# Tenofovir disoproxil fumarate intravaginal ring for HIV pre-exposure prophylaxis in sexually active women: a phase 1, single-blind, randomised, controlled trial

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## Summary

**Background** An intravaginal ring that releases the tenofovir prodrug, tenofovir disoproxil fumarate, provided 100% protection in macaques against simian HIV and was safe in a 14-day clinical trial in sexually abstinent women. We aimed to assess the safety and pharmacokinetics of this intravaginal ring over 90 days in sexually active women.

**Methods** We did a phase 1, single-blind, randomised, placebo-controlled trial to assess safety, pharmacokinetics, and acceptability of a tenofovir disoproxil fumarate intravaginal ring used continuously with monthly ring changes for 3 months. Sexually active women who were HIV negative were randomly assigned (3:1) to a tenofovir disoproxil fumarate ring or placebo ring. Primary safety endpoint was the proportion of women who had grade 2 or higher genitourinary adverse events judged related to study product and any grade 2 or higher adverse event as defined by the Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events. We quantified tenofovir disoproxil fumarate and tenofovir concentrations in cervicovaginal fluid, tenofovir in plasma, and tenofovir diphosphate, the active metabolite, in cervical tissue and dried blood spots 1 month after each ring insertion. We compared changes over time in cervicovaginal fluid cytokine and chemokine concentrations and vaginal microbiota. The study was electively stopped early and is registered with ClinicalTrials.gov, number NCT02762617.

**Findings** Between Feb 24 and July 20, 2017, 17 women were enrolled before study termination. 12 were assigned to receive the tenofovir disoproxil fumarate ring and five were assigned to receive the placebo ring. Two participants in the tenofovir disoproxil fumarate ring group completed 3 months of continuous ring use; eight were asked to discontinue ring use early because of ulcerations (grade 1) near the ring; in the remaining two women, rings were electively removed by study staff on day 20 and day 23. Ulcers were detected a mean of 32 days after ring use (range 23–56). Four of eight participants with ulcers were symptomatic with vaginal discharge; four had ulcers identified when examined; three had two ulcers; all ulcers resolved after ring removal. No participants in the placebo group developed ulcers. No grade 2 product-related adverse events were reported in either group and four non-product-related grade 2 adverse events were reported in the tenofovir disoproxil fumarate ring group. Cervicovaginal fluid tenofovir concentrations did not differ at day 14 (p=0·14) comparing the eight patients who did (median $1·0\times10^5$ ng/mL [IQR $9·1\times10^4$–$1·1\times10^5$]) with the four who did not ($6·0\times10^4$ ng/mL [$5·6\times10^4$–$1·1\times10^5$]) develop ulcers. No significant changes in vaginal microbiota were detected in either group. Concentrations of multiple inflammatory cytokines and chemokines were significantly higher at days 14 and 28 compared with baseline in the tenofovir disoproxil fumarate ring group but not the placebo group.

**Interpretation** Future studies are needed to establish whether the unanticipated finding of ulcerations is specific to this tenofovir disoproxil fumarate ring or generalisable to other sustained topical release formulations of tenofovir or its prodrugs.

**Funding** National Institutes of Health.

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## Introduction

Nearly half of the 36·7 million people living with HIV worldwide are women and adolescent girls, and young women are among the most vulnerable to HIV.[1] Although oral pre-exposure prophylaxis (PrEP) is effective in men who have sex with men and in HIV-serodiscordant couples and vaginally delivered PrEP has shown some efficacy in subpopulations of women, clinical trial results in adolescent girls and young women have been disappointing. These discrepant results indicate that biological, virological, or behavioural factors, or a combination of these, limit the efficacy of PrEP in this population. For example, although a 39% reduction in HIV acquisition was observed with pericoital dosing of 1% vaginal tenofovir gel,[2] neither tenofovir gel, oral tenofovir disoproxil fumarate, or the oral combination of tenofovir disoproxil fumarate

*Lancet HIV* 2019

Published Online July 15, 2019 http://dx.doi.org/10.1016/S2352-3018(19)30145-6

See Online/Comment http://dx.doi.org/10.1016/S2352-3018(19)30187-0

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## Research in context

### Evidence before this study

Daily oral pre-exposure prophylaxis (PrEP) with tenofovir disoproxil fumarate and emtricitabine is recommended by WHO and the US Centers for Disease Control and Prevention as an additional prevention tool for people at substantial risk for HIV infection, including men who have sex with men, heterosexual men and women, intravenous drug users, and HIV-negative partners in serodiscordant couples. Although oral PrEP has proven effective in HIV-1-serodiscordant heterosexual couples, two clinical trials did not show efficacy in young women at high risk of HIV, which has fostered the development of alternative topical formulations, including vaginal rings, films, and fast-dissolve tablets. We searched PubMed and abstracts from major international AIDS conferences with combinations of the terms "tenofovir disoproxil fumarate" or "tenofovir", "pre-exposure prophylaxis", and "vaginal ring" for safety analyses of HIV prevention products in women, with no restrictions on language or publication date. A previously published phase 1 study of this polyurethane reservoir-type intravaginal ring designed for 30-day delivery of tenofovir disoproxil fumarate showed that the ring was safe when used for 14 days in women who were asked to abstain from sexual activity during ring use. A different polyurethane reservoir-type vaginal ring designed to deliver tenofovir (not the prodrug tenofovir disoproxil fumarate) was also safe in sexually abstinent women for up to 1 month. Additionally, sustained tenofovir disoproxil fumarate delivery from a silicone pod-type vaginal ring was safe when used for 7 days in sexually abstinent women.

