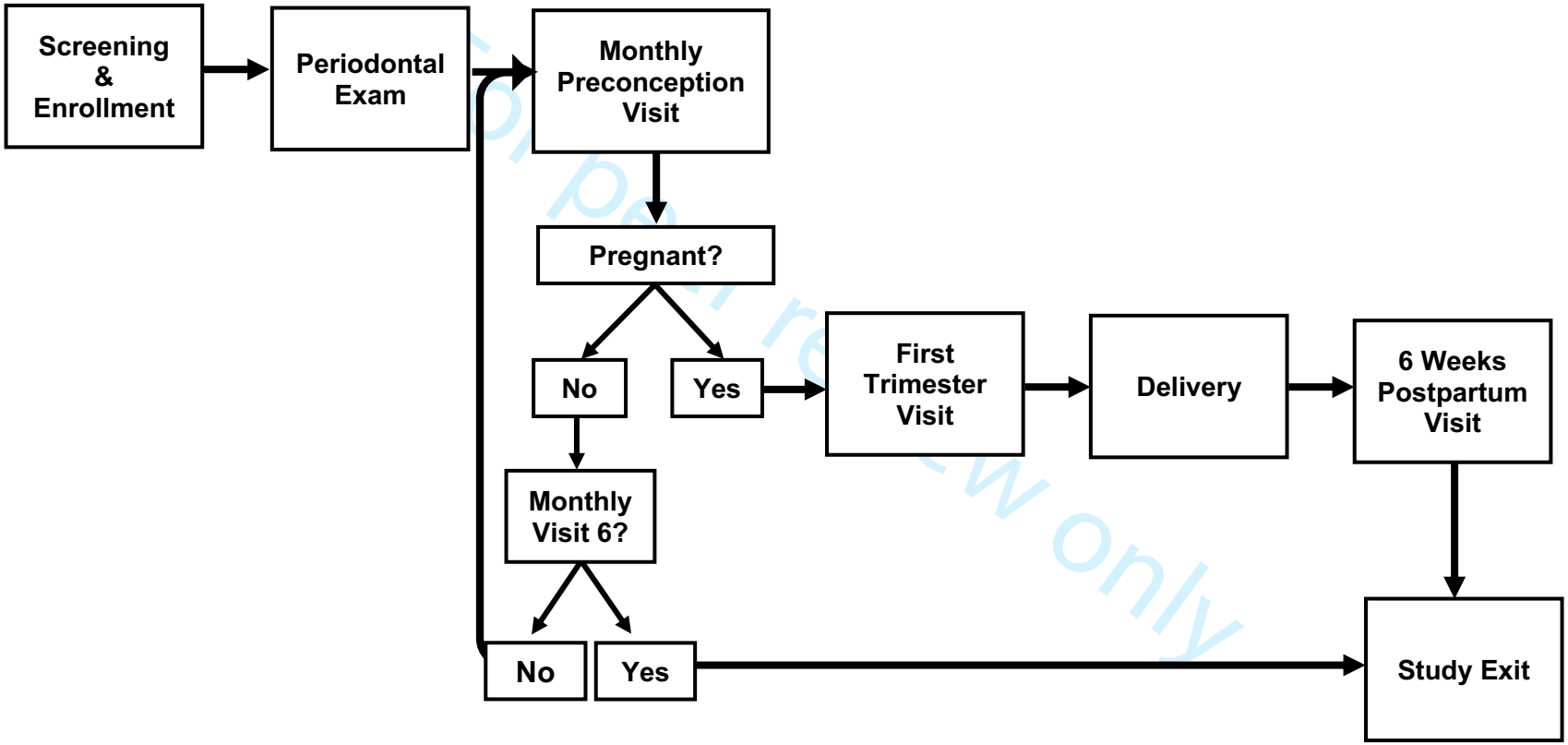
Table 2. Periodontal examination, first trimester obstetric ultrasound, and fetal membrane sample training and continuous quality control procedures

Procedure	Initial Training	Continuous Quality Control Procedures
Periodontal examination	• The UoN periodontist conducted an in-person training with the CPGH dentist that included teaching, observation, and calibration.	• Inter-examiner calibration bi-annually - The UoN periodontist and a second UoN experienced periodontist conduct periodontal exams on a series of patients to compare results. The UoN periodontist serves as the gold standard comparator for the CPGH dentist. • Intra-examiner calibration once per 50 examinations – Examination is repeated on one participant to compare results between the first and second examination.
First Trimester Obstetric Ultrasound	• All sonographers received a standardized refresher training on ultrasound determination of gestational age, early pregnancy complications, and MPTB study specific procedures by the Kenyan study radiologist.	• For each participant with a first trimester ultrasound, an image of the crown rump length is saved and quality is assessed by the study radiologist according to criteria developed for the International Fetal and Newborn Growth Consortium for the 21st Century [62]. (For participants with a crown rump length measurement of >84mm, images are not saved and gestational age is assessed using biometric measurements.) • Sonographers access the results of quality assessments for ongoing feedback. • Annually, sonographers in Nairobi meet with the study radiologist to review quality metrics and receive refresher training on study procedures. The radiologist provides ongoing one-on-one support to the Mombasa sonographers by phone.
Fetal Membrane Sample	• Initial training of labor and delivery nurses and KNH based Kenyan obstetrician was performed by Dr. Lannon, who developed the fetal membrane collection technique [43]. • Trainees were required to demonstrate proficient sample collection technique and verbalize study procedures.	• Subsequent trainings are conducted as needed and led by a Kenyan obstetrician-gynecologist based at KNH who participated in the initial training.

Table 3. Potential key covariates in analysis of the association between preconception vaginal microbiota and spontaneous preterm birth

Variable	Timing	Method
Demographics		
Maternal Age	Baseline	FTFI
Educational level	Baseline	FTFI
Socioeconomic status	Baseline	FTFI
Obstetrical history (e.g., number of pregnancies, time since last pregnancy, prior preterm birth)	Baseline	FTFI
Prior contraceptive history	Baseline	FTFI
Current pregnancy		
Maternal hemoglobin level, blood pressure	Throughout pregnancy	ANC records
Fetal health (e.g., Apgar scores, spontaneous breathing, birthweight, gender)	Delivery	Delivery records, FTFI or phone interview
Maternal infection	Throughout pregnancy	ANC and delivery records, FTFI or phone interview
Maternal pregnancy complications	Throughout pregnancy	ANC and delivery records, FTFI or phone interview
General health status		
Body mass index	Baseline	Measure weight & height
Smoking	Baseline & follow-up	FTFI
Alcohol use	Baseline & follow-up	Alcohol use disorders identification test (AUDIT)
Depressive symptoms	Baseline	Patient health questionnaire-9 (PHQ-9)
Periodontitis	Within 4 weeks of baseline	Oral examination
Genital tract infection/STI	Baseline	Detection of gonorrhea, chlamydia, and *T. vaginalis* by NAAT (Hologic). Wet prep, vaginal and cervical Gram stain.
Lactobacilli	Baseline & follow-up	Culture on Rogosa and TMB agar
Unprotected sex	Baseline & follow-up	FTFI
Prostate specific antigen	Baseline & follow-up	ABACard PSA Detection (Abacus Diagnostics)
Intravaginal practices	Baseline & follow-up	FTFI

