Clinical Study Protocol
VMRC4Africa -Pilot

Protocol Title:	Vaginal Microbiome Research Consortium for Africa
Short Title:	VMRC4Africa - Pilot
Investment ID:	INV-037612
Protocol Version:	2.3
Protocol Date:	09 June 2023

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Signature Page

Protocol Title: *Vaginal Microbiome Research Consortium for Africa - Pilot* **Investment ID:** *INV-037612* Sponsor

Herewith we approve the protocol INV-037612 (VMRC4Africa - Pilot) version 2.3, dated 29 May 2023, and confirm that it contains all information necessary to conduct the study according to the ethical principles laid down in the Declaration of Helsinki, Good Clinical Practice, and all applicable local regulations.

DocuSigned by:
Jo-Ann Passmore
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1/23/2023

Sponsor Responsible Person
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Mucosal Infection Group
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Clinical Study Partners

Herewith I confirm that I have read and understood the protocol INV-037612 (VMRC4Africa - Pilot) version 2.3, dated 29 May 2023, and agree to conduct this observational trial accordingly, including all statements regarding compliance with the ethical principles laid down in the Declaration of Helsinki, Good Clinical Practice and all applicable local regulations.

I will provide copies of the protocol and all information to the site personnel under my supervision required to conduct the observational study duly. I will discuss this material with them and ensure they are fully informed on all study requirements.

DocuSigned by:
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Protocol Synopsis

Protocol Title	Vaginal Microbiome Research Consortium for Africa - Pilot
Investment ID	INV-037612
Short Title	VMRC4Africa - Pilot
Sponsor	Mucosal Infection Group Division of Medical Virology, Faculty of Health Sciences, University of Cape Town
Study Centres	Desmond Tutu Health Foundation, Masiphumelele, Cape Town, South Africa CIS clinic, Lumumba, and Kargeno Research and Policy Hub along Karume road, Kisumu all under Research Care and Training Program, Kenya Medical Research Institute, Kenya
Objectives	1. To characterise vaginal microbial community dynamics (bacterial and fungal) from different geographies in Africa to understand the microbial diversity that occurs in women with stable *L. crispatus*-dominant versus unstable vaginal microbiota. 2. To identify vaginal communities associated with low levels of inflammation in women from different geographies in Africa 3. To examine prevalence and diversity of HPV types circulating in the different geographies and their interaction with the vaginal microbiota 4. To create a biobank of stored samples that can be used in future studies and for the isolation of regionally representative bacterial strains.
Study Design	A pilot observational cohort study
Population	Female at birth, 18-35 years old

Inclusion/Exclusion Criteria	Inclusion Criteria ● Female at birth ● Willing and able to provide informed consent for screening and cognitive ability to understand sampling procedures ● Not pregnant ● HIV negative on testing performed by study staff ● 18-35 years old ● Planning to stay in the area for the next 10 weeks ● Able and willing to provide adequate locator information for study retention purposes ● Willing and able to return for all 3 nurse visits and return self-swabs to the clinic weekly ● Sexually active for the last 3 months defined as penetrative penile-vaginal intercourse at least once in the last 3 months
	Exclusion Criteria ● Male at birth ● Not willing to provide consent ● Pregnant or actively trying to conceive/become pregnant in the next 10 weeks ● Living with HIV or untreated STIs (CT, NG, TV) or bacterial vaginosis ● Currently taking antibiotics or having been on antibiotic treatment in the previous four weeks ● <18 or >35 years old ● Planning on moving, going away out of area within the next 10 weeks ● On chronic disease management for gynaecological conditions ● Unable to agree to adhere to all study visits and procedures ● Any medical condition or other factors which would preclude study participation as per principal Investigator's or designee's decision, including but not limited to cancer of the cervix ● Any mental health condition which, in the opinion of the investigator, would preclude comprehension of informed consent, or preclude study participation ● Not sexually active, defined as penetrative penile-vaginal intercourse at least once in the last three months

Evaluation Criteria	● Questionnaires and daily diaries to record exposure to sex and antibiotics ● STI testing ● Assessment of vaginal microbiota Total 16S rRNA gene copy number measurement ● CST assignment ● Measurement of cervicovaginal cytokines
Study Timeline	Estimated date of first participant enrolled: 01 June 2023 Estimated date of last participant enrolled: 31 May 2024 Estimated date of last participant completed: 12 October 2024 Total duration of study: ~ 60 months (12 months enrolment period + 10-16 weeks follow-up)

Schedule of Events

Fig1. VMRC4Africa Observational Trial – Schedule of Procedures sample (100ppts at each Clinical Research Site for 10 weeks intensive follow-up)

		Nurse-Collected				Self-Collected
Procedure	**Sample processing**	**Screening**	**Enrolment** *within 4 weeks from screening if STI/BV- and up to 6 weeks if treated for STI*	**Week 5** *±7-day window* **MID-POINT**	**Week 10** *±7-day window* **EXIT**	**Weekly (Week 1-4, 6-9)**
Pre-screen for eligibility	n/a	X				
Informed Consent	n/a	X	X^	X^	X^	
Participant locator info	n/a	X	X	X		
Eligibility assessment	n/a	X	X			
Pre-test counselling	n/a	X	X	X	X	
Questionnaire	n/a	X	X	X	X	
Urine Pregnancy test	tested in country / at CRS	X	X	X	X	

HIV rapid test	tested in country / at CRS	X	X	X	X	
*HIV + confirmatory test	tested in country / at CRS	#	#	#	#	
Post-test counselling	n/a	X	X	X	X	
Targeted physical exam	n/a	X		X	X	
Speculum exam	n/a	X	X	X	X	
1x Vulvo-vaginal swab for STI testing	tested in country / at CRS‡	X	X	X	X	
1x high vaginal wall swab for storage	shipped to central storage	X	X	X	X	
1x lateral vaginal wall swab to be applied to pH indicator and rolled on a glass slide for Nugent scoring	tested in country / at CRS	X	X	X	X	
1x lateral vaginal wall swab for qPCR and 16S rRNA (bacterial) & ITS (fungal) amplicon sequencing (Molecular transport medium [Qiagen])	shipped to central Lab	X	X	X	X	
1x soft cup (supernatant-cytokines and pellet - HPV)	shipped to central Lab		X	X	X	
1x Lateral wall swab for culture (Amies-based transport solution, eg E-Swab [Copan]) (culture)	shipped to central Lab	X	X	X	X	

1 x Lateral wall swab for storage (future proteomics)	Shipped to central storage		X	X	X	
1 x Lateral wall swab for storage (future metabolomics)	Shipped to central storage		X	X	X	
1 x Cytobrush for storage (future transcriptomics)	Shipped to central storage		X	X	X	
STI/BV treatment	in country according to local procedure	*	*	*	*	
Exposure to sex and antibiotics Questionnaire	n/a					X weekly by phone/SMS/WhatsApp
Self-collected low vaginal swab (Molecular transport medium [Qiagen]) for qPCR and 16S rRNA (bacterial) & ITS (fungal) amplicon sequencing†	shipped to central Lab					X 2 weekly
Shipping to the Central Lab		X *batch ship twice during study*	X *batch ship twice during study*	X *batch ship twice during study*	X *batch ship twice during study*	

a. ^Document continuing consent for procedures

b. #Confirmatory HIV test only for positive HIV rapid test

c. *Referral for pregnancy, HIV+ and symptomatic ST/BVI detection

d. 2x self collected swabs in the week on different days. To be returned to the clinic for storage weekly and batch shipping twice during the study.

*No blood contaminated nurse collected samples. Blood contaminated self-collected swabs acceptable.