### Added value of this study

We are the first group, to our knowledge, to report safety and pharmacokinetics of vaginal ring delivery of tenofovir disoproxil fumarate in sexually active women. Our phase 1 trial was stopped early because of vaginal ulcerations that developed in eight of 12 women in the tenofovir disoproxil fumarate ring group. No ulcers occurred in the five women who had a placebo ring. Multiple inflammatory cytokines and chemokines were significantly higher at 14 and 28 days after ring insertion in women who received tenofovir disoproxil fumarate but not placebo vaginal rings. Additionally, an inflammatory gene signature was observed in cervical tissue from women assigned to the tenofovir disoproxil fumarate ring, but not placebo group, which occurred in women with and without ulcers.

### Implications of all the available evidence

Our findings were not predicted by extensive preclinical data, including macaque studies or the 14-day clinical study in sexually abstinent women. Results suggest that sustained concentrations of intracellular tenofovir diphosphate or other metabolites might induce inflammation in sexually active women. The findings emphasise the need for more predictive preclinical models to assess topical PrEP safety. Moreover, results suggest caution with ongoing and planned trials of vaginal release formulations of tenofovir and its prodrugs (tenofovir disoproxil fumarate or tenofovir alafenamide).

and emtricitabine were protective in subsequent phase 3 trials.[3,4] Similarly, although a dapivirine intravaginal ring provided overall HIV protection in 27%[5] and 31%[6] of women in two phase 3 trials, no protection was recorded in women younger than 21 years in either study. Poor adherence, which was assessed by quantifying plasma tenofovir concentrations[3,5] or residual dapivirine in used rings[6] contributed to the outcomes.[3,5–7] However, even with high adherence, protection was incomplete.

The ability of PrEP to protect against HIV depends on virological factors (viral load and transmission efficiency), host susceptibility, and drug potency and pharmacokinetics. Tenofovir enters human vaginal and cervical epithelial and immune cells by an endocytic pathway whereas the prodrug, tenofovir disoproxil fumarate, passively diffuses into cells.[8] These differences in transport mechanisms are reflected in the relative potency: tenofovir disoproxil fumarate inhibits HIV-1 and herpes simplex virus (HSV) at 100-times lower concentrations than tenofovir.[9,10] Once inside a cell, tenofovir disoproxil fumarate is hydrolysed by carboxylesterases to the monoprotected moiety and then by phosphodiesterases into tenofovir, which is subsequently phosphorylated by kinases to tenofovir diphosphate. Tenofovir diphosphate has a long intracellular half-life and competes with cellular 2′-deoxyadenosine triphosphate for incorporation into the viral DNA chain. Tenofovir disoproxil fumarate that is not metabolised intracellularly passively diffuses out of cells where it is hydrolysed to tenofovir within vaginal fluid. The pharmacokinetics of dapivirine, a non-nucleoside reverse transcriptase inhibitor that blocks HIV but not HSV replication, are much simpler. Dapivirine passively diffuses into and out of cells without modifications, and thus has a short intracellular half-life compared with tenofovir diphosphate. These pharmacological differences also contribute to the ability of the vaginal environment and its microbiota to modulate drug pharmacokinetics. For example, tenofovir endocytosis is reduced as the pH increases to more than 6·0, a pH common in the presence of anaerobic dysbiosis, compared with uptake at a healthy vaginal pH of 3·5–4·5.[10] Additionally, *Gardnerella vaginalis* and other bacteria secrete adenine, which competitively inhibits tenofovir endocytosis.[10] *G vaginalis* might also degrade tenofovir.[11] Dapivirine binds irreversibly and non-specifically to individual bacteria and semen, which renders it less forgiving with intermittent adherence.[10,12] If a dapivirine ring is removed, the drug will rapidly diffuse out of cells where it might be sequestered by bacteria or semen, leaving the cells unprotected. By contrast, neither pH nor microbiota adversely affect passive diffusion of tenofovir

disoproxil fumarate into cells and intracellular tenofovir diphosphate concentrations persist for days.[10]

Thus, given the realities of incomplete adherence and these different pharmacokinetics properties, we hypothesised that a sustained-release formulation of tenofovir disoproxil fumarate would provide greater and more consistent HIV protection than tenofovir or dapivirine, as well as the potential added benefit of HSV prevention. A reservoir polyurethane intravaginal ring was engineered to deliver 5–7 mg/day of tenofovir disoproxil fumarate for 1 month based on in vitro release studies.[13] Macaque-sized rings completely protected non-human primates from repeated low-dose challenges with simian HIV and provided significant protection in a stringent model of medroxyprogesterone-treated macaques.[13,14] In a first-in-human clinical study, the tenofovir disoproxil fumarate ring was well tolerated in sexually abstinent women with 14 days of continuous use.[15] Building on these early results, we initiated a phase 1 randomised, placebo-controlled trial of 3 months of continuous tenofovir disoproxil fumarate ring versus placebo ring use in sexually active women, with monthly ring changes.