Abbreviations: ANC-antenatal clinic; FTFI-face-to-face interview; NAAT-nucleic acid amplification test; PHQ-9-patient health questionnaire version 9; PSA-prostate specific antigen; TMB-tetramethylbenzidine; STI-sexually transmitted infections



BMJ Open

Impact of preconception vaginal microbiota on women's risk of spontaneous preterm birth: Protocol for a prospective case-cohort study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2019-035186.R1
Article Type:	Protocol
Date Submitted by the Author:	07-Dec-2019
Complete List of Authors:	Lokken, Erica; University of Washington, Mandaliya, Kishorchandra ; University of Nairobi Srinivasan, Sujatha ; Fred Hutchinson Cancer Research Center, Vaccine and Infectious Diseases Division Richardson, BA; University of Washington, Department of Biostatistics; University of Washington, Department of Global Health Kinuthia, John; Kenyatta National Hospital, Research and Programs; Kenyatta National Hospital, Obstetrics and Gynaecology Lannon, Sophia; Northwest Perinatal Jaoko, Walter; University of Nairobi, Medical Microbiology Alumera, Hudson; University of Nairobi School of Dental Sciences Kemoli, Arthur; University of Nairobi School of Dental Sciences Fay, Emily; University of Washington, Obstetrics and Gynecology John-Stewart, G; University of Washington, Departments of Epidemiology, Global Health, and Medicine Fredricks, DN; Fred Hutchinson Cancer Research Center, Vaccine and Infectious Diseases Division; University of Washington, Medicine McClelland, Scott; University of Washington, Departments of Epidemiology, Global Health, and Medicine
Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Global health, Infectious diseases, Epidemiology
Keywords:	Epidemiology < INFECTIOUS DISEASES, OBSTETRICS, MICROBIOLOGY

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Impact of preconception vaginal microbiota on women's risk of spontaneous preterm birth: Protocol for a prospective case-cohort study

Authors:
Erica M Lokken[1]
Kishorchandra Mandaliya[2]
Sujatha Srinivasan[3]
Barbra A Richardson[4,5]
John Kinuthia[6,7]
Sophia Lannon[8]
Walter Jaoko[9]
Hudson Alumera[10]
Arthur Kemoli[10]
Emily Fay[11]
Grace John-Stewart[1,5,12]
David N Fredricks[3,12]
R Scott McClelland[1,5,12]

Affiliations: Department of Epidemiology, University of Washington, Seattle, WA[1]; University of Nairobi Institute of Tropical & Infectious Diseases (UNITID), Nairobi, Kenya[2]; Vaccine and Infectious Diseases Division, Fred Hutchinson Cancer Research Center, Seattle, WA[3]; Department of Biostatistics, University of Washington, Seattle, WA[4]; Department of Global Health, University of Washington, Seattle, WA[5]; Research and Programs, Kenyatta National Hospital, Nairobi, Kenya[6]; Obstetrics and Gynaecology, Kenyatta National Hospital, Nairobi, Kenya[7]; Northwest Perinatal, Portland, OR[8]; Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya[9]; School of Dental Sciences, University of Nairobi, Nairobi, Kenya[10]; Department of Obstetrics and Gynecology, University of Washington, Seattle, WA[11]; Department of Medicine, University of Washington, Seattle, WA[12]

Corresponding Author:
Scott McClelland
University of Washington
Box 359909
325 9th Avenue
Seattle, WA 98102
Tel: 206-543-4278
Email: mcclell@uw.edu

Word Count-Abstract: 293/300
Word Count-Main Text: 3988/4000

ABSTRACT

INTRODUCTION

Bacterial vaginosis (BV) and vaginal microbiota disruption during pregnancy are associated with increased risk of spontaneous preterm birth (SPTB), but clinical trials of BV treatment during pregnancy have shown little or no benefit. An alternative hypothesis is that vaginal bacteria present around conception may lead to SPTB by compromising the protective effects of cervical mucus, colonizing the endometrial surface before fetal membrane development, and causing low-level inflammation in the decidua, placenta, and fetal membranes. This protocol describes a prospective case-cohort study addressing this hypothesis.

METHODS AND ANALYSIS

HIV-seronegative Kenyan women with fertility intent are followed from preconception through pregnancy, delivery, and early postpartum. Participants provide monthly vaginal specimens during the preconception period for vaginal microbiota assessment. Estimated date of delivery is determined by last menstrual period and first trimester obstetrical ultrasound. After delivery, a swab is collected from between the fetal membranes. Placenta and umbilical cord samples are collected for histopathology. Broad-range 16S rRNA gene PCR and deep sequencing of preconception vaginal specimens will assess species richness and diversity in women with SPTB versus term delivery. Concentrations of key bacterial species will be compared using quantitative PCR (qPCR). Taxon-directed qPCR will also be used to quantify bacteria from fetal membrane samples and evaluate the association between bacterial concentrations and histopathological evidence of inflammation in the fetal membranes, placenta, and umbilical cord.

ETHICS AND DISSEMINATION

This study was approved by ethics committees at Kenyatta National Hospital and the University of Washington. Results will be disseminated to clinicians at study sites and partner institutions,

presented at conferences, and published in peer-reviewed journals. The findings of this study could shift the paradigm for thinking about the mechanisms linking vaginal microbiota and prematurity by focusing attention on the preconception vaginal microbiota as a mediator of SPTB.

ARTICLE SUMMARY

STRENGTHS AND LIMITATIONS OF THIS STUDY

- This prospective case-cohort study enrolls Kenyan women with fertility intent, enabling follow-up and exposure measurement from preconception through pregnancy and the early postpartum period.
- Monthly specimen collection during the preconception period allows for examination of the vaginal microbiota close to the time of conception.
- Fetal membrane swabs and placental samples are collected at delivery, enabling assessment of the association between detection and concentrations of bacteria in the fetal membranes, inflammation, and preterm birth.
- A combination of broad-range 16S rRNA gene PCR with next generation sequencing and quantitative PCR assays provide both the relative and absolute quantities of bacteria in vaginal secretions and in the fetal membranes.
- Because this is an observational study, it cannot definitively establish a causal relationship between vaginal bacteria and preterm birth.

INTRODUCTION

Globally, approximately 10% of births are preterm (<37 weeks of gestation), but the prevalence can be as high as 18% in low-resource countries [1]. Preterm birth and its sequalae are the leading causes of death among children under five [2]. The majority of preterm deliveries are spontaneous preterm births (SPTB) [3]. The cause of SPTB is often unknown, but up to 40% may be associated with intrauterine infections [3,4]. Other risk factors include other infections (urinary tract, sexually transmitted, systemic), socio-demographic characteristics (age, race, education) [3], extremes of body mass index [5], periodontal disease [6], inter-pregnancy interval <6 months, prior SPTB, stress, depression, and smoking [3]. Further elucidation of the causes may provide insight into novel approaches for reducing SPTB.

Bacterial vaginosis (BV) is a common vaginal condition characterized by a shift from an optimal *Lactobacillus*-predominant vaginal microbiota to one characterized by high concentrations of diverse anaerobic species [7]. Numerous studies indicate that BV during pregnancy is associated with increased risk of SPTB [8]. However, a meta-analysis of clinical trials concluded that while antibiotic treatment prior to 20 weeks gestation successfully treated BV, the risk of SPTB was not substantially reduced [9].