*Schedule to be adjusted to each ppt monthly cycle.

e. †Samples will be self-collected weekly (including the weeks of the study visits) by participants and returned to the lab during study visits

f. ‡ Participants with STIs at screening will attend a post-treatment visit to confirm test-of-cure prior to enrolment

List of Abbreviations

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BV	bacterial vaginosis
CAB	community advisory board
CRF	Case report form
CRS	Clinical Research Site
CT	Chlamydia trachomatis
DTHF	Desmond Tutu Health Foundation
EC	Ethic committee
ELISA	enzyme-linked immunosorbent assay
FGD	focus group discussion
GCP	Good Clinical Practice
HCP	healthcare professional
HIV	Human Immunodeficiency Virus
HPV	human papillomavirus
IC	Informed Consent
IDI	in-depth interview
KEMRI	Kenya Medical Research Institute
LMICs	low- and middle-income countries
MG	Mycoplasma genitalium
NAATs	nucleic acid amplification tests
NG	Neisseria gonorrhoeae
NHLS	National Health Laboratory Services
SOP	standard operating procedure
STIs	sexually transmitted infections
TV	Trichomonas vaginalis
WHO	World Health Organization

Background Information

This investment forms part of the BMGF Calestous Juma Scientific Leadership (CJSL) Fellowship to Dr Jo-Ann Passmore, to pilot VMRC4Africa and establish a collaborative regional network with African partners and Centres of Excellence with capacity and expertise to conduct clinical trials and vaginal microbiome research in Africa. With this CJSL Fellowship investment, Dr Passmore and her collaborators aim to enrol parallel cohorts of women from two sites in two African countries (South Africa: Desmond Tutu HIV Foundation [DTHF] and Kenya Medical Research Institute [KEMRI]) to evaluate detailed temporal fluctuations in vaginal microbiota in young, generally healthy women from Southern and Eastern Africa. These parallel cohorts will be intensively followed for 10 weeks, to create detailed profiles of vaginal microbial community state types (CSTs; by 16S rRNA gene sequencing) and fungal communities [by internal transcribed spacer (ITS) sequencing], to identify women with stable *Lactobacillus*-dominated microbiota, with no evidence of genital inflammation. Through the establishment of an "African vaginal microbiome biorepository", the intention will be to create a biobank from which to ultimately select geographically diverse *Lactobacillus crispatus* strains with health promoting characteristics that can be co-formulated into live biotherapeutic products (LBPs) to treat bacterial vaginosis (BV) for women globally.

This investment aims to pilot the formation of an African network, collecting key information about geographic variation in vaginal microbiotas across the African continent. This will include (1) characterization of vaginal microbiotas, mycobiota and infection with human papillomavirus (HPV) and cervicovaginal inflammation in South African and Kenyan women by 16S rRNA and ITS gene sequencing, HPV Direct Flow CHIP, and Luminex, and (2) building a well curated biorepository including samples from women with stable *Lactobacillus*-dominated microbiotas from which *L. crispatus* strains with health promoting properties can be isolated, alongside providing meta-data to support these samples. Downstream proposed experiments not included in the current investment include whole genome sequencing and laboratory characterisation of isolates (*Lactobacillus* spp. as well as BV-associated bacteria), human vagina on chip models, metabolomics and transcriptomics analyses. Central to this investment application is the aim that these observational trials will pilot the broader concept of VMRC4Africa, which will eventually enrol women from Central and West Africa as well, to understand the impact of geography on the vaginal microbiome and contribute *L. crispatus* strains for inclusion in a combination live biotherapeutic (LBP) consortium to treat and/or prevent BV and preterm birth on this continent and globally.

Although not budgeted for here, and contingent on raising additional funding, this consortium ultimately aims to use the vaginal microbiome biobank to isolate primary vaginal *L. crispatus* strains from women in South Africa and Kenya with persistently community state type I (*L. crispatus*-dominated) communities (over 10 weeks); and BV-associated bacteria (including *Gardnerella vaginalis* and *Prevotella bivia*) from women transitioning from one community state type to another, to understand functional and metabolic aspects of these vaginal microbial communities that determine their interaction (including bacterial, fungal and viral

strains), and contribute to stability or dysbiosis in genetically diverse women from different geographics within Africa.

This CJSL fellowship is based on the rationale that US studies have shown that daily changes in the composition of the vaginal microbiota is common (Mayer et al., 2015). While VMRC4Health hypothesizes that health-promoting vaginal *Lactobacillus* strains should be isolated from women with a stable vaginal microbiota who are consistently genital healthy (no symptoms; *L. crispatus*-dominated microbiota; no BV, bacterial STIs and vulvovaginal yeast infections), it is unclear how geography will serve this hypothesis and whether phenotypic and genotypic characteristics of the *Lactobacillus* strains that successfully and stably colonize the vaginas of women from different regions in Africa will differ geographically within the continent as well as with US strains. This fellowship hypothesizes that the best *Lactobacillus* strain combination for a novel BV treatment for women from different regions would likely include isolates with the greatest phenotypic and genotypic breadth and come from a diversity of populations. This VMRC4Africa initiative proposes to first enrol cohorts of Kenyan and South African women (and additional countries at a later stage as funds become available) using standardized enrolment criteria and sampling methods, to create a biorepository of African vaginal samples for the development of a probiotic for vaginal health that will serve women in Africa and globally. In particular, this CJSL fellowship proposes that:

(1) Characterization of vaginal microbial community interactions and obtaining bacterial isolates from different geographies in Africa will contribute to the global effort to understand the functional microbial diversity that occurs in women with stable *L. crispatus* dominant versus unstable vaginal microbiota.

(2) Vaginal *L. crispatus* strains from different regions of Africa will have better anti-microbial properties against local BV-associated bacteria (such as *G. vaginalis* and *P. bivia*).

(3) Addition of a diverse range of *L. crispatus* isolates from different regions in Africa into the global sequence database will contribute substantively to our understanding of geographic adaption of vaginal microbiomes, and metagenomes that are publicly available.

(4) Designing a live biotherapeutic product (LBP) which includes a diverse but genetically complimentary consortium of *L. crispatus* isolates will more likely result in stable *L. crispatus* vaginal colonization in women in Africa and globally.

Rationale for the Study

High rates of bacterial vaginosis (BV) and sexually transmitted infections (STIs), frequently asymptomatic in women, contribute significantly to HIV risk in women in Africa. Genital inflammation associated with these conditions, even when clinically asymptomatic, contribute significantly to this risk (*Mlisana et al. 2012; Masson et al. 2015*). BV treatment leads to a decrease in the presence of BV-associated species, but re-colonization with *Lactobacillus* species is often slow, and recurrence rates of BV following treatment are high (*Mtshali et al. 2021*). After antibiotic treatment of BV, several studies have shown a modest

reduction in bacterial counts of BV-associated microorganisms, inversely associated with increased relative abundances of *Lactobacillus* species *(Joag et al. 2019; Verwijs et al. 2020*). Given the relationship between BV, genital tract inflammation and HIV risk, there is an urgent need to rethink BV treatment strategies.

Globally and in Africa, female reproductive health is characterized by cervicovaginal colonization with *Lactobacillus* species (*L. crispatus* in particular, but possibly *L. jensenii, L. vaginalis, L. mucosae,* and *L. gasseri*) and a vaginal pH below 4.5 *(O'Hanlon et al. 2013; Lennard et al. 2018; McClelland et al. 2018*). Studies have shown that commensal *Lactobacillus* spp. support mucosal barrier function and protect the host from challenge by (i) lowering the vaginal pH while being relatively acid tolerant themselves; (ii) by competitively antagonizing pathogens; (iii) by stimulating host antimicrobial factor production; (iv) by modulating genital epithelial barrier integrity; and (v) by moderating mucosal immune cell function by inducing tolerizing regulatory T cells (Tregs). While several *Lactobacillus* spp. have been shown to lower pH and produce active metabolites (like D- or L-Lactic acid [LA]), *L. crispatus* appears to most consistently map to healthy outcomes (*Lambert et al. 2013*). Studies conducted in the US have shown that African- American women are less likely to be colonized with *L. crispatus* than their Caucasian American counterparts and tend to have more heterogenous vaginal microbiota and higher pH (*Ravel et al. 2011*), although studies in South Africa showed that *L. crispatus*-dominant vaginal communities (community state type [CST]1) and vaginal pHs ≤4.0 were evident in a subset (~25%) of women in Africa (*Anahtar et al. 2015; Lennard et al. 2018*)

In the gut, lactobacilli-containing probiotic products have been shown to improve gut epithelial function by promoting competitive exclusion of pathogens; enhancing gut epithelial barrier function by modulating signalling pathways that increase mucus or defensin production, by increasing tight junction function of the barrier, or by preventing apoptosis; or by directly modulating the host mucosal immune system, by promoting immune regulation and down modulating inflammatory processes (*Bron et al. 2011*). Based on the strong impact of targeted and broad microbiome interventions (faecal microbiome transplants) to restore gut health in patients with irritable bowel disease (IBD) and Crohn's Disease, live biotherapeutic or microbiome interventions to restore vaginal health and improve BV treatment outcomes in women are being explored by several research groups, including the BMGF-funded VMRC and the MIG lab at UCT over the past 5 years (*Chetwin et al. 2019; Happel et al. 2020a, 2020b*). To date, the efficacy of probiotics to treat BV have been mixed *(Li et al. 2019*) although the majority of commercial biotherapeutic products tested do not contain species commonly found in the lower reproductive tract of women *(Happel et al. 2017*).