## Methods

### Study design and participants

This phase 1, single-blind, randomised, placebo-controlled trial was designed to assess the safety, pharmacokinetics, and acceptability of a tenofovir disoproxil fumarate ring in sexually active women (minimum of four vaginal sex acts per month) who were using any form of hormonal contraception exclusive of an intravaginal ring. The planned enrolment was 80 women at two sites (Albert Einstein College of Medicine in Bronx, New York, USA and Partners in Health Research and Development site in Thika, Kenya) with an initial review of 1-month safety data in 20 US women before enrolment at Thika. The study protocol is available online.

Inclusion criteria were age 18–45 years, general good health, willingness to avoid intercourse for 1 week after each biopsy, no HIV infection, using a copper intrauterine device or hormonal contraceptive other than an intravaginal ring for at least 2 months before study participation, and no use of PrEP or post-exposure prophylaxis for HIV in the 3 months before screening. Exclusion criteria were sex with a partner who is HIV positive or has an unknown HIV status in the preceding 3 months, known adverse reaction to polyurethane or other components of the study product, hepatitis B infection, vulvar or vaginal symptoms, known bleeding disorder, pregnant or intending to become pregnant, breastfeeding, menopause, HIV infection, sexually transmitted infection in the preceding 3 months, unexplained or unresolved intermenstrual bleeding in the preceding 3 months, gynaecological procedures in the preceding 14 days, hysterectomy, abnormal Papanicolaou (Pap) test, abnormal renal or liver function, and systemic use of corticosteroids, anticoagulants, or antiretrovirals in the preceding 2 weeks.

The study was approved by the Albert Einstein College of Medicine Institutional Review Board. All participants provided written informed consent.

### Randomisation and masking

Women were randomly assigned (3:1) to the tenofovir disoproxil fumarate ring or placebo group. A block randomisation scheme with a block size of four was computer generated by a study statistician. Participants and laboratory staff were masked to treatment group, but the study was not double-blind because the tenofovir disoproxil fumarate and placebo rings differ in appearance (drug filled *vs* only sodium chloride [NaCl] filled).

### Procedures

At screening, participants had a urine pregnancy test, gynaecological examination, Pap test, and nucleic acid amplification testing (NAAT) for *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Trichomonas vaginalis* (Gen-Probe, Inc, San Diego, CA, USA). Vaginal swabs were collected for pH (Whatman pH paper) and Nugent scoring. Blood was collected for HSV-1 and HSV-2 antibodies (BioPlex 2200, Bio-Rad Laboratories, Inc, Hercules, CA, USA), hepatitis B serologies, complete blood count, and kidney and liver function tests. An oral swab was collected for the OraQuick ADVANCE Rapid HIV-1/2 antibody test (OraSure Technologies, Inc, Bethlehem, PA, USA).

Enrolment (visit 2) occurred within 28 days of screening. HIV and pregnancy testing were repeated and vaginal swabs were collected for pH, Nugent score, and vaginal microbiota. Additional swabs were individually held at the proximal vaginal walls near the cervix for 2 min for assessment of baseline drug concentrations and immune mediators. Additional sexually transmitted infection testing was done if women developed symptoms such as vaginal discharge or genital ulcers.

For the **study protocol** see https://www.heroldlab.org/bedside

The first ring was inserted at enrolment and scheduled for clinician replacement every 30 days. Study visits 3–8 were scheduled every 2 weeks to assess safety. Visit 9 was scheduled 5–7 days after the third ring removal. Sampling at each of these visits included blood for pharmacokinetics and vaginal swabs for pH, Nugent score, pharmacokinetics, and immune mediators. Additional swabs for vaginal microbiota were collected at day 28 (before second ring insertion), day 56 (before third ring insertion), and 5–7 days after final ring removal. Two ectocervical tissue biopsy samples and dried blood spots were collected monthly. Participants were randomly assigned to additional pharmacokinetics sampling at one of six timepoints (1 h, 4 h, 1 day, 1 week, 2 weeks, or 3 weeks after initial ring insertion). Adverse events were collected at each visit.

Rings were designed as previously described[15] and manufactured by Particle Science (Bethlehem, PA, USA).

Tenofovir disoproxil fumarate and placebo rings were formulated using hydrophilic elastomer HydroThane AL 25–93A tubing (AdvanSource Biomaterials, Inc, Wilmington, MA, USA). The inner core compartment of the drug rings was comprised of 360 mg tenofovir disoproxil fumarate and 55 mg NaCl. The placebo rings contained 55 mg NaCl. The ring dimensions were similar to those of the commercially available NuvaRing with outer diameter of 55 mm and a cross-sectional diameter of 5·5 mm. The daily tenofovir disoproxil fumarate in vitro release rate was 5·5±1·5 mg/day in acetate buffer at pH 4·2 and 37°C.