Molecular microbiology has transformed the understanding of vaginal microbiota to a broader spectrum of phenotypes ranging from low-diversity *Lactobacillus*-dominated bacterial communities to a heterogeneous group of high-diversity BV-associated communities [10,11]. Studies exploring the relationship between the vaginal microbiota and SPTB have yielded conflicting findings. Some found significant associations between increased species diversity and preterm birth [12–16], while others have not [17–19]. In addition, some studies have found that women with a low relative abundance of *Lactobacillus* species and higher relative abundance of BV-related taxa may be at higher risk of preterm birth [16,19–22]. Others found no association

between vaginal bacterial community type and preterm birth [12,14,17]. Detection and higher concentrations of specific vaginal bacteria have also been associated with preterm birth including *Leptotrichia/Sneathia* [16,23], BV-associated bacterium 1 (BVAB1) [16,23], *Megasphaera* [23], *Gardnerella vaginalis* [24,25], *Atopobium vaginae* [24], TM7-H1 [16], and some *Prevotella* species [16].

A novel hypothesis to explain the lack of success of BV treatment during pregnancy on SPTB risk is that preconception vaginal microbiota may be a more important risk factor for SPTB than vaginal bacteria during pregnancy. Vaginal bacteria present around the time of conception could compromise the protective effects of cervical mucus [26], gaining access to the endometrium before embryo implantation, formation of the cervical mucus plug, and development of the fetal membrane. These bacteria could colonize and cause chronic, low-level inflammation in the decidua, placenta, fetal membranes, or amniotic cavity [27,28]. In this scenario, antibiotic treatment during pregnancy may be too late to influence a woman's risk of SPTB associated with intrauterine bacteria.

The Microbiota and Preterm Birth Study (MPTB) enrolls HIV-negative Kenyan women trying to become pregnant into a prospective case-cohort study to address the following aims:

1) Compare the species diversity and richness of the vaginal microbiota sampled close to the time of conception in women with SPTB versus term delivery using broad-range 16S rRNA gene PCR and next generation sequencing.
2) Compare the presence and concentrations of select bacterial genera/species (based on published data [23] and results of Aim 1) using targeted qPCR assays in vaginal specimens sampled close to the time of conception in women with SPTB versus term births.

3) Perform species-specific qPCR assays on samples collected from fetal membranes and histological examination of membranes and umbilical cord to determine if vaginal bacteria ascend to the upper genital tract and cause inflammation in the fetal membranes and umbilical cord.

METHODS AND ANALYSIS

STUDY DESIGN, SETTING, AND TIMELINE

The MPTB Study is a prospective case-cohort study. Eligible women enroll prior to conception and are followed through preconception, pregnancy, delivery, and early postpartum. Participants will be selected into the case-cohort sample as detailed in the Statistical Analysis section. Study sites are located at Kenyatta National Hospital (KNH) in Nairobi and at Ganjoni Health Center and Coast Provincial General Hospital (CPGH) in Mombasa.

Enrollment began in Nairobi in April 2017 and Mombasa in April 2018. Preconception enrollment of participants will continue through approximately June 2019 with the last study deliveries occurring in 2021, approximately 18 months after the last enrollment.

ELIGIBILITY CRITERIA AND RECRUITMENT

The target population is HIV-negative women who are currently planning to become pregnant. Additional eligibility criteria include being ≤45 years old, having a menstrual period in the prior three months or recently discontinued contraceptive methods that induce amenorrhea (e.g., implant, hormonal intrauterine device), willing to comply with study procedures, and able to provide informed consent. Minors aged 14-17 are eligible if emancipated under Kenyan law. Exclusion criteria include current pregnancy, continuing contraception other than condoms for HIV/STI prevention, having a depo medroxyprogesterone acetate injection in the last three months, history of cervical or uterine surgery (other than cesarean section), known autoimmune disease, antibiotic use in the prior four weeks, and history of infertility care-seeking. For women

with known HIV-positive male partners, their partner must have an undetectable HIV viral load, or the participant must be taking pre-exposure prophylaxis.

Participants are recruited by study staff or referred by healthcare providers at sites providing reproductive or maternal health services. Women discontinuing contraception for the purpose of becoming pregnant are a key recruitment population.

STUDY VISITS AND PROCEDURES

Study participation occurs in six phases including screening/enrollment, periodontal examination, preconception, pregnancy, delivery, and postpartum (Figure 1). Specimen collection, laboratory testing, and other clinical procedures are summarized in Table 1.

Screening and Enrollment

Following written informed consent for screening, study staff perform urine pregnancy testing and rapid HIV testing according to Kenyan guidelines [29], and conduct an eligibility interview. Eligible women can enroll immediately upon consent. If enrollment occurs at a later date, pregnancy testing is repeated to re-confirm eligibility.

Enrollees complete a structured face-to-face interview regarding demographics, sexual behavior, substance use, depression symptoms (Patient Health Questionnaire-9), and reproductive and medical history. A study clinician performs a physical examination and speculum-assisted pelvic examination. If a woman is menstruating, the examination is deferred to avoid sampling during this interval when the vaginal microbiota undergoes rapid changes [30,31]. Two vaginal fluid specimens are collected by rolling push-off Dacron swabs (FitzCo, Inc) three rotations against the lateral vaginal wall; these are stored for vaginal microbiota and inflammatory response evaluation. Additional genital specimens are collected for STI diagnosis [*Neisseria gonorrhoeae, Chlamydia*

trachomatis, and *Trichomonas vaginalis* by nucleic acid amplification testing (NAAT)], vaginal and cervical Gram stains, detection of prostate specific antigen (PSA) and elevated sialidase using a point-of-care diagnostic test for BV (Diagnosit BVBLUE, Gryphus Diagnostics). A vaginal specimen is inoculated onto Rogosa agar for detection of *Lactobacillus*. Syndromic management is provided as indicated for genital syndromes including vaginal discharge [32]. Additional therapy is provided at the first preconception visit based on STI NAAT results.

After the examination, women receive counseling on healthy behaviors during preconception and pregnancy including smoking cessation, refraining from vaginal washing, and a healthy diet. Study clinicians discuss participants' menstrual cycle and identify the probable fertile window using calendar-based methods. Ovulation is estimated to occur 14 days prior to the first day of the next predicted menses, with the most fertile days emphasized as the five days before and day of ovulation [33]. All participants receive prenatal vitamins.

Periodontal Exam

Periodontitis has been associated with SPTB [6] so participants undergo a periodontal examination. Since pregnancy increases gingival inflammation [34], examinations occur within four weeks of enrollment. The oral examination includes assessment for periodontitis using periodontal pocket depth and clinical attachment measurements, and the Decay-Missing-Filled Index and Gingival Index assessments [35]. Presence and severity of periodontitis are defined using the 2007 Centers for Disease Control and Prevention/American Academy of Periodontology case definitions [36]. Participants also complete a modified version of the WHO's oral health questionnaire [35].

Monthly Preconception Visits

Participants return for study visits at one-month intervals while trying to become pregnant. Retention staff call participants in advance of each appointment. A structured interview is conducted to update sexual behavior and medical history. Women self-collect vaginal swabs for microbiota and inflammatory response analysis, vaginal Gram stain, PSA detection, *Lactobacillus* culture, and sialidase detection. Participants with genital symptoms receive syndromic management [32].