A significant amount of microbiome research has been published from cohorts across Africa, including 16S rRNA sequencing of the microbiota from the gut, vagina, lung, and oral cavity (*Allali et al. 2021*), although African scientists have only been first or senior authorship on <21% of the manuscripts, with the vast majority of microbiome research from Africa, including South Africa, being led by scientists from the US. There are several African Centres of

Excellence in HIV prevention, with the expertise to conduct high quality microbiome research in Africa, to understand determinants of reproductive health for women on our continent and lead the development of novel products to improve BV outcomes. This is the time for African Centres of Excellence and African PIs to work together, to lead strategic microbiome research studies on this continent, and to contribute directly to development of novel microbiome-directed treatments for BV in African women. It is unclear whether protective vaginal *Lactobacillus* isolates from US women would confer a health advantage to women in Africa and vice versa. There is thus an urgent need to understand the temporal dynamics of vaginal microbiomes from women in Africa, across consecutive menstrual cycles, focusing on the major determinants of *Lactobacillus* strain stability or resilience on our continent. Identifying African women with stable/resilient vaginal microbiomes will ultimately be critical for selecting vaginal lactobacilli strains to promote vaginal health and treat BV in African women.

Study Objectives

Primary outcomes:

1. Collaboration agreement between two African countries focusing on vaginal health and microbiome interventions for women – initiation of broader collaboration between a network of investigators and Centres of Excellence with necessary skills in conducting reproductive health focused clinical trials across the continent and ability to analyse microbiome data.
2. Publicly available 16S rRNA and ITS gene sequences, comprehensive HPV typing and cytokine data comparing the temporal dynamics of vaginal micro- and mycobiotas (using 16S rRNA gene and ITS sequencing), HPV prevalence and diversity (using the Master Diagnostica direct flow CHIP) and host inflammation (using Luminex) in women (18-35 years) from South Africa (n=100) and Kenya (n=100) who will be intensively followed over two menstrual cycles.
3. Creation of a biorepository of genital tract samples and standardized metadata from the 200 women (including extracted DNA from 5000 self-collected vaginal swabs; and 800 cervicovaginal swabs).

Contingent on additional funding being raised, this biorepository will enable the isolation of *L. crispatus* strains from women with persistently CST1 vaginal communities over 10 weeks that can be compared and characterized [by whole genome sequencing (WGS), antimicrobial susceptibility testing, ability to produce D/L-lactic acid and other metabolites) and evaluated for their ability to compete or displace regionally matched pathobionts (BV-associated bacteria including but not limited to *Prevotella* spp. and *Gardnerella* spp.).

Sustainability:

The long-term vision for this Project is to provide feasibility and infrastructure for African investigators and Centres of Excellence across Africa (bringing onboard partners from Nigeria, Senegal, Zambia, and Rwanda) to expand the ongoing activities of the Gates Foundation

Reproductive Health Technologies Focused research across the continent (VMRC4Africa). We will be leveraging existing networks of African sites and Centres already working together through ongoing and previous investments (through Wellcome Trust and NIH – H3Africa and H3ABionet (PI: Nicola Mulder, UCT); CAPRISA Research Administration & Management Training Program (G11TW010553; PI: Devenie Latchmanan, CAPRISA). Although this pilot includes only two sites (Cape Town, and Kisumu), there is interest, expertise and commitment from other sites in Kenya (U. Nairobi), Rwanda (U. Rwanda), Zambia (UNC Global and CIDRZ), Nigeria (Institute of Human Virology Abuja and Nnamdi Azikiwe University, Awka), and Senegal (Institute for Health Research, Epidemiological Surveillance and Training [IRESSEF], Dakar) to participate, either from the perspective of conducting clinical trials or lab-based microbiome research or both. These centres are currently participating in the BMGF planning grant (PI: Passmore, Mansoor; CAPRISA-UCT). While this CJSL fellowship is an individual award to Jo-Ann Passmore, the broader VMRC4Africa team and vision involves several highly talented early and mid-career biomedical, clinical and bioinformatics researchers from South Africa and other African partner Centres that are committed to vaginal microbiome and HIV prevention research and are positioned to take leadership positions through this initiative. JoAnn Passmore is committed to training the next generation of HIV prevention researchers with expertise in mucosal immunology and biomedical laboratory science and will continue to use this award to nurture and mentor younger African researchers. This is an amazing opportunity to establish an African network focused on vaginal microbiomes and reproductive health, with intrinsic training and translational opportunities for the broader consortium that Passmore represents. This fellowship further aims to leverage the existing informatics training infrastructure created through H3ABionet and the BMGF VMRC4Health.

Scope of Work

1. To demonstrate feasibility for basic collaborative infrastructure (between South African and Kenyan research sites) for conducting clinical studies focused on understanding and comparing regional temporal fluctuations in vaginal microbiota in generally healthy women from Southern and Eastern Africa.
2. To enrol parallel cohorts in South Africa and Kenya. These parallel cohorts will be intensively followed for 10 weeks, to create detailed profiles of cervicovaginal microbiota, mycobiota, and HPV prevalence, and identify women with stable *Lactobacillus*-dominated microbiota, with no STIs and no evidence of genital inflammation.
3. To establish the basic infrastructure for an “African vaginal microbiome biorepository” that can be used for isolation of *L. crispatus* strains from women with persistently CSTI dominant vaginal communities and vaginal pathobiont strains from women who transition between stable CSTI and BV-associated states during follow-up.

Timeline

5 years total; January 2022 – December 2026.

Phase 1 – initiation of the Leadership Fellowship and agreements between BMGF, UCT (primary), DTHF (subaward 1) and KEMRI (subaward 2): January 2022 – December 2022 (year 1)
Phase 2 – Observational Pilot studies in South Africa (DTHF subaward), and Kenya (KEMRI subaward): January/February 2023 – December 2024 (years 2 and 3)
Phase 3 – Laboratory analysis of cervicovaginal 16S rRNA and ITS gene sequencing, HPV typing, and cytokines at UCT: January 2024 – December 2025 (year 3 and 4)
Phase 4 – [Contingent on funding] Isolation of *L. crispatus* and BV-associated bacteria from women in Cape Town and Kenya and evaluation *in vitro:* June 2025 - March 2026 (year 4 and 5)

Study population and eligibility criteria

We propose to enrol 200 young women (18-35 years) at two Sites (100 at the Desmond Tutu Health Foundation, Masiphumelele CRS, 100 at the Kenya Medical Research Institute, Lumumba clinic, and Kargeno Research and Policy Hub, Kisumu CRS). Eligibility criteria will include being HIV-uninfected with no other serious comorbid illnesses, having no clinical or microbiological evidence of BV/STIs at screening, not being pregnant, not having taken antibiotics in the past month, and having no history of cervical disease. All CRSs have extensive experience enrolling women into HIV-related and reproductive health research in Africa. After enrolment, written informed consent will be obtained from participants before performing any procedures. In addition, we will seek consent that vaginal lactobacilli strains isolated through this study can potentially be included in a formulation to treat BV for women in Africa. We will also seek consent for the isolation of additional species (e.g. *Prevotella bivia*, *Gardnerella vaginalis*, etc) for research purposes. At enrolment, a detailed interviewer-assisted questionnaire will be administered assessing demographics, behaviour (sexual and vaginal practices), menstrual and health history, contraceptive choice, based on our previous Questionnaires.