Cervical tissue and swab-collected fluids were weighed, placed in separate cryovials, flash frozen in liquid nitrogen and stored at –80°C before analysis. Tenofovir disoproxil and tenofovir concentrations were quantified using liquid chromatographic-tandem mass spectrometric methods.[16] The lower limits of quantification (LLOQ) for tenofovir in plasma is 0·31 ng/mL, in tissue it is 0·25 ng/sample, and in cervicovaginal fluid it is 0·625 ng/swab. For tenofovir disoproxil, the LLOQ was 0·0625 ng/swab. For swabs and tissue, concentrations were converted to ng/mg on the basis of the net weight of the specimen swab or biopsy sample. Cervicovaginal fluid drug concentrations were converted to ng/mL. Values below the LLOQ were reported as below the limit of quantification. Tenofovir diphosphate concentrations were also measured in tissue homogenates using validated liquid chromatographic-tandem mass spectrometric assays with LLOQ of 50 fmol/sample.[16] Final concentrations were converted to ng/mg on the basis of the net weight of tissue. Tenofovir diphosphate concentrations in dried blood spots were measured as described with LLOQ of 25 fmol/sample.[15,17] After ring removal, residual drug was assayed as described.[9]

Concentrations of of interleukin-1α (IL-1α), IL-1β, IL-8, CXCL10 (interferon-γ-induced protein 10), CXCL9 (monokine induced by gamma interferon), CCL4 (macrophage inflammatory protein [MIP] 1β), CCL5 (RANTES), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-17, tumour necrosis factor (TNF)-α, IL-6, IL-10, CCL2 (monocyte chemoattractant protein 1), and CCL20 (MIP-3α) in vaginal swabs were measured by Luminex (Luminex Corp, Austin, TX, USA) with beads from Chemicon International (Billerica, MA, USA) and analysed using StarStation (Applied Cytometry Systems, Sacramento, CA, USA). Concentrations below the LLOQ were set at the midpoint between zero and the LLOQ.

DNA was extracted from vaginal swabs using the MoBio Bacteremia extraction kit (MoBio, Carlsbad, CA, USA). Broad-range PCR targeting the V3–V4 hypervariable region of the 16S rRNA gene coupled with sequencing on the Illumina MiSeq instrument (Illumina, San Diego, CA, USA) was done to characterise the vaginal microbiota.[18] Sequences were classified using the phylogenetic placement tool *pplacer* and a curated reference set of vaginal bacteria.[19,20] An average of 42 400 reads per sample was generated. α diversity was measured using Shannon Diversity Index and β diversity is represented using a non-metric multi-dimensional scaling plot using implementations of the R microbiome package.

RNA-Seq and bioinformatics were performed at the Yerkes Non-Human Primates Genomics Core at Emory University (Atlanta, GA, USA). A section of 48 snap-frozen ectocervical tissue samples were cut and placed into 350 µL of RLT lysis buffer (Qiagen; Germantown, MD, USA) and 1% 2-mercaptoethanol (Sigma-Aldrich; St Louis, MO, USA). The tissues were bead milled with a stainless-steel bead and Tissuelyser II (Qiagen) and RNA extracted using RNeasy Micro Kit with DNase digestion (Qiagen). RNA quantity and quality were assessed with Nanodrop 2000 Spectrophotometer and Agilent's 4200 Bioanalyzer Capillary electrophoresis. 1 ng of total RNA was used for mRNA amplifications using Clontech Smarter V4 chemistry (Takara Bio; Mountain View, CA, USA). Amplified mRNA was fragmented and barcodes appended using Illumina's Nextera XT kits. Amplified libraries were validated with an Agilent 4200 Tapestation and quantified using a Qubit fluorimeter. Libraries were normalised, pooled, and clustered on an Illumina HiSeq 3000/4000 flowcell using an Illumina cBOT. Libraries were sequenced on an Illumina HiSeq 3000 system in 101-base single-read reactions with multiplexing to achieve about 20 million reads per sample. Reads were aligned to the Human Reference Genome Sequence and transcripts annotated with Genome Reference Consortium Build 38 using STAR software (version 2.5.2b).[21] Unsorted bam files were sorted and indexed using samtools and converted to HTSeq-count format. Estimates of gene-wise and isoform-wise expression levels for individual genes were performed using the R package DESeq2 with R, version 3.5.0.[22] Differentially expressed transcripts (n=457, p≤0·02, fold change >1·5) between tenofovir disoproxil fumarate and placebo recipients were identified by negative binomial generalised linear models (DESeq2). To identify pathways differentially modulated between tenofovir disoproxil fumarate ring and placebo recipients, Gene Set Enrichment Analysis (GSEA) was performed using the desktop module available from the Broad Institute.[23] For each contrast, transcripts were ranked by differential expression with the Signal2Noise metric and GSEA was done for the ranked transcript lists using 1000 gene set permutations, collapse of duplicates to Max probe, and random seeding. Gene sets used included the MSigDB Hallmark and Gene Ontology Biological Processes[23] and co-transcriptional networks (Blood Transcriptome Modules).[24]