A urine pregnancy test is performed, and participants with a positive test are scheduled for a first trimester visit between 9-12 weeks of gestation. Most women who remain non-pregnant after six months exit the study; those who discontinued DMPA less than six months prior to enrollment are eligible for nine months of preconception 'trying time' due to delayed return to fertility after DMPA discontinuation [37,38].

First Trimester Visit (9-12 weeks of gestation)

At the first trimester visit, a structured interview is conducted to update information on sexual behavior and medical history. Women self-collect vaginal swabs for microbiota and inflammatory response analysis, vaginal Gram stain, PSA detection, and *Lactobacillus* culture. Women then undergo an obstetrical ultrasound to confirm gestational age. The ultrasound is conducted by sonographers at KNH in Nairobi and at a private radiology facility in Mombasa (training and quality control described in Table 2). The American College of Obstetrics & Gynecology's 2014 guidelines are used to estimate gestational age if the ultrasound derived estimate differs from that calculated using the last menstrual period by >7 days before 16 weeks [39]. If a later ultrasound is obtained, the sonographic dates are used if they differ from last menstrual period dates by >10 days between 16 and 22 weeks, >14 days between 22 and 28 weeks, and >21 days after 28 weeks.

Pregnant participants are referred to routine antenatal care (ANC) and enrolled into a short message service (SMS) program to support retention (see Retention During Pregnancy section). Routinely collected antenatal data (e.g., syphilis test results, blood pressure) are abstracted from participants' ANC facility records and the Mother and Child Health Booklet provided to all pregnant Kenyan women.

If a participant suffers a miscarriage, management of the pregnancy loss is conducted by non-study clinicians according to standard of care. Women who miscarry at <20 weeks of gestation may remain in the study for six additional monthly preconception visits if they would like to try for another pregnancy.

Retention During Pregnancy

To improve retention, participants are offered enrollment into an adaptation of a two-way SMS program that was initially designed to support HIV-positive Kenyan women during pregnancy and postpartum [40]. Messages are sent at 16, 20, and 24 weeks gestation, bi-weekly beginning at 28 weeks, and weekly from 38 weeks through six weeks postpartum. In addition, study nurses call participants weekly starting at week 35 to confirm pregnancy status and planned delivery location. Participants are instructed to call at the onset of labor so study staff can coordinate collection of delivery samples for deliveries at KNH or CPGH, and to assist with identification of the delivery date for births occurring elsewhere.

Delivery Procedures

Deliveries follow standard obstetrical procedures and are not conducted by study staff. Swabs from between the fetal membranes and placental samples are collected for births occurring at KNH and CPGH by trained labor and delivery nurses (training and quality control described in Table 2). Upon vaginal or caesarean delivery of the placenta, it is placed in a sterile container.

Samples are collected as soon as feasible and within two hours of delivery. Using sterile technique, a pair of nurses collects samples from between the amnion and chorion [41]. A sterile push-off swab (FitzCo, Inc) is collected from between the membranes, placed in a cryovial, and temporarily stored in a -4°C (Nairobi) or -20°C (Mombasa) freezer. The placenta is placed in 10% neutral-buffered formalin. Laboratory staff transport the fetal membrane swab to a -80°C freezer in study laboratories at KNH and CPGH within one working day. Laboratory staff also collect placental samples for histopathology. These include a four-centimeter fetal membrane roll from the ruptured edge of the membranes to the edge of the placental disc, a one-centimeter section of umbilical cord starting three centimeters from the placental disk, and a one-centimeter block of placenta with overlying membranes adjacent to the site of cord insertion. All pathological specimens are stored in 10% neutral-buffered formalin.

Study staff abstract data from delivery records, including type of delivery (i.e., vaginal or caesarean; labored or did not labor; spontaneous or induced labor), complications, live birth or stillbirth, and birthweight.

Postpartum Visit

At the six-week postpartum visit, clinicians abstract any ANC and delivery details not previously captured from the participant's delivery discharge report and Mother & Child Health Booklet. Participants complete an interview about the infant's heath and immunization status.

Incentives

Participants receive KSh 300 (about $3.00) at each study visit. This amount is consistent with other studies in Kenya and is provided to cover transportation costs. Women who become

pregnant receive a free obstetrical ultrasound. An incentive of 1000 KSH is provided for deliveries at KNH or CPGH.

LABORATORY METHODS

Microscopy, *Lactobacillus* culture, STI testing, and detection of sialidase and PSA in vaginal fluids

Vaginal Gram stained slides are evaluated for BV using the criteria developed by Nugent and Hillier [42]. Saline and potassium hydroxide wet mounts are examined for the presence of motile trichomonads, clue cells, yeast, and sperm. Endocervical Gram stained slides are scanned at low power, and polymorphonuclear leukocytes in three nonadjacent oil immersion fields are counted and averaged to evaluate cervical inflammation. Clinicians inoculate vaginal specimens directly on Rogosa agar for detection of cultivable *Lactobacillus* and store the plate in a candle jar until transportation to the laboratory within four hours [43]. Hydrogen peroxide production is evaluated by subculture of *Lactobacillus* isolates on tetramethylbenzidine agar containing horseradish peroxidase [44]. A vaginal specimen is tested for *N. gonorrhoeae, C. trachomatis,* and *T. vaginalis* by NAAT (Aptima Combo-2 CT/NG Detection System, Aptima *Trichomonas vaginalis* assay; Hologic Corporation). One vaginal swab is used for detection of sialidase using a commercially available BV diagnostic test (Diagnosit BVBLUE; Gryphus Diagnostics). Testing for PSA in vaginal samples is performed with a commercially available assay (ABAcard, Abacus Diagnostics), which can detect semen for 24-48 hours after condomless sex [45].

Molecular methods for identification of bacteria in vaginal and fetal membrane samples

Stored vaginal and fetal membrane swabs for bacterial PCR will be transported on dry ice to the Fredricks Laboratory at the Fred Hutchinson Cancer Research Center in Seattle, WA. Qiagen QIAamp BiOstic Bacteremia DNA Isolation Kits (Qiagen, Germantown, MD) will be used to extract DNA from vaginal swabs. This protocol uses bead beating and chaotropic lysis to break apart

bacterial cells and recover DNA that is free of PCR inhibitors. Swabs that have not contacted a human surface will be processed in parallel to serve as sham DNA extraction (negative) controls. Extracted DNA will be subjected to broad-range 16S rRNA gene PCR using primers that anneal with highly conserved regions of the small subunit rDNA gene, amplifying a 470 base pair segment that contains a highly variable sequence useful for species identification. Bar coded primers will be used to multiplex samples [46]. Libraries of 16S rDNA gene amplicons will be mixed for sequencing on the Illumina MiSeq platform using 300 bp paired-end reads. The assembled reads will be binned into individual study samples using the nucleic acid bar codes. Approximately 10,000-30,000 sequence reads will be generated per sample, providing robust detection of minority species. Raw sequence reads will be demultiplexed using Illumina's on-board bclfastq conversion software v1.8.4 with zero mismatches in either forward or reverse indices. The DADA2 software package will be used to quality control, filter, pair and cluster the amplicon sequence reads [47]. Sequence variants will be assigned taxonomy using the phylogenetic placement tool *pplacer* [48] and a custom vaginal reference set [11]. Sham extraction controls will also be processed in parallel to exclude bacterial contamination of reagents. Bacterium-specific qPCR assays will also be performed. This study focuses on species hypothesized to be associated with increased risk of SPTB, such as BVAB1, *Megasphaera,* and *Sneathi*a. The final set of species/genera tested will be fine-tuned following analysis of the deep sequencing data from Aim 1, selecting additional bacteria based on a comparison of the relative abundance of individual taxa in women with and without SPTB. A standard exogenous jellyfish qPCR amplification control will be used to assess for PCR inhibitors [49]. A broad-range bacterial 16S rDNA gene qPCR assay will be used to measure total bacterial load in each sample.