Fig 2. Inclusion/Exclusion

Inclusion Criteria	**Exclusion Criteria**
Female at birth	Male at birth
Willing and able to provide informed consent and cognitive ability to understand sampling procedures	Not willing to provide informed consent
Not pregnant	Pregnant or planning to become pregnant in the next 10 weeks
HIV-	HIV+
18-35 years old	<18 or >35 years old

Sexually active for the last 3 months defined as penetrative penile-vaginal intercourse at least once in the last 3 months	No penetrative penile-vaginal intercourse in the last 3 months
Planning to stay in the area for the next 10 weeks	Planning on moving, going away out of area within the next 10 weeks
Willing and able to return for all 3 nurse visits and return self-swabs to the clinic weekly	On chronic disease management for gynaecological conditions
Able and willing to provide adequate locator information for study retention purposes	Unable to return for all 3 nurse visits and return self-collected swabs to the clinic weekly / Unable to agree to adhere to all study visits and procedures
	Bacterial vaginosis
	Untreated STI
	Currently taking an antibiotic or antibiotic use in the last four weeks
	Any medical condition or other factors which would preclude study participation as per principal Investigator's or designee's decision, including but not limited to cancer of the cervix
	Any mental health condition which, in the opinion of the investigator, would preclude comprehension of informed consent, or preclude study participation.

Sample Size

Prior studies by our groups have demonstrated the vaginal microbiota of reproductive-aged women can be clustered into 5 major CSTs *(Lennard et al. 2018; Balle et al. 2020*). In order to ensure that our study yields interpretable results, enough women need to be sampled so that each of the 5 CSTs are represented. To determine the number of women that must be sampled, we propose to follow an approach adapted from Abdo et al. *(Abdo et al. 2006*). Assuming that the proportions reported in our previous studies (including the uCHOOSE cohort (*Balle et al. 2020*) and the WISH Cohort (*Lennard et al. 2018*), correspond very closely to the population frequencies of CSTs and that they are preserved over time, Abdo et al. derived an equation $\sum_{i=1}^{5} p_i^n = \alpha$ which when solved for n, determines that the study needs at least 100 women in order to obtain data on all five CSTs with 80% probability, where α=0.05 and p_i is the probability of missing the i-th CST estimated as 1 – (frequency of the i-th CST). In particular, we estimate that ~20% of young women enrolled through both CRSs would have

CST I vaginal microbiota that appears to be relatively stable over time. Thus, with a cohort of 200 women, we therefore expect ~40/200 of the participants will cluster into CST I.

Questionnaires and daily diaries to record exposure to sex and antibiotics

A detailed interviewer-assisted questionnaire will be administered assessing demographics, behaviour, sexual and vaginal practices, menstrual and health history, contraceptive choice, based on questionnaires from our previous studies in the same populations *(Barnabas et al. 2018*). In addition, daily diaries will model those previously used by Gajer et al. (*Gajer et al. 2012*) in the form of a yes/no check-off list to report menstrual bleeding; vaginal douching; sexual activity; use of medications or contraceptives, diaphragm, and sanitary care (including sanitary pads, cloths, newspaper, menstrual cups and/or tampons). Like the swabs, behavioural diaries will be submitted to the study site weekly. Data will be captured using RedCap.

STI testing

Vulvovaginal swabs will be tested in-country, for multiplex PCR of CT, NG, TV (SA Clinical site) and additionally for HSV and MG (Kenyan clinical sites). Lateral vaginal wall swabs will be used to screen for BV (Nugent scoring) and yeast (hyphae and spores). SoftCup pellets will be used for HPV genotyping and to run Hybrispot HPV Direct flow chip assays.

Assessment of vaginal microbiota

Sample Preparation and Illumina 16S iTag library preparation. - DNA will be extracted using the PowerSoil DNA kit (MoBio), after mechanical and enzymatic disruption using lysozyme, mutanolysin and bead beating. 16S rRNA gene V3-V4 region will be amplified using universal 319F/806R primers, and quality checked with Bioanalyzer (Agilent). Pooled triplicate samples will be purified with Agencourt AMPure XP beads (Beckman Coulter). Amplicons will be pooled in equal quantities. Purified libraries consisting of ~120 pooled samples will be sequenced with the Illumina MiSeq platform (300 bp paired-end with v3 chemistry).

The nuclear ribosomal ITS2 region will be amplified using universal fITS7/ ITS4 primers, and PCR products will be purified with Agencourt AMPure XP beads (Beckman Coulter). Nested PCR will be run to add the barcodes and Illumina NexteraXT indexed adapters and the amplicon pool will be sequenced with the Illumina MiSeq platform (300 bp paired-end with v3 chemistry). A commercial standard containing two fungal species, *Saccharomyces cerevisiae* and *Cryptococcus neoformans*, will be used as a positive control.

16S rRNA gene sequence data analysis. - The Divisive Amplicon Denoising Algorithm (DADA) 2 algorithm will be used to infer amplicon sequence variants (ASVs). The SILVA rRNA gene sequence database will be used for taxonomic assignment. The resulting phylogenetic tree,

ASV table and taxonomic table combined with relevant metadata will be consolidated into a phyloseq object using the phyloseq R package to perform community compositional analyses and ordination.

ITS sequence data analysis. – The RDP Initial Process tool will be used to perform data quality control of raw sequences and to infer parameters for downstream processing. CD-HIT will be used to infer amplicon sequence variants (ASVs). The RDP Classifier against the Warcup database, a manually-curated subset of sequences from the UNITE database, will be used for taxonomic assignment. Taxa will be reported as relative abundance (% of total sequences).

Rarefaction Analysis, Diversity Estimates, and Sample Ordinations. - To assess quality of microbial diversity sampling, multiple rarefactions at different sequencing depths will be performed. α-diversity estimates, richness (Chao1), evenness (Simpson's E) and phylogenetic diversity (Faith's PD) will be calculated using the R vegan library. β-diversity between samples will be estimated by UniFrac distances. An all-by-all pairwise distance matrix based on UniFrac distances will be generated and used to hierarchically cluster and ordinate samples. The ordinations will be performed using Nonmetric Multidimensional Scaling (NMDS). Distinct community types will be defined based on hierarchical clustering and examination of speciesslevel classifications.

Total 16S rRNA gene copy number measurement

The total number of 16S rRNA gene copies in each DNA sample will be measured using the TaqMan®Bact-Quant assay targeting the V3–V4 regions of the gene. The total number of 16S rRNA gene copies will be expressed as copy per swab and is used as an estimate of bacterial load (total count of bacterial cells present in a sample). An estimate of absolute abundance of each taxon for each sample will be calculated by multiplying the total 16S rRNA gene copies obtained by qPCR and the relative abundance of that taxa obtained by 16S rRNA gene sequencing.

CST assignment

According to VMRC4Health best practice, taxonomic profiles of vaginal microbiota will be sorted using VALENCIA, a tool which instead classifies a sample to a CST based on the sample's similarity scores to a reference community derived from bacterial taxonomic profiles from 13,161 vaginal samples, including women from different ethnicity and ages *(France et al. 2020)*.

Measurement of cervicovaginal cytokines

We will measure inflammatory cytokines in menstrual cup secretions in all women at 3 visits (enrolment, 5 and 10 weeks). Samples will be stored at -80°C until the assay is performed. Cytokine concentrations in genital secretions will be assessed using Luminex®. Data will be

collected using a Bio-PlexTM Suspension Array Reader (Bio-Rad Laboratories Inc®) and a 5 PL regression formula used to calculate cytokine concentrations from the standard curves, and analyzed using BIO-plex manager software (version 4; Bio-Rad Laboratories Inc®). As we have previously defined, genital tract inflammation in this study will be considered as women having >5/9 of the following cytokines/chemokines in the top 75th percentile of the population: IL-1α, IL-1β, IL-6, TNF-α, IL-8, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β. All women below the 75th percentile of these cytokines will be considered to be not inflamed.