### Outcomes

The primary safety endpoint was the proportion of women who had grade 2 or higher genitourinary adverse events related to the study product, as defined

by the Female Genital Grading Table for Use in Microbicide Studies and any grade 2 or higher adverse event as defined by the Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events. For vaginal lesions, grade 0 was defined as normal variants (skin tags, moles, and scars); grade 1 adverse events were mild ulcerations with no treatment indicated; grade 2 were moderate with treatment indicated; and grade 3 was severe epithelial disruption requiring hospital admission. Secondary pharmacokinetic endpoints included measurement of tenofovir disoproxil fumarate and tenofovir concentrations in cervicovaginal fluid, tenofovir in plasma, and tenofovir diphosphate in dried blood spots and cervical tissue 1 month after ring insertion. We assessed ring acceptability as a secondary endpoint, which we plan to report in a separate manuscript. Prespecified exploratory outcomes included assessment of the vaginal microbiota and cervicovaginal fluid immune mediators before, during, and after ring use. Post-hoc analyses included analysis of changes in ectocervical tissue gene expression (RNA-Seq).

### Statistical analysis

The sample size of 40 women at each of two sites was chosen with the goal of achieving 32 participants with complete data (24 in the tenofovir disoproxil fumarate ring group and eight in the placebo group). An interim safety analysis was planned when 20 US women completed 1 month of ring use. Demographic and clinical characteristics were compared between participants in the tenofovir disoproxil fumarate ring group or placebo ring group using Fisher's exact tests for categorical variables. Continuous data were not normally distributed; therefore, we tested for differences between groups using the non-parametric Mann Whitney U test. Vaginal pH, Nugent score, and cytokine and chemokine concentrations were compared by Friedman's test to examine changes over time while accounting for repeated measures taken from the same women. Holm–Bonferroni adjustments were applied for post-hoc comparisons between timepoints. α diversity between the tenofovir disoproxil fumarate ring and placebo groups was compared using the Wilcoxon rank-sum test. Statistical analyses were performed using GraphPad Prism, version 7 (GraphPad Software, La Jolla, CA, USA) and Stata version 15.1 (StataCorp LLC, College Station, TX, USA).

A data safety monitoring committee reviewed all adverse events monthly and when requested by the study team. The study was registered at ClinicalTrials.gov, number NCT02762617.

### Role of the funding source

The funder of the study had a role in study design but had no role in data collection, analysis, and interpretation, or writing of this report. MJK and BCH had access to all the data in the study. All authors had final responsibility for the decision to submit for publication.

| | Tenofovir disoproxil fumarate group (n=12) | Placebo group (n=5) |
|---|---|---|
| Age (years) | 28·3 (22·2–33·5) | 33·6 (28·8–38·1) |
| Race | | |
| White | 8 (67%) | 2 (40%) |
| Black | 3 (25%) | 0 |
| Asian | 0 | 1 (20%) |
| Mixed race | 1 (8%) | 0 |
| Not reported | 0 | 2 (40%) |
| Ethnicity | | |
| Hispanic | 2 (17%) | 1 (20%) |
| Non-Hispanic | 10 (83%) | 4 (80%) |
| Relationship status | | |
| Married | 1 (8%) | 2 (40%) |
| Single | 2 (17%) | 1 (20%) |
| Cohabitating | 5 (42%) | 1 (20%) |
| In relationship, not living together | 4 (33%) | 1 (20%) |
| Contraception | | |
| Oral contraceptive pills | 7 (58%) | 3 (60%) |
| DMPA | 1 (8%) | 1 (20%) |
| Intrauterine device | 3* (25%) | 1† (20%) |
| Implant | 1 (8%) | 0 |
| Self-report of condom use | | |
| Never | 8 (67%) | 4 (80%) |
| Sometimes | 4 (33%) | 1 (20%) |
| Sex acts month before screening | 6·5 (4·2–10) | 12 (10·5–17·5) |
| Partner circumcised | 7 (58%) | 4 (80%) |
| History of receptive anal intercourse | 6 (50%) | 4 (80%) |
| History of sexually transmitted infection | 4 (33%) | 1 (20%) |
| HSV-2 seropositive | 2 (17%) | 1 (20%) |
| Previous NuvaRing use | 2 (17%) | 0 |
| Ever pregnant | 3 (25%) | 2 (40%) |
| Vaginal pH at enrolment | 4·6 (3·8–5·1) | 4·9 (3·9–5·5) |
| Nugent score at enrolment | 2·5 (1–3·2) | 7 (0·5–8) |

Data are median (IQR) or n (%). DMPA=depot medroxyprogesterone acetate. HSV-2=herpes simplex virus type 2. *Two copper, one levonorgestrel. †One levonorgestrel.