Histopathological examination of fetal membranes, placenta, and umbilical cord samples

Hematoxylin and eosin staining of fetal membrane rolls, placental samples, and umbilical cord sections will be graded according to published guidelines by an experienced pathologist (Dr.

Mandaliya) [50]. Acute and subacute inflammatory lesions will be graded and staged for both maternal and fetal components. Chronic inflammatory lesions will be characterized including any observation of chronic deciduitis or the presence of decidual plasma cells.

STATISTICAL ANALYSIS

Sample size estimate and case-cohort population generating

Aim 2 requires the largest sample size and was used to guide sample size estimation for this case cohort study [51]. The prospective case-cohort analysis set will include three women who delivered at term for each one with SPTB. Assuming three primary species/genera of interest, Simes' methodology was utilized to fix a type-1 error rate adjusted for three tests [52]. A sample of 80 SPTB and 240 term births has ≥80% power to detect a statistically significant ≥2.8-fold difference in the odds of detecting a preconception vaginal bacterial species/genus in women with SPTB versus term delivery, assuming ≥10% prevalence of the organism at preconception visits for term deliveries.

To accrue 80 SPTB cases including women with spontaneous preterm labor or preterm premature rupture of membranes, a cohort of approximately 1100 women will be enrolled. Once 80 SPTB cases occur, the sample for the case-cohort will be defined. First, cases of spontaneous abortion (<20 weeks gestation) and preterm births without spontaneous labor or preterm premature rupture of membranes will be excluded. Next, a random sample of the remaining pregnancies will be selected such that when added to the remaining SPTB cases, the case-cohort sample will have a term birth to SPTB ratio of 3:1. A sampling fraction *f*, with *f* solved using the formula: $nf + (1-f)*80=320$, where *n* is the total number of women (cases and non-cases) with delivery data, will be used to select the random sample. Lastly, all of the remaining SPTB cases will be added to the random sample, creating the full case-cohort sample.

Statistical analysis plan

The goal of Aim 1 is to characterize and compare preconception vaginal species diversity and richness between women with SPTB versus term birth. All women with SPTB and a random sample of the same number of women with term birth from the case-cohort sample will be included. To describe the overall frequency and relative abundance of species, cumulative rank abundance plots will be generated for each group [53]. We will compare the cumulative distribution of preconception vaginal bacterial taxa between women with SPTB versus term birth using the Kolmogorov-Smirnov test. Rarefaction curves will be used to evaluate species richness (number of taxa at a 97% sequence similarity cutoff defining an operational taxonomic unit) in women with SPTB versus term birth. Finally, we will assess species diversity (Shannon Diversity Index [54]) and species richness (Chao1 richness estimator [55]) by comparing the mean values between women with SPTB versus term birth. We will perform logistic regression with SPTB status as the outcome and species rank abundance percentage for each species. We will first determine the score statistics (SPTB status as the outcome) for each variable, then rank the variables from largest to smallest score statistic. Next, we will perform the logistic modeling on each of these in rank order of score statistic until reaching a p-value of 0.2 in univariate logistic regressions. These data will be examined to refine targets for the primary hypothesis test in Aim 2.

For Aim 2, based on the literature and informed by Aim 1 results, bacterial species/genera will be selected for evaluation using qPCR assays, comparing their presence and concentrations in vaginal specimens sampled before conception. These analyses will utilize data from the full case-cohort sample and will be weighted to account for the sampling scheme. For each bacterial taxon, we will perform unadjusted logistic regression to examine the association between the presence of that taxon and the risk of SPTB. Multivariable logistic regression analysis will be used to determine the independent contributions of bacterial species to the risk of SPTB. Species

associated with SPTB in univariate analyses ($p<0.10$) will be included in the multivariable model after addressing collinearity. A manual forward stepwise model building approach will be used to address confounding. Potential confounding factors are listed in Table 3. For the three species selected for the primary hypothesis test, $p<0.033$ will be considered statistically significant, using the Simes' correction for multiple comparisons. Other bacterial taxa may be evaluated as exploratory analyses. Additional exploratory analyses will be performed to investigate the relationship between preconception quantities of specific bacteria and risk of SPTB.

Aim 3 explores the association between bacteria detected in vaginal preconception samples and fetal membrane samples to further support the role of ascending vaginal bacteria in SPTB. This analysis will also evaluate the association between bacteria identified in the fetal membranes and histological evidence of deciduitis, chorioamnionitis, and funisitis. The analysis will use data from women in the case-cohort sample with delivery samples. The exposures are preconception detection of each of the three vaginal species tested for the primary hypothesis in Aim 2. Outcomes includes detection of the same bacterial species in fetal membranes, deciduitis, chorioamnionitis, and funisitis. Odds ratios will be estimated for each exposure using unadjusted logistic regression models, and multivariable logistic regression analyses will be utilized to adjust for potential confounding factors (Table 3).

PATIENT AND PUBLIC INVOLVEMENT

Neither patients nor the public were involved in the design of this research. Healthcare providers providing reproductive/maternal health services and study participants can refer potentially eligible women to the study.

ETHICS AND DISSEMINATION

This study was approved by the ethics committees at KNH and University of Washington. There are inherent risks to women and fetuses during pregnancy and delivery, but participation in this observational study does not increase these risks. Study participation risks include discomfort associated with the fingerstick for HIV testing, sensitive questions about sexual behavior, and the pelvic examination; stress associated with HIV or STI diagnosis; and breach of confidentiality. To minimize these risks, experienced Kenyan research staff fluent in both English and Kiswahili conduct a robust consent process. At screening, the screening procedures are explained and written informed consent is obtained with forms available in English and Kiswahili. Eligible women who opt to participate in the study undergo a separate consenting process for study enrollment, including verbal explanation of the procedures and written informed consent. Participants are counselled on potential risks and are informed that they can withdraw from the study at any time. The risk of breach of confidentiality is minimized by following Good Clinical Practice procedures for data security. Signed consents are secured in locked cabinets in a restricted area. Study identification numbers are assigned to de-identify hard copies of the case report forms, which are locked in a restricted area. Data are entered into a password-protected database using encrypted computers. Results will be disseminated to clinicians at study sites and partner institutions, presented at conferences, and published in peer-reviewed journals.