Data analysis

The daily dynamics of relative abundance of the 30 most abundant taxa, and CST distribution will be visualized using stream graph representation and the measure of community constancy over time used by Gajer et al. (*Gajer et al. 2012*) will be applied. The average population-wide deviation from constancy of vaginal bacterial communities will be calculated for each woman across both sites. In addition, we will use data generated on genital inflammation/cytokines to assign inflammation categories to women with cytokines in the >75th percentile of the cohort. Integration of microbiota and cytokine data will be performed via Bayesian network learning. We will also control for epidemiological variables such as age, site, hormone contraceptive use, STI/BV status.

Participating Sites

UCT

Dr Passmore's Mucosal Infections Group at UCT and the IDM (www.passmore-lab.org.za; http://www.idm.uct.ac.za/Jo-Ann_Passmore) was established in 2003, with >100 published studies in HIV and HPV prevention and pathogenesis research involving women in Africa, including both immune biomarker and microbial research approaches to address genital inflammation which drives both increased HIV and HPV risk and pathogenesis.

Dr Heather Jaspan (http://www.idm.uct.ac.za/Heather_Jaspan) is an ID Paediatrician MD who set up several clinical trials through her affiliation with UCT and the IDM over the past 15 years, establishing vaginal microbiome expertise within the IDM since 2013. Heather has published a significant number of high impact vaginal microbiome papers in the past 5 years, demonstrating the central role of vaginal dysbiosis and genital inflammation in adolescent and young women. With Anna Happel, Heather will lead analysis of 16S rRNA gene sequencing and metagenomics related to samples from these cohorts.

Dr Anna Happel (https://www.linkedin.com/in/anna-happel-627859112) is an early-career researcher working with Heather Jaspan with more than 5 years' experience isolating and characterizing vaginal *Lactobacillus* strains for developing novel treatments for BV (the focus on her PhD and Post-Doctoral research; Happel et al., 2020a). Anna has experience with SAHPRA around conducting a pilot probiotic trial at UCT, completed during her PhD, demonstrating the regulatory pathway surrounding live bio-therapeutic treatment in South Africa (Happel et al., 2020b).

Dr Brian Kullin (https://scholar.google.com/citations?user=IfEFtkkAAAAJ&hl=en&oi=ao) is an anaerobic microbiologist and Senior Research Scientist working with Jo-Ann at UCT, leading the establishment of the IDMs Microbiome and Anaerobe Core facility since joining in 2018. Brian completed his post-graduate degrees at UCT focusing on developing probiotics for the prevention of kidney stones and subsequently expanding to other gut-associated pathogenic anaerobes like *Clostridioides difficile*.
Drs Nicola Mulder (http://www.idm.uct.ac.za/Nicola_Mulder) is a IDM full member and Professor at the Computational Biology Unit at UCT. Dr Mulder heads H3ABionet (https://www.h3abionet.org/) that we will leverage for computing infrastructure, training and microbiome pipeline support for this project.

KEMRI

Prof Elizabeth Bukusi FAAS (https://www.aasciences.africa/fellow/bukusi-elizabeth-anne) is a research professor working within the field of obstetrics and gynaecology, and global health, with her main areas of research focus around sexually transmitted infections, women's health, reproductive health, and HIV care, prevention and treatment. Prof Bukusi serves as a senior principal clinical research scientist at KEMRI and a research professor at the University of Washington and has led several pivotal studies on the use of PrEP in Kenya. Completing her PhD in 2006 which focused on BV recurrence and randomized clinical trials to prevent BV, she has been a champion for improved BV treatment for women in LMICs.

Dr Serah Gitome (https://www.researchgate.net/profile/Serah-Gitome) is a clinical research scientist and public health expert at the Kenya Medical Research Institute (KEMRI) with over 14 years of experience in designing and implementing HIV prevention and sexual reproductive health research in Kenya. Her research interests focus on optimizing women's access to selfcare contraceptives and other products using human centered design approaches, femaleinitiated HIV prevention methods and safer conception strategies for HIV-discordant couples. Dr Gitome is passionate about ethical conduct of research in Kenya and serves as a member of the KEMRI IRB. Dr. Gitome obtained her MBChB degree from the University of Nairobi, Kenya, and MPH from the University of California, Berkeley.

DTHF

Prof Linda-Gail Bekker (http://www.idm.uct.ac.za/Linda-Gail_Bekker) is the Chief Operating Officer for the Desmond Tutu HIV Centre and Deputy Director at the Desmond Tutu HIV Foundation; Full Member at the IDM), and immediate ex-President of the International AIDS Society (IAS). She is a physician-scientist with a keen interest in HIV, tuberculosis and related diseases. Her research interests include programmatic and Health Service research around antiretroviral roll-out and TB integration, prevention of HIV in women, youth and men who have sex with men. She is passionate about community development and engagement; her most recent community projects have included community-based HIV treatment, peer-led community education, mobile health services (Tutu testers) to the neediest populations, a comprehensive youth centre providing recreation, education and SRH services to youth from

peri urban settings and dedicated adolescent HIV care services. She has been collaborating with Dr Passmore for more than 15 years on projects related to mucosal HIV risk and HIV biomedical mechanisms.

Dr Katherine Gill (https://www.linkedin.com/in/katherine-gill-81b27277) is a Clinician researcher at DTHF and Clinical Research Site Director at Masiphumelele, Cape Town where the South Africa part of the study will be done. Katherine has more than 10 years of experience working in HIV/TB clinical management, grant management, and clinical research, running the Masiphumelele research site which specializes in HIV Prevention Clinical Trials and COVID research. Katherine Gill has been collaborating closely with Dr Passmore for more than 5 years on the uCHOOSE study, which focused on hormone contraceptive choices in adolescents in a randomized cross-over trial.

Data Collection and Handling

The investigator agrees to maintain accurate source documentation and case report forms. For each participant screened, an electronic CRF (eCRF) will be completed, even if the participant drops out at any time during the study. All eCRF information for the completed visits must be completed.

All applicable eCRF pages must be completed for each subject who has completed the study. For participants who are prematurely withdrawn, the visits up to withdrawal plus the withdrawal visit need to be completed. In the case of necessary corrections, all changes will be effectuated such that the first version is still legible, and the change is initialled and dated by the correcting person.

Data entered into the eCRF is, on saving the eCRF, stored in a web-based database, hosted on a secure server at UCT (RedCap). The database will have user access control, and all changes are tracked in an audit trail. Further, data quality is ensured by a series of pre-programmed edit checks to ensure plausibility, and by manual curation by a professional data manager. The server is subject to regular backups and access control.

Source Documents

Source data is defined as all information in original records and certified copies of original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source documents will include, but are not limited to, progress notes, electronic data, screening logs, and recorded data from automated instruments. Study data will be directly entered into the electronic CRF, in which case the eCRF is the source. Some data may still be captured entirely or partially on paper source documents, and may then manually be entered into an eCRF, including but not limited to:

- Informed Consents
- Sample collection data (may be on Lab Forms), and

- Laboratory results and their review by the investigator
- Participant locator information

All paper source documents and the electronic source documents pertaining to this trial will be maintained by the investigators. The investigator has to permit trial-related monitoring, audits, Independent Ethics Committee/Institutional Review Board (IEC/IRB) review, providing authorized persons direct access to source data/documents.

Study Assessments

Demographic and Background Assessment

- Written informed consent
- Eligibility assessment
- Demographic data: date of birth, gender
- Targeted physical exam including Weight in kilograms (kg) and Height in meters (m)
- Social history (sexual risk behaviour)
- Information about the recent or current antibiotic use
- "Key informant" socio-economic questionnaire on the household
- Locator information including address and contact information (names and phone numbers)
- Clinically significant medical and treatment history (including HIV status, ART, IPT and previous TB episodes, diabetes)
- Questionnaire on socio-economic, exposure and risk parameters
- STI symptom screen

Pre-test counselling

The counselling process will be conducted to address individual HIV risk in the language that the participant understands. The information session will include the following key components if appropriate to the circumstances:

- Information about HIV acquisition and transmission.
- Emphasis on the importance and advantages of early HIV testing to facilitate diagnosis, positive living, and healthy lifestyle.
- Information about the HIV testing process.
- Discussion on confidentiality and shared confidentiality.
- Discussion on the option not to take the test.
- Offer an opportunity to test at a later date should the client decline the test.
- The importance of TB symptomatic screening during pre- and post-test counselling.
- Referral to HIV and AIDS related services
- Importance of partner testing, especially in ANC settings.