***Table 1:*** **Demographic and clinical characteristics of participants**

## Results

Between Feb 24 and July 20, 2017, we assessed 27 women for eligibility and randomly assigned 17 participants to either tenofovir disoproxil fumarate (n=12) or placebo (n=5) intravaginal ring. Demographic and clinical characteristics of participants were similar between the groups except the number of reported sex acts in the month before screening, which was higher in the placebo group (table 1, figure 1). Only two women in the

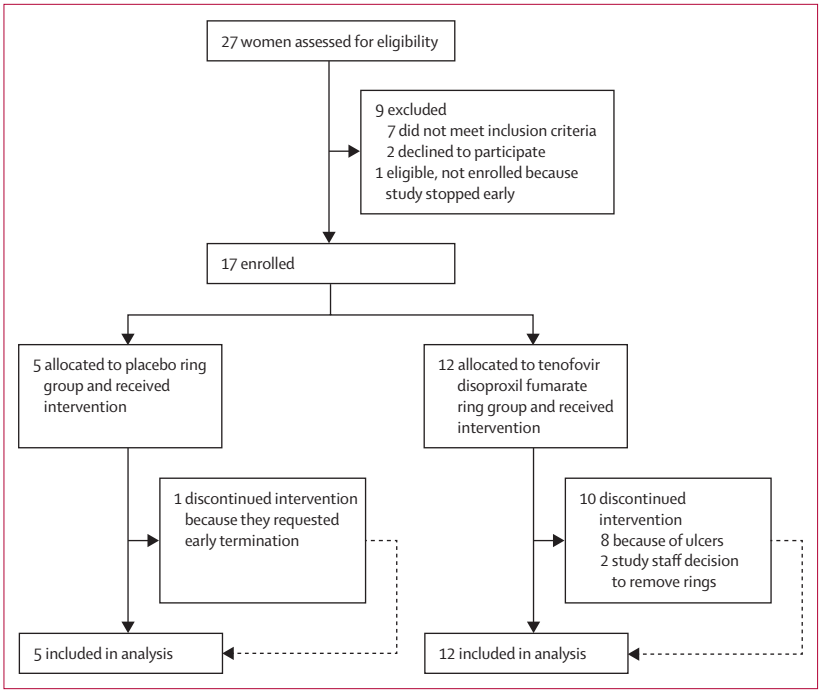


*Figure 1:* **Participant recruitment and follow-up**

| | Ulcer onset | Intravaginal ring terminated | Ulcer resolution | Symptoms | Comment |
|---|---|---|---|---|---|
| 904 | Day 34 | Day 45 | Day 38 | Vaginal discharge | Second ring was removed for ulceration; replaced on day 38; erythema on day 42; ring electively removed |
| 908 | Day 56 | Day 56 | Day 65 | None | Second ring removed, not replaced at scheduled visit |
| 912 | Day 31 | Day 28 | Day 36 | None | Second ring replacement delayed for erythema; ulcer identified on follow-up visit; no second ring placed |
| 915 | Day 28 | Day 28 | Day 42 | Vaginal discharge | First ring removed as scheduled, not replaced |
| 916 | Day 28 | Day 28 | Day 34, day 44* | Vaginal discharge | First ring removed as scheduled, not replaced |
| 918 | Day 23 | Day 23 | Day 36 | None | First ring removed early for ulcer noted at scheduled visit |
| 919 | No ulcer | Day 23 | .. | Vaginal discharge | Ring removed pre-emptively by study staff |
| 920 | Day 29 | Day 29 | Day 72 | Vaginal discharge | First ring removed as scheduled, not replaced |
| 921 | No ulcer | Day 20 | .. | None | Ring removed pre-emptively by study staff |
| 922 | Day 29 | Day 29 | Day 43 | None | First ring removed as scheduled, not replaced |

*One ulcer resolved on day 34 and second ulcer resolved on day 44.

*Table 2:* **Participants with premature tenofovir disoproxil fumarate ring discontinuation**

See Online for appendix

tenofovir disoproxil fumarate ring group (participant ID 902 and 903) completed the 3 months of ring use. Eight women who were assigned to the tenofovir disoproxil fumarate ring group developed grade 1 genital ulcers, which occurred a mean of 32 days after first ring insertion (range 23–56 days; table 2). Because of this unanticipated finding, the study team, in collaboration with the data safety monitoring committee, elected to remove the rings from the other two actively enrolled participants in the tenofovir disoproxil fumarate ring group (919 on day 20 and 921 on day 23) and to discontinue the trial. None of the women assigned to the placebo ring developed ulcers. Four placebo ring participants completed 3 months of continuous ring use and one discontinued the study at day 67 because of unanticipated travel. All participants were followed up until Oct 17, 2017.

All ulcerations occurred near the ring at the apex of the vagina and lateral to the cervix. All ulcers resolved over time after ring removal (table 2). Three women had two ulcers (one on each vaginal wall) and one had both a vaginal and cervical ulcer. The ulcers ranged in size from 2 mm to 2 cm in length, 2 mm to 1·5 cm in height, and 1 mm to 5 mm in diameter. Four women with ulceration were symptomatic with vaginal discharge (904, 915, 916, and 920). The other four participants with ulceration (908, 912, 918, and 922) were asymptomatic and had ulcers identified at scheduled study visit examinations (table 2). Participants 908, 915, and 920 had vaginal swabs tested for HSV DNA by PCR at the time ulcers were observed; results were negative. All participants with vaginal discharge had additional testing, including wet mount and NAATs for gonorrhoea, chlamydia, and trichomonas. All results were negative.

The frequency of vaginal sex reported during the first month of ring use did not differ between women with (median 5; IQR 3–8) or without (median 6; 4–7) ulcers or between women in the tenofovir disoproxil fumarate ring group (median 6; 4–8) versus those in the placebo group (median 6; 4–6).