DISCUSSION

Most cases of SPTB occur without a known cause [3]. Bacterial vaginosis during pregnancy is associated with increased risk of SPTB, but clinical trials of BV treatment in pregnancy have shown minimal or no reduction in SPTB [9]. The MPTB Study addresses the hypothesis that the vaginal microbiota detected during the preconception period may be linked to SPTB. The findings could shift the paradigm for thinking about the mechanisms linking vaginal microbiota and preterm birth, and could be used to guide the development and evaluation of interventions aimed at

lowering the risk of SPTB by identifying and eradicating high-risk vaginal bacteria prior to conception [56–58].

REFERENCES

1 Beck S, Wojdyla D, Say L, *et al.* The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bull World Health Organ* 2010;**88**:31–8. doi:10.2471/BLT.08.062554

2 Liu L, Oza S, Hogan D, *et al.* Global, regional, and national causes of under-5 mortality in 2000–15: an updated systematic analysis with implications for the Sustainable Development Goals. *Lancet* 2016;**388**:3027–35. doi:10.1016/S0140-6736(16)31593-8

3 Goldenberg RL, Culhane JF, Iams JD, *et al.* Epidemiology and causes of preterm birth. *Lancet* 2008;**371**:75–84. doi:10.1016/S0140-6736(08)60074-4

4 Goldenberg RL, Culhane JF, Johnson DC. Maternal infection and adverse fetal and neonatal outcomes. *Clin Perinatol* 2005;**32**:523–59. doi:10.1016/j.clp.2005.04.006

5 Hendler I, Goldenberg RL, Mercer BM, *et al.* The Preterm Prediction study: Association between maternal body mass index and spontaneous and indicated preterm birth. *Am J Obstet Gynecol* 2005;**192**:882–6. doi:10.1016/j.ajog.2004.09.021

6 Daalderop LA, Wieland B V., Tomsin K, *et al.* Periodontal disease and pregnancy outcomes: Overview of systematic reviews. *JDR Clin Transl Res* 2018;**3**:10–27. doi:10.1177/2380084417731097

7 Hillier S, Marrazzo J, Holmes KK. Bacterial Vaginosis. In: Holmes KK, Sparling P, Stamm W, *et al.*, eds. *Sexually Transmitted Infections*. New York, NY: : McGraw Hill Companies 2007. 737–68.

8 Leitich H, Kiss H. Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. *Best Pract Res Clin Obstet Gynaecol* 2007;**21**:375–90. doi:10.1016/j.bpobgyn.2006.12.005

9 Brocklehurst P, Gordon A, Heatley E, *et al.* Antibiotics for treating bacterial vaginosis in pregnancy. *Cochrane Collab* 2013;:1–123. doi:10.1002/14651858.CD000262.pub4.

10 Ravel J, Gajer P, Abdo Z, *et al.* Vaginal microbiome of reproductive-age women. *Proc*

Natl Acad Sci 2011;**108**:4680–7. doi:10.1073/pnas.1002611107

11 Srinivasan S, Hoffman NG, Morgan MT, *et al.* Bacterial communities in women with bacterial vaginosis: High resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PLoS One* 2012;**7**:e37818. doi:10.1371/journal.pone.0037818

12 Hyman RW, Fukushima M, Jiang H, *et al.* Diversity of the vaginal microbiome correlates with preterm birth. *Reprod Sci* 2014;**21**:32–40. doi:10.1177/1933719113488838

13 Stout MJ, Zhou Y, Wylie KM, *et al.* Early pregnancy vaginal microbiome trends and preterm birth. *Am J Obstet Gynecol* 2017;**217**:356.e1-356.e18. doi:10.1016/j.ajog.2017.05.030

14 Freitas AC, Bocking A, Hill JE, *et al.* Increased richness and diversity of the vaginal microbiota and spontaneous preterm birth. *Microbiome* 2018;**6**:117. doi:10.1186/s40168-018-0502-8

15 Brown RG, Al-Memar M, Marchesi JR, *et al.* Establishment of vaginal microbiota composition in early pregnancy and its association with subsequent preterm prelabor rupture of the fetal membranes. *Transl Res* 2019;**207**:30–43. doi:10.1016/j.trsl.2018.12.005

16 Fettweis JM, Serrano MG, Brooks JP, *et al.* The vaginal microbiome and preterm birth. *Nat Med* 2019;**25**:1012–21. doi:10.1038/s41591-019-0450-2

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

18 Nelson DB, Shin H, Wu J, *et al.* The gestational vaginal microbiome and spontaneous preterm birth among nulliparous African American women. *Am J Perinatol* 2016;**33**:887–93. doi:10.1055/s-0036-1581057

19 Elovitz MA, Gajer P, Riis V, *et al.* Cervicovaginal microbiota and local immune response

modulate the risk of spontaneous preterm delivery. *Nat Commun* 2019;**10**:1305. doi:10.1038/s41467-019-09285-9

20 DiGiulio DB, Callahan BJ, McMurdie PJ, *et al.* Temporal and spatial variation of the human microbiota during pregnancy. *Proc Natl Acad Sci* 2015;**112**:11060–5. doi:10.1073/pnas.1502875112

21 Callahan BJ, DiGiulio DB, Goltsman DSA, *et al.* Replication and refinement of a vaginal microbial signature of preterm birth in two racially distinct cohorts of US women. *Proc Natl Acad Sci* 2017;**114**:9966–9971. doi:10.1073/pnas.1705899114

22 Stafford GP, Parker JL, Amabebe E, *et al.* Spontaneous preterm birth is associated with differential expression of vaginal metabolites by lactobacilli-dominated microflora. *Front Physiol* 2017;**8**:615. doi:10.3389/fphys.2017.00615

23 Nelson DB, Hanlon A, Nachamkin I, *et al.* Early pregnancy changes in bacterial vaginosis-associated bacteria and preterm delivery. *Paediatr Perinat Epidemiol* 2014;**28**:88–96. doi:10.1111/ppe.12106

24 Menard JP, Mazouni C, Salem-Cherif I, *et al.* High vaginal concentrations of Atopobium vaginae and Gardnerella vaginalis in women undergoing preterm labor. *Obstet Gynecol* 2010;**115**:134–40. doi:10.1097/AOG.0b013e3181c391d7

25 Nelson DB, Hanlon A, Hassan S, *et al.* Preterm labor and bacterial vaginosis-associated bacteria among urban women. *J Perinat Med* 2009;**37**:130–4. doi:10.1515/JPM.2009.026

26 Rahkonen L, Rutanen EM, Unkila-Kallio L, *et al.* Factors affecting matrix metalloproteinase-8 levels in the vaginal and cervical fluids in the first and second trimester of pregnancy. *Hum Reprod* 2009;**24**:2693–702. doi:10.1093/humrep/dep284

27 de Andrade Ramos B, Kanninen TT, Sisti G, *et al.* Microorganisms in the female genital tract during pregnancy: Tolerance versus pathogenesis. *Am J Reprod Immunol* 2015;**73**:383–9. doi:10.1111/aji.12326

28 Prince AL, Antony KM, Chu DM, *et al.* The microbiome, parturition, and timing of birth:

More questions than answers. *J Reprod Immunol* 2014;**104–105**:12–9. doi:10.1016/j.jri.2014.03.006

29 National AIDS & STD Control Programme. Guidelines on Use of Antiretroviral Drugs for Treating and Preventing HIV in Kenya. Nairobi, Kenya: 2018.