- Information about effective HIV prevention measures, including consistent and correct use of condoms, partner reduction, and other options.

In addition, if time permits the provider may also discuss the following components as appropriate to the circumstances:

- Assessment of individual risk and when a possible exposure may have occurred.
- Determining whether there is a history of domestic violence.
- Discussion on partner involvement and referral for testing.

Pregnancy urine test

A urine pregnancy test will be used to identify raised HCG. The test will be performed in the lab and read at 3 minutes or as test manufacturer recommendations.

HIV rapid

A single-use rapid immunoassay for the qualitative detection of antibodies to HIV-1 and HIV2 intended for use in point of care settings as an aid in diagnosis of HIV-1 and HIV-2 infection will be used and read at 20 minutes or as test manufacturer recommendations.

*HIV + confirmatory test

A confirmatory test in cases where the screening test is reactive will be ordered. The preliminary result will be verified with a second rapid test that works in a slightly different way.

Post-test counselling

All participants, regardless of the outcome of the HIV test, will be offered and receive post-test counselling based on their test result. All results will be given clearly in a language the participant is most comfortable with. The length of the post-test counselling session will vary based on the test result. The post-test counselling prevention package will include information and advantages of TB screening, risk reduction and correct and regular use of condoms. Depending on the participant's circumstances there may be a need for them to retest for HIV. The participant may be encouraged to bring their partner in for couples testing, depending on the client's circumstances. there may be a need for them to re-test for HIV. See section 4.5 for when to recommend re-testing. The participant may further be referred for additional prevention counselling if the participant reports ongoing risk.

Physical Examination

The physical examination may include:

- height and weight measurements
- nose, mouth, throat, and ear examination with a torch or scope

- Vitals
- checking the body's reflexes
- listening to the heart and lungs with a stethoscope
- feeling the lymph nodes in the neck, underarms, or groin
- feeling the abdomen to check for abnormalities

Amsel Test

- 1x lateral wall vaginal wall swab to be applied to a pH indicator, used for wet mount preparation and applied to a second glass slide for the whiff test (addition of a few drops of 10% potassium hydroxide solution).
- Description of any vaginal discharge

Speculum Exam

Will include:

- physical examination of the vagina, vulva
- Insertion of a speculum for examination of the cervix
- checking for any abnormalities that may be indicative of cancer, sexually transmitted disease, or other benign conditions
- Swabs for STI and BV testing

STI testing

1x Vulvo-vaginal swab and 1x high vaginal wall swab will be taken for STI and BV testing Testing will include:

- Chlamydia (SA + Kenya)
- Gonorrhoea (SA + Kenya)
- Trichomoniasis (SA + Kenya)
- Herpes Type 1 & 2 (Kenya)
- Mycoplasma genitalium (Kenya)

Nugent scoring

1x lateral vaginal wall swab to be applied to the pH indicator and rolled on a glass slide. Nugent score on Gram smears will be used as the diagnostic test that defines normal vaginal flora, BV, or intermediate abnormal flora.

ITS and 16S rRNA microbiome sequencing

1x lateral vaginal wall swab for qPCR and 16S rRNA (bacterial) & sequencing (Molecular transport medium [Qiagen]). ITS of nuclear DNA will be used as a target for analysing fungal diversity in the genital samples, as the standard marker for fungal DNA barcoding.

Cytokine Measurement and HPV genotyping

1x soft cup will be used for the cytokine measurements (supernatant) and HPV genotyping (pellet).

Long-term sample storage (Bio-bank)

1 X lateral vaginal wall swab for culture and storage (Amies-based transport solution, eg Eswab for culture)
2 X lateral vaginal wall swabs stored for use in future studies (proteomic and metabolomic, swabs stored in protective buffer and dry respectively)
1 X cytobrush stored for use in future studies (transcriptomic)
Samples from VMRC4Africa Pilot participants will be stored for three purposes:
Perform experimental assays and sub-studies, after end of recruitment and follow-up of participants
Long-term storage for further research on yet undefined diagnostics and other infectious disease research questions

Product formulation

Both short- and long-term storage samples will be cryopreserved at the recruiting health research institutions or designated laboratories in their countries. Long-term storage of samples will only be permitted for samples of participants who have signed the sample storage and future use consent.

Participant enrolment

A total of 200 women will be enrolled at two sites (Cape Town, South Africa, and Kisumu Kenya). Participants who meet all of the inclusion criteria and none of the exclusion criteria will be followed intensively for 10 weeks. Eligible participants will be enrolled in line with their menstrual cycle to avoid blood contaminated genital samples for processing and storage. Those who are deemed eligible but present with recent antibiotic use or treatable STIs will wait 28 days post antibiotic treatment before enrolment. Thus, screening to exit may be extended to 16 weeks if appropriate.

Recruitment process

Participants will be recruited via the CRS standard recruiting process. Participants will be informed about the study via social media, flyers and posters.
Each site will use a variety of recruitment approaches that works best for the local setting. Recruitment may be conducted through the following possible approaches: community events and mobilisation, partnerships with appropriate programs and via popular social media platforms. Recruitment materials will educate women about HIV, sexual health, and risks in their community, the effectiveness of PrEP for HIV prevention, and the benefits of HIV prevention services. Recruitment will occur over approximately 12 months.

Recruitment strategy DTHF.

The Masiphumelele Research Office is strategically positioned on the outskirts of a discreet, relatively moderate sized township community (Masiphumelele) and in close proximity to a second low socioeconomic, high-density community (Ocean View). It has easy access to a population of >50 000 people. In close proximity, there are also a number of middle-class suburbs with a combined population of >50 000 people. The site is planning to recruit in all of these areas via the two primary care clinics in Masiphumelele and Ocean View and the district hospital in Fish Hoek.

The site actively tracks research visits and sends reminders to participants before each visit window opens. The site also pro-actively follows up with participants who have missed target dates to avoid missed visits. The site will also do home visits and transport participants to and from the site as needed.

Recruitment strategy KEMRI.

Recruitment will take place in Kisumu city and its environs. We will work with staff at strategic pharmacies and maternal child health/family planning (MCH/FP) clinics to identify potential participants and refer them to the study staff for recruitment based on our eligibility criteria. At the community level, study staff members will work with community health volunteers (CHVs) where possible, to sensitize potential participants about the study and directly refer potential participants to the study staff or give phone contacts to those interested to participate in the study to call the study team for additional information about the study. Study staff will also carry out door-to-door recruitment of women within the community to complement the recruitment done by CHVs. Study staff will leverage community meetings/gatherings to sensitize the community on the study and identify potential participants for recruitment. Recruitment activities will be conducted in line with current COVID-19 infection prevention measures and guidelines to ensure safety of both participants and staff.

All materials to advertise the study (leaflets and posters) will be used only upon approval of the ethics committees and/or regulatory authorities relevant for this study. The investigator or delegate may discuss certain aspects of the study with potential participants before consenting, e.g. to determine eligibility/ pre-screening.

Participant informed consent

Participants who fit the eligibility criteria will be invited to join the study. They will be given an informed consent form (ICF) about the study and will be explained the anticipated benefits and the potential risks associated with the protocol procedures. The principal investigator (PI) or designee will fully inform the participant, or the participant's legally acceptable representative. The language used will be as non-technical as possible and the participant will not unduly be influenced to participate in the study. Participants will be informed that they may voluntarily withdraw from the study for any reason at any time, and a withdrawal will have no negative effects on them receiving standard care afterwards.

Written consent must be obtained from every participant prior to any procedures being done specifically for the study.

An impartial witness is required for the entire informed consent process for any participant who is illiterate or whose literacy is limited. Documentation of the presence of a witness will be achieved through their signature on the informed consent document. Illiterate participants will indicate their consent via use of their mark (finger/thumb print) on the informed consent documents.