57 adverse events (including the 12 genital ulcers) occurred in all 12 women assigned to the tenofovir disoproxil fumarate ring group and 13 adverse events occurred in four of the five women in the placebo group (table 3; appendix p 1). Most adverse events involved the reproductive tract and 36 were judged to be study-product related. One reproductive tract adverse event was related to study procedures (bleeding after biopsy). All product-related adverse events were mild (grade 1). Four grade 2 adverse events occurred, which were judged not to be product related, and no grade 3, grade 4, or serious adverse events occurred. There were three laboratory abnormalities, which were grade 1 and judged not to be product related in one participant in the tenofovir disoproxil fumarate ring group and two in the placebo group.

Because tenofovir disoproxol fumarate is hydrolysed to tenofovir by vaginal fluid, tenofovir provides a more consistent measure of extracellular drug concentrations than the prodrug. Cervicovaginal fluid tenofovir concentrations on days 14 and 28 were similar in this study to concentrations observed on day 14 in the previous 2-week study[15] in sexually abstinent women. The concentrations did not differ at days 14 or 28 between those who did

and did not develop ulcers; figure 2A). Tissue tenofovir diphosphate concentrations were more variable, possibly reflecting the smaller number of biopsy tissue samples available at each timepoint (figure 2B) but were also similar to tissue concentrations detected on day 14 in the previous study in sexually abstinent women.[15] Based on drug recovered from used rings, the calculated average in-vivo release rate was 7·1 mg/day (IQR 5·6–7·9; appendix p 2). Plasma concentrations were low but detectable in all nine women who had a tenofovir disoproxil fumarate ring in place on day 28 and tenofovir diphosphate in red blood cells, a potential biomarker of recent and cumulative adherence, was also detected in these nine participants. Additional pharmacokinetic data are summarised in the appendix (p 2).

To explore potential mechanisms that might have contributed to ulcerations in women assigned to the tenofovir disoproxil fumarate intravaginal ring, differences in genital tract pH, microbiota, cytokines, chemokines, and gene expression were assessed. Vaginal pH (p=0·049) and Nugent score (p=0·01) increased over time in the tenofovir disoproxil fumarate ring group, but not in the placebo group (comparing days 0, 14, and 28). In post-hoc analyses, vaginal pH was significantly different between day 28 and day 0 (p=0·03), as was Nugent score (p=0·01; appendix p 3). However, the magnitude of these differences was small. For example, the median Nugent score in women in the tenofovir disoproxil fumarate ring group increased from a median baseline score of 2·5 (IQR 1·0–3·2) to 4·0 (3·2–5·0) on day 28. No major changes in the vaginal microbiota were noted when we compared the relative abundances of bacterial taxa using 16S rRNA gene PCR coupled with deep sequencing on day 28 (range day 26–30) to those at enrolment, in either the tenofovir disoproxil fumarate ring or placebo groups (figure 3; sequencing data were not available for one tenofovir disoproxil fumarate ring participant, 918). Among the participants in the tenofovir disoproxil fumarate ring group who developed ulcers, two (904 and 908) maintained a *Lactobacillus iners*-dominant profile and one (912) maintained a *Lactobacillus crispatus*-dominant profile throughout the study; one (915) shifted from *L iners* dominance to a more diverse profile; two (916 and 920) had high diversity with the appearance of several species associated with bacterial vaginosis, and in one participant (922), the microbiota shifted from *G vaginalis* and *Atopobium vaginae* dominance to *L crispatus* dominance. Among the five placebo participants, three had diverse vaginal bacterial communities at enrolment (reflected in their higher Nugent scores), which were maintained throughout the study and two had *Lactobacillus*-dominant communities, which were also maintained throughout the study (figure 3).

The Shannon Diversity Index, which provides a measure of the number of bacterial taxa and the evenness of distribution of these taxa, was not significantly different in either the placebo or the tenofovir disoproxil fumarate ring groups between visits 2 and 4 (median 1·5 at visit 2 *vs* 1·52 at visit 4 in the placebo group, p=0·75; median 0·6 at visit 2 *vs* 0·63 at visit 4 in the tenofovir disoproxil fumarate ring group, p=0·28; figure 3; appendix p 18). Large shifts in the bacterial community were noted in two participants in the tenofovir disoproxil fumarate ring group, but the compositions shifted in opposite directions. Participant 920 went from a *Lactobacillus*-dominant to a diverse microbiota, whereas participant 922 went from a diverse microbiota to a *Lactobacillus*-dominant microbiota, both after use of the tenofovir disoproxil fumarate ring for 1 month (appendix p 19).