30 Gajer P, Brotman RM, Bai G, *et al.* Temporal dynamics of the human vaginal microbiota. *Sci Transl Med* 2012;**4**:132ra52. doi:10.1126/scitranslmed.3003605

31 Srinivasan S, Liu C, Mitchell CM, *et al.* Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PLoS One* 2010;**5**:e10197. doi:10.1371/journal.pone.0010197

32 National AIDS & STI Control Programme of Kenya. Kenya National Guidelines for Prevention, Management and Control of Sexually Transmitted Infections. Nairobi, Kenya: 2018.

33 Lynch CD, Jackson LW, Buck Louis GM. Estimation of the day-specific probabilities of conception: current state of the knowledge and the relevance for epidemiological research. *Paediatr Perinat Epidemiol* 2006;**20**:3–12. doi:10.1111/j.1365-3016.2006.00765

34 Laine MA. Effect of pregnancy on periodontal and dental health. *Acta Odontol Scand* 2002;**60**:257–64. doi:10.1080/00016350260248210

35 World Health Organization. Oral Health Surveys - Basic Methods: Fifth Edition. Geneva, Switzerland: 2013.

36 Page RC, Eke PI. Case definitions for use in population-based surveillance of periodontitis. *J Periodontol* 2007;**78**:1387–99. doi:10.1902/jop.2007.060264

37 Pardthaisong T, Gray R, McDaniel E. Return of fertility after discontinuation of dept medroxyprogesterone acetate and intra-uterine devices in northern Thailand. *Lancet* 1980;**315**:509–12. doi:10.1016/s0140-6736(80)92765-8

38 Schwallie P, Assenzo J. The effect of depo-medroxyprogesterone acetate on pituitary and ovarian function, and the return of fertility following its discontinuation: a review.

Contraception 1974;**10**:181–202. doi:10.1016/0010-7824(74)90073-0

39 Committee opinion no 611: Method for estimating due date. *Obs Gynecol* 2014;**124**:863–6. doi:10.1097/01.AOG.0000454932.15177.be

40 Odeny TA, Newman M, Bukusi EA, *et al.* Developing content for a mHealth intervention to promote postpartum retention in prevention of mother-to-child HIV transmission programs and early infant diagnosis of HIV: A qualitative study. *PLoS One* 2014;**9**:e106383. doi:10.1371/journal.pone.0106383

41 Lannon SMR, Adams Waldorf KM, Fiedler T, *et al.* Parallel detection of lactobacillus and bacterial vaginosis-associated bacterial DNA in the chorioamnion and vagina of pregnant women at term. *J Matern Neonatal Med* 2019;**32**:2702–10. doi:10.1080/14767058.2018.1446208

42 Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *J Clin Microbiol* 1991;**29**:297–301.

43 Martin HL, Richardson BA, Nyange PM, *et al.* Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. *J Infect Dis* 1999;**180**:1863–8. doi:10.1086/315127

44 Eschenbach DA, Davick PR, Williams BL, *et al.* Prevalence of hydrogen peroxide-producing lactobacillus species in normal women and women with bacterial vaginosis. *J Clin Microbiol* 1989;**27**:251–6.

45 Hobbs M, Steiner M, Rich K, *et al.* Good performance of rapid prostate-specific antigen test for detection of semen exposure in women: Implications for qualitative research. *Sex Transm Dis* 2009;**36**:501–6. doi:10.1097/OLQ.0b013e3181a2b4bf

46 Golob JL, Pergam SA, Srinivasan S, *et al.* Stool Microbiota at Neutrophil Recovery Is Predictive for Severe Acute Graft vs Host Disease after Hematopoietic Cell Transplantation. *Clin Infect Dis* 2017;**65**:1984–91. doi:10.1093/cid/cix699

47 Callahan BJ, McMurdie PJ, Rosen MJ, *et al.* DADA2: High-resolution sample inference

from Illumina amplicon data. *Nat Methods* 2016;**13**:581–3. doi:10.1038/nmeth.3869

48 Matsen F, Kodner R, Armbrust E. pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. *BMC Bioinformatics* 2010;**11**:538. doi:10.1186/1471-2105-11-538

49 Khot PD, Ko DL, Hackman RC, *et al.* Development and optimization of quantitative PCR for the diagnosis of invasive aspergillosis with bronchoalveolar lavage fluid. *BMC Infect Dis* 2008;**8**:73. doi:10.1186/1471-2334-8-73

50 Kraus F, Redline R, Gersell D, *et al. Atlas of Nontumor Pathology - Placental Pathology*. Washington, DC: : American Registry of Pathology 2004.

51 Kim M, Xue X, Du Y. Approaches for calculating power for case-cohort studies. *Biometrics* 2006;**62**:929–33. doi:10.1111/j.1541-0420.2006.00639_1.x

52 Simes R. An improved Bonferroni procedure for multiple tests of significance. *Biometrika* 1986;**73**:751–4.

53 Hughes JB, Hellman JJ, Ricketts TH, *et al.* Counting the Uncountable: Statistical Approaches to Estimating Microbial Diversity. *Appl Environ Microbiol* 2001;**67**:4399–406. doi:10.1128/AEM.67.10.4399–4406.2001

54 Krebs C. *Ecological Methodology*. New York: : Harper Collins 1989.

55 Chao A. Non-parametric estimation of the number of classes in a population. *Scand J Stat* 1984;**11**:265–70.

56 McClelland RS, Balkus JE, Lee J, *et al.* Randomized trial of periodic presumptive treatment with high-dose intravaginal metronidazole and miconazole to prevent vaginal infections in HIV-negative women. *J Infect Dis* 2015;**211**:1875–82. doi:10.1093/infdis/jiu818

57 McClelland RS, Richardson BA, Hassan WM, *et al.* Improvement of vaginal health for Kenyan women at risk for acquisition of human immunodeficiency virus rype 1: results of a randomized trial. *J Infect Dis* 2008;**197**:1361–8. doi:10.1086/587490

58 Sobel JD, Ferris D, Schwebke J, *et al.* Suppressive antibacterial therapy with 0.75% metronidazole vaginal gel to prevent recurrent bacterial vaginosis. *Am J Obstet Gynecol* 2006;**194**:1283–9. doi:10.1016/j.ajog.2005.11.041

59 Wanyonyi SZ, Napolitano R, Ohuma EO, *et al.* Image-scoring system for crown-rump length measurement. *Ultrasound Obstet Gynecol* 2014;**44**:649–54. doi:10.1002/uog.13376

AUTHORS' CONTRIBUTIONS: RSM is the principal investigator. RSM and DF generated the idea for this study and supervised study protocol development and implementation. GJS contributed to essential elements of the protocol related to recruitment and retention of pregnant and postpartum women in Kenya. JK and WJ served as site principal investigators, overseeing study staff and implementation at Kenyatta National Hospital and Ganjoni Health Center. EML, SL, EF, KM, AK, and HA participated in designing the study, protocol, data collection tools, and staff training. BAR, RSM, DF, and SS developed the statistical analysis plan for the study. DF, SS, and KM oversaw laboratory methods. EML coordinates the study and wrote the first draft of the manuscript. All authors reviewed and approved the final manuscript.