The signed original ICF will be kept with the Investigator Site File at the site. A second original of the signed informed consent form, will be given to the participant or to the participants' legally acceptable representative. The site will document the name and position of those persons at the site who are responsible for obtaining informed consent and no other staff will perform this.

In the event any modifications to the ICF are proposed, the consent form will be re-submitted to the ethics committee for approval. The ICF will be revised whenever new important information becomes available. All active participants will additionally sign all revised ICFS, after ethics approval is received.

Incentives and expenses

Participants will not receive any incentives for their participation in the study. However, participants will be reimbursed for their time and transport costs to the research clinics. The exact amount will be decided by each of the sites and informed by the community advisory committees.

Participant withdrawal and/or loss to follow-up

A participant may decide to withdraw from the study at any time and for any reason. The investigator may also withdraw a participant for any of the following reasons:

- Participant is unwilling or unable to comply with required study procedures
- If, for any reason, the investigator concludes that continued participation in the study would not be in the participant's best interest
- Early study termination

When a participant withdraws or becomes lost to follow-up from the study, the reason(s) for withdrawal or lost to follow-up shall be recorded by the investigator or designee in participants' study records.

Should a participant become HIV positive or pregnant after enrolment, they will be allowed to continue to attend clinic visits and participate in the interviews/questionnaires if they choose to do so. This is to reduce the potential impact of stigma associated with a positive HIV diagnosis and/or unplanned pregnancy. However, no further mucosal samples will be collected, since for HIV positive participants this poses an additional risk to study personnel who will need to handle and process potentially infectious samples and for pregnant participants, the additional discomfort and risk associated with speculum exam and sampling are difficult to justify given that they will not be included in the study analysis.

Participants who become infected with an STI after enrolment (TV, NG, CT) will be referred for treatment, but can continue to provide samples should they choose to do so.

Study procedure

Screening

Potential participants identified will be invited to be screened for inclusion in the study. Before any study-specific screening procedures will be performed, all participants will sign and date (or thumbprint) the latest version of the ICF. Illiterate participants will be encouraged to have an independent witness present. Once a participant has signed a consent form, their details, including date and participant number, will be registered on a screening log.

After informed consent has been obtained, the following screening procedures will be performed and documented:

- Participant locator information
- Pre-test counselling
- Sexual risk behaviour questionnaire
- Pregnancy urine dipstick

- HIV rapid
- *HIV + confirmatory test (in the event of an HIV+ rapid test)
- Post-test counselling
- Targeted Physical examination
- Speculum examination and collection of
    - 1x Vulvo-vaginal swab for STI testing
    - 1x lateral vaginal wall swab to be applied to pH indicator and rolled on a glass slide for Nugent scoring
    - 1x lateral vaginal wall swab for qPCR and 16S rRNA (bacterial) & ITS (fungal) amplicon sequencing (Molecular transport medium [Qiagen])
    - 1x Lateral wall swab for culture (Amies-based transport solution, eg E-Swab [Copan]) (culture)
- STI/BV treatment as indicated by local symptomatic treatment protocol

If the in-/exclusion criteria are fulfilled, the participant will be enrolled in the study. All information will be recorded on a case report form (CRF or eCRF). In addition, contact information will be recorded on a locator form.

The screening and enrolment may not be done at the same visit.

Additional Screening Test (Amsel test for BV)

Participants who attend the screening visit, but who are not enrolled in the longitudinal study due to having bacterial vaginosis will still provide samples during their screening examination. Once approximately 100 such participants (screened but not enrolled) have been screened at each site, an additional pre-screening test (Amsel test for bacterial vaginosis) will be included to enrich for participants without BV. This will be done during the physical exam after the pregnancy and HIV tests and prior to the speculum examination. Participants who test positive for 3 or more of the Amsel criteria (thin homogenous/milky vaginal discharge, vaginal pH greater than 4.5, positive whiff test [fishy amine odour on addition of 10% KOH] and presence of clue cells on wet mount microscopy), will be screened out and not undergo the speculum exam.

Enrolment

The enrolment visit has to be conducted within 6 weeks of the screening visit. Before any study-specific screening procedures will be performed, all continuing consent will be obtained and documented. The investigator or delegate may discuss certain aspects of the study e.g. to determine continued eligibility. The following information will be collected and documented:

- Participant locator information re-confirmed
- Pre-test counselling
- Sexual risk behaviour questionnaire

- Pregnancy urine dipstick
- HIV rapid
- *HIV + confirmatory test (in the event of an HIV+ rapid test)
- Post-test counselling
- Speculum collection of
    - 1x Vulvo-vaginal swab for STI testing
    - 1x high vaginal wall swab for storage
    - 1x lateral vaginal wall swab to be applied to pH indicator and rolled on a glass slide for Nugent scoring
    - 1x lateral vaginal wall swab for qPCR and 16S rRNA (bacterial) & ITS (fungal) amplicon sequencing (Molecular transport medium [Qiagen])
    - 1x soft cup (cytokines) inserted for 30 mins
    - 1x Lateral wall swab for culture (Amies-based transport solution, eg E-Swab
    - [Copan]) (culture)
    - 1 x lateral vaginal wall swab for storage (proteomic)
    - 1 x lateral vaginal wall swab for storage (metabolomic)
    - 1 x cytobrush for storage (transcriptomics)

Week 5

The mid-way week 5 visit has to be conducted when the participant is not menstruating, a ±7day visit window is allowable to account for this. Before any study-specific screening procedures will be performed, all continuing consent will be obtained and documented. The investigator or delegate may discuss certain aspects of the study e.g. to determine continued eligibility. The following information will be collected and documented:

- Participant locator information re-confirmed
- Pre-test counselling
- Sexual risk behaviour questionnaire
- Pregnancy urine dipstick
- HIV rapid
- *HIV + confirmatory test (in the event of an HIV+ rapid test)
- Post-test counselling
- Speculum collection of
    - 1x Vulvo-vaginal swab for STI testing
    - 1x high vaginal wall swab for storage
    - 1x lateral vaginal wall swab to be applied to pH indicator and rolled on a glass slide for Nugent scoring
    - 1x lateral vaginal wall swab for qPCR and 16S rRNA (bacterial) & ITS (fungal) amplicon sequencing (Molecular transport medium [Qiagen])
    - 1x soft cup (cytokines) inserted for 30 mins

- 1x Lateral wall swab for culture (Amies-based transport solution, eg E-Swab
- [Copan]) (culture)
- 1 x lateral vaginal wall swab for storage (proteomic)
- 1 x lateral vaginal wall swab for storage (metabolomic)
- 1 x cytobrush for storage (transcriptomics)

Exit

The exit visit has to be conducted when the participant is not menstruating, a ±7-day visit window is allowable to account for this. Before any study-specific screening procedures will be performed, all continuing consent will be obtained and documented. The investigator or delegate may discuss certain aspects of the study e.g. to determine continued eligibility. The following information will be collected and documented:

- Pre-test counselling
- Sexual risk behaviour questionnaire
- Pregnancy urine dipstick
- HIV rapid
- *HIV + confirmatory test (in the event of an HIV+ rapid test)
- Post-test counselling
- Physical examination
- Speculum collection of
    - 1x Vulvo-vaginal swab for STI testing
    - 1x high vaginal wall swab for storage
    - 1x lateral vaginal wall swab to be applied to pH indicator and rolled on a glass slide for Nugent scoring
    - 1x lateral vaginal wall swab for qPCR and 16S rRNA (bacterial) & ITS (fungal) amplicon sequencing (Molecular transport medium [Qiagen])
    - 1x soft cup (cytokines) inserted for 30 mins
    - 1x Lateral wall swab for culture (Amies-based transport solution, eg E-Swab [Copan]) (culture)
    - 1 x lateral vaginal wall swab for storage (proteomic)
    - 1 x lateral vaginal wall swab for storage (metabolomic)
    - 1 x cytobrush for storage (transcriptomics)

Self-collected samples

Self-collected low vaginal swabs in molecular transport medium [Qiagen] for qPCR and 16S rRNA (bacterial) & ITS (fungal) amplicon sequencing will be collected weekly and stored in a cool dry place and dropped at the CRS as soon as possible or at the next study visit.