FUNDING STATEMENT: This work was supported by a National Institutes of Health grant (NICHD R01 HD087346-RSM). RSM received additional support for mentoring (NICHD K24 HD88229). EML was supported by a pre-doctoral fellowship (NIAID T32 AI07140-Lukehart). Data collection and management are made possible using REDCap electronic data capture tools hosted at the University of Washington's Institute of Translational Health Science supported by grants from NCATS/NIH (UL1 TR002319, KL2 TR002317, and TL1 TR002318).

COMPETING INTERESTS STATEMENT: RSM has received honoraria for consulting from Lupin Pharmaceuticals and receives research funding, paid to the University of Washington, from Hologic Corporation.

ACKNOWLEDGEMENTS: We would like to acknowledge the clinic, laboratory, and administrative study staff in Nairobi, Mombasa, and Seattle for their dedication and teamwork. We are also grateful to Kenyatta National Hospital, Coast Provincial General Hospital, and the Mombasa County Department of Health for supporting this research and providing clinical and laboratory space. We thank the study participants whose commitment has made the ongoing

implementation of this protocol possible. Development and implementation of the ultrasound quality assurance measures has been generously and skillfully supported by Dr. Robert Nathan (University of Washington) and Dr. Christine Mamai (Kenyatta National Hospital). Lastly, the authors thank Dr. Anne Pulei, Dr. Lydia Okutoyi, Raheli Mukhwana and Grace Wang'ombe (Kenyatta National Hospital), Charity Muthoni and Josephine Mwangi (Coast Provincial General Hospital), and the labor and delivery nurses at KNH and CPGH for their contributions to the successful training, oversight, and implementation of delivery sample collection.

Figure Title & Caption:

Figure 1. Microbiota and Preterm Birth Study Phases – Additional participant pathways: 1) Participants who discontinued DMPA injectable contraception within 6 months of study enrollment are eligible for 9 months of preconception follow-up. 2) Participants who miscarry are eligible to re-enter preconception follow-up for a second pregnancy attempt.

Table 1. Specimen collection, laboratory testing, and other procedures by study visit

Procedure Type	Screening	Enrollment	Periodontal Exam	Pre-conception	9-12 Week Gestation	Delivery[i]	Postpartum
Specimen Collection							
Urine pregnancy test	X	X		X			
Genital swabs							
APTIMA swab		X					
Push-off swabs[ii]		X		X	X		
Cotton & Dacron swabs		X		X	X		
Sialidase test swab		X		X			
Fetal membrane swab						X	
Placental punch						X	
Fetal membrane roll						X	
Umbilical cord						X	
Laboratory Testing							
HIV Rapid Test	X						
N. gonorrhoeae, C. trachomatis, & T. vaginalis		X					
Cervical Gram stain		X					
Vaginal pH		X					
Vaginal wet mount		X					
Vaginal Gram stain		X		X	X		
Lactobacillus culture[iii]		X		X	X		
Sialidase test		X		X			
PSA test		X		X	X		
Participant Questionnaires							
Eligibility screen	X						
Demographics		X					
Reproductive history		X					
Medical history & current symptoms		X		X	X		
Sexual behavior		X		X	X		
Substance use		X		X	X		
Dental health history			X				
Neonatal heath							X
Obstetric Ultrasound					X		

Abbreviations: PSA-prostate specific antigen

[i] Delivery follows standard obstetrical procedure and is not conducted by study staff
[ii] Storing for vaginal microbiota and vaginal inflammatory response testing
[iii] Rogosa agar, followed by sub-culture for hydrogen peroxide production on tetramethylbenzidine agar containing horseradish peroxidase

Table 2. Periodontal examination, first trimester obstetric ultrasound, and fetal membrane sample training and continuous quality control procedures

Procedure	Initial Training	Continuous Quality Control Procedures
Periodontal examination	• The UoN periodontist conducted an in-person training with the CPGH dentist that included teaching, observation, and calibration.	• Inter-examiner calibration bi-annually - The UoN periodontist and a second UoN experienced periodontist conduct periodontal exams on a series of patients to compare results. The UoN periodontist serves as the gold standard comparator for the CPGH dentist. • Intra-examiner calibration once per 50 examinations – Examination is repeated on one participant to compare results between the first and second examination.
First Trimester Obstetric Ultrasound	• All sonographers received a standardized refresher training on ultrasound determination of gestational age, early pregnancy complications, and MPTB study specific procedures by the Kenyan study radiologist.	• For each participant with a first trimester ultrasound, an image of the crown rump length is saved and quality is assessed by the study radiologist according to criteria developed for the International Fetal and Newborn Growth Consortium for the 21st Century [59]. (For participants with a crown rump length measurement of >84mm, images are not saved and gestational age is assessed using biometric measurements.) • Sonographers access the results of quality assessments for ongoing feedback. • Annually, sonographers in Nairobi meet with the study radiologist to review quality metrics and receive refresher training on study procedures. The radiologist provides ongoing one-on-one support to the Mombasa sonographers by phone.
Fetal Membrane Sample	• Initial training of labor and delivery nurses and KNH based Kenyan obstetrician was performed by Dr. Lannon, who developed the fetal membrane collection technique [41]. • Trainees were required to demonstrate proficient sample collection technique and verbalize study procedures.	• Subsequent trainings are conducted as needed and led by a Kenyan obstetrician-gynecologist based at KNH who participated in the initial training.

Table 3. Potential key covariates in analysis of the association between preconception vaginal microbiota and spontaneous preterm birth

Variable	Timing	Method
Demographics		
Maternal Age	Baseline	FTFI
Educational level	Baseline	FTFI
Socioeconomic status	Baseline	FTFI
Obstetrical history (e.g., number of pregnancies, time since last pregnancy, prior preterm birth)	Baseline	FTFI
Prior contraceptive history	Baseline	FTFI
Current pregnancy		
Maternal hemoglobin level, blood pressure	Throughout pregnancy	ANC records
Fetal health (e.g., Apgar scores, spontaneous breathing, birthweight, gender)	Delivery	Delivery records, FTFI or phone interview
Maternal infection	Throughout pregnancy	ANC and delivery records, FTFI or phone interview
Maternal pregnancy complications	Throughout pregnancy	ANC and delivery records, FTFI or phone interview
General health status		
Body mass index	Baseline	Measure weight & height
Smoking	Baseline & follow-up	FTFI
Alcohol use	Baseline & follow-up	Alcohol use disorders identification test (AUDIT)
Depressive symptoms	Baseline	Patient health questionnaire-9 (PHQ-9)
Periodontitis	Within 4 weeks of baseline	Oral examination
Genital tract infection/STI	Baseline	Detection of gonorrhea, chlamydia, and *T. vaginalis* by NAAT (Hologic). Wet prep, vaginal and cervical Gram stain.
Lactobacilli	Baseline & follow-up	Culture on Rogosa and TMB agar
Unprotected sex	Baseline & follow-up	FTFI
Prostate specific antigen	Baseline & follow-up	ABACard PSA Detection (Abacus Diagnostics)
Intravaginal practices	Baseline & follow-up	FTFI

Abbreviations: ANC-antenatal clinic; FTFI-face-to-face interview; NAAT-nucleic acid amplification test; PHQ-9-patient health questionnaire version 9; PSA-prostate specific antigen; TMB-tetramethylbenzidine; STI-sexually transmitted infections