Self-collected samples will also be taken at the week 5 study and week 10 exit visits. These will be compared to the nurse collected swabs at the same visits to evaluate differences between 16S rRNA and ITS fungal sequences possibly related to collection method (nurse collected vs self-collected)

Weekly questionnaire

Weekly yes/no questionnaires will be administered by telephone or WhatsApp along with reminders for self-collected samples. The questionnaires will collect sexual behaviour and exposure to antibiotics data to inform the study data.

The questionnaire will be administered at weeks 3-4 or 5 if the week 5 visit is delayed due to menstruation. As well as weeks 6-9 or 10 if exit is delayed due to menstruation.

Dissemination of study results

The dissemination strategy and activities will follow principles and best practices successfully tested by the partners in other projects and in line with the South African Guidelines for successful dissemination:

- All research results/reports will be duly reviewed, and a copy will be sent to relevant collaborators and partners involved in the project before these are disseminated and/or published.
- When appropriate, the reports will refer to other research projects and build on the existing results and literature.
- Research will be conducted following sound analysis and scientific practice principles, taking into account as much as possible policy requirements and needs.
- All public results will be accessible from the project website and usable for all parties who may benefit from them.

Dissemination timing

Dissemination activities are planned in accordance with the stages of the project. A number of dissemination activities will take place during the first 18 months of the project, the most significant dissemination activities will take place as final research results are available.

The dissemination activities are to be performed according to the following logical schedule:

1) Initial awareness phase (month 12-15): this establishes community awareness of the VMRC4Africa initiative (posters, pamphlets, peer educators, local press

releases, and community engagement in a variety of forms) and analysis of relevant information resources in terms of identification of dissemination opportunities.

2) Targeted dissemination phase (month 24-30): the consortium will enrich their websites, update the project communication kit, attend selected events and organize workshops. Host a participant dissemination event to present the study results.
3) Post study (month 60-63): this represents the period when VMRC4Africa consortium partners will start dissemination to the scientific community. This phase will also focus on informing the target audience of the VMRC4Africa exploitable outputs.

Statistical considerations

Sample size consideration

As outlined above.

Statistical methodology

A Statistical Analysis Plan will be developed before analysis.

Baseline characteristics, such as demographic and analytical data will be summarised using descriptive statistical methods. Continuous data will be summarized using the mean, the median, standard deviation, and the range (minimum and maximum value). Categorical values will be summarized using frequency counts and percentages. Sensitivity and specificity of tests predicting CST 1 will be determined and ROC curves calculated. Positive and negative predictive values will be calculated.

Other analysis

The daily dynamics of relative abundance of the 30 most abundant taxa, and CST distribution will be visualized using stream graph representation and the measure of community constancy over time used by Gajer et al. (*Gajer et al. 2012*) will be applied. The average population-wide deviation from constancy of vaginal bacterial communities will be calculated for each woman across both sites. In addition, we will use data generated on genital inflammation/cytokines to assign inflammation categories to women with cytokines in the >75th percentile of the cohort. Integration of microbiota and cytokine data will be performed via Bayesian network learning. We will also control for epidemiological variables such as age, site, hormone contraceptive use, STI/BV status.

Data Management

Data collection

The investigator agrees to maintain accurate source data and electronic Case Report Forms. For each participant enrolled, a CRF will be completed, even if the participant withdraws or is lost to follow-up at any time during the study. All information from a performed visit will be entered in the CRF/eCRF.

Source documents

The investigator agrees to maintain accurate source documents as part of the case histories and permits direct access for domestic and foreign regulatory authorities, monitors and auditors. Some data will be recorded directly into the CRF/eCRF and will be considered source data.

Record retention

The investigator will keep essential study documents (including CRFs/eCRFs) other than participant medical files:

1. for at least 15 years after completion or discontinuation of the study
2. or until at least two years after the last approval of a marketing application in an ICH region and until there are no pending or contemplated marketing applications in an ICH region
3. or at least 2 years have elapsed since the formal discontinuation of clinical development biotherapeutic products

Confidentiality of personal data

All participant records, lab specimens etc. will be identified in a manner to maintain participant confidentiality and will be kept in a secure storage area with limited access. Each participant will be assigned a pseudonymous identification number. No data that could identify the participant other than this identification number (and the sex and date of birth) will appear on the CRFs or resultant publications.

Data of study participants will only be used as defined in the ICF and in line with applicable data privacy regulations. Accordingly, participant records may be reviewed by inspectors of regulatory authorities or ethics committees, who ensure the quality of the study.

An individual's study data will not be released without the written agreement of the participant (or their legal guardian), except as necessary for monitoring and auditing by regulatory authorities or ethics committees, or in case of medical emergencies when written

consent cannot be obtained, as deemed in the participant' s best interest by the investigator. Results of any tests will not be disclosed to anybody not involved with the study, in particular not to immediate relatives without prior consent of the participant.

Ethical consideration

Basic principals

This study will be performed in accordance with the study protocol, the ICH-Harmonised Tripartite Guideline for GCP E6 (R2) (2016)/Declaration of Helsinki (October 2013)/CIOMS International Ethical Guidelines for Biomedical Research Involving Human Subjects (November 2002) as well as any other applicable national and other regulatory guidelines.

Involvement of Ethics Committees and Regulatory Authorities

The protocol and the informed consent document to be used in this study must be submitted to the responsible investigator's ethics committee and regulatory authority, and also to the sponsor's local EC for approval. The country-specific ethics' committees are stated in the sitespecific protocols. Written documentation of approval of both the protocol and the informed consent must be provided to the sponsor before starting the study.
The investigator will promptly report to the EC deviations from the protocol and all unanticipated problems involving risks to human subjects or others, and will not make changes in the research without EC approval, except where necessary to eliminate apparent immediate hazards to human subjects.

Protocol Amendment Policy

Any substantial change to the protocol will be effected by means of a protocol amendment and will have to be submitted to ethics committees and regulatory authorities. No amendment will be implemented until approved and signed by all required parties. Exceptions to this are when the investigator considers that the subject's safety is compromised. Protocol amendments detailing minor administrative changes should be submitted by the investigator to ethics committees and regulatory authorities for notification purposes as appropriate.

Administrative concerns

Financing

The study sponsor is Mucosal Infection Group, Division of Medical Virology, Faculty of Health Sciences, University of Cape Town.

The study is funded collaboratively by the Bill and Melinda Gates Foundation

Study Registration

As the study is an observational pilot study, it does not need to be registered with the ClinicalTrials.gov study registry or SAHPRA.

Participant Insurance and Compensation

The investigators certify that clinical study insurance for this study is not necessary, as this study does not include an experimental intervention that has an influence on patient management. This does not relieve the investigators of the obligation to maintain their own liability insurance as required by applicable law. The investigator does not assume any obligation for the medical treatment of other injuries and illnesses.

Training for Staff Involved in the Study

The site investigators will ensure all personnel are trained on all procedures relevant for their study responsibilities. Particular emphasis will be put on informed consent procedures, confidentiality, speculum examination, samples collection, sample storage and data handling.

Publication Policy

After completion of the study, the data may be considered for presenting at a scientific conference or for publication in a scientific journal. The sponsor will be responsible for these activities and will collaborate with the investigators to determine how the manuscript/s are written and edited, the number and order of authors, the journal to which it will be submitted and other related issues. The results of the study will be published independent of the outcome – positive or negative - of the study. Under certain circumstances, i.e. when the publication of particular findings (of an epidemiological, sociological or genetics study) may present a risk to the interest of a community or population or a racially or ethnically defined group of people, it may be considered inappropriate to publish findings.

Infection Control and Prevention

Local and national guidelines for infection prevention and control (ICP) will be strictly adhered to and will be described in detail in site specific infection prevention SOPs. ICP procedures will aim to prevent airborne disease transmission. Social distancing will be observed; face to face interactions will be conducted in a well-ventilated space. Participants will be asked to wait in the open air with appropriate social distancing observed. Staff will wear personal protective equipment.

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