Although, as in previous studies,[25] baseline concentrations of cytokines and chemokines in cervicovaginal fluid varied between participants, the median concentrations of IL-1α, IL-1β, TNF-α, GM-CSF, IL-6, IL-8, CXCL9, CXCL10, RANTES, MIP-1β, MIP-3α, MCP-1, IL-10, and

| | Tenofovir disoproxil fumarate group (n=12) | | Placebo group (n=5) | |
|---|---|---|---|---|
| | Events | Participants | Events | Participants |
| All adverse events | 57/70 (81%) | 12 | 13/70 (19%) | 4 |
| Grade 1 | 53/57 (93%) | 12 | 13/13 (100%) | 4 |
| Grade 2 | 4/57 (7%) | 4 | 0 | 0 |
| Product or procedure-related genitourinary adverse events | 33/57 (58%) | .. | 4/13 (31%) | .. |
| Cervical discharge | 2/33 (6%) | 2 | 0 | 0 |
| Cervical ulcer | 1/33 (3%) | 1 | 0 | 0 |
| Dyspareunia | 1/33 (3%) | 1 | 0 | 0 |
| Postcoital bleeding | 1/33 (3%) | 1 | 0 | 0 |
| Vaginal bleeding | 1/33 (3%) | 1 | 0 | 0 |
| Vaginal dryness | 0 | 0 | 2/4 (50%) | 2 |
| Vaginal discharge* | 7/33 (21%) | 7 | 2/4 (50%) | 2 |
| Vaginal erythema | 8/33 (24%) | 6 | 0 | 0 |
| Vaginal itching | 1/33 (3%) | 1 | 0 | 0 |
| Vaginal ulcer | 11/33 (33%) | 8 | 0 | 0 |
| Common adverse events not related to study product | 23/57 (40%) | .. | 7/13 (54%) | .. |
| Gastrointestinal disorders | 1/23 (4%) | 1 | 2/7 (29%) | 2 |
| Respiratory disorders | 5/23 (22%) | 3 | 1/7 (14%) | 1 |
| Psychiatric disorders | 1/23 (4%) | 1 | 0 | 0 |
| Reproductive system and breast disorders | 3/23 (13%) | 2 | 2/7 (29%) | 1 |
| Renal and urinary disorders | 5/23 (22%) | 4 | 0 | 0 |
| Eye disorders | 3/23 (13%) | 3 | 1/7 (14%) | 1 |
| Nervous system disorders | 1/23 (4%) | 1 | 0 | 0 |
| Infections and infestations | 4/23 (17%) | 3 | 0 | 0 |
| Musculoskeletal disorders | 0 | 0 | 1/7 (14%) | 1 |
| Laboratory abnormalities | 1/57 (2%) | .. | 2/13 (15%) | .. |
| Increased alanine aminotransferase | 0 | 0 | 1/13 (8%) | 1 |
| Increased non-fasting glucose | 0 | 0 | 1/13 (8%) | 1 |
| Reduced haemoglobin | 1/57 (2%) | 1 | 0 | 0 |

Data are n/N (%) for events and n for participants. *Five were self-reported (three in the tenofovir disoproxil fumarate group and two in the placebo group) and not observed on examination.

***Table 3:*** **Summary of adverse events**

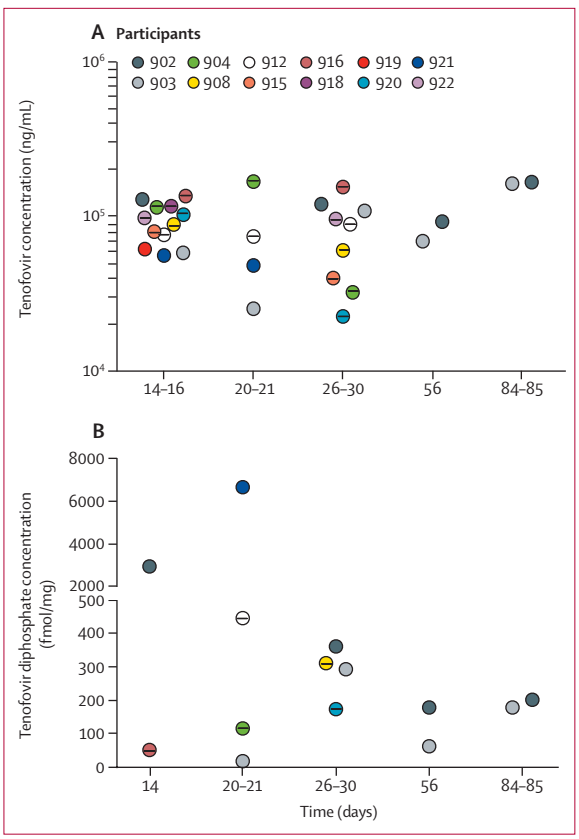


***Figure 2:*** **Tenofovir in vaginal swab fluid and tenofovir diphosphate in ectocervical tissue**

(A) Cervicovaginal fluid; collected by swabbing the vaginal wall proximal to intravaginal ring and assayed for tenofovir concentrations. Day 14 median concentration $1{\cdot}0 \times 10^5$ ng/mL (IQR $9{\cdot}1 \times 10^4$–$1{\cdot}1 \times 10^5$) in eight participants who developed ulcers compared with $6{\cdot}0 \times 10^4$ ng/mL (IQR $5{\cdot}6 \times 10^4$–$1{\cdot}1 \times 10^5$) in the four who did not (p=0·14). Day 28 median concentration $6{\cdot}0 \times 10^4$ ng/mL (IQR $3{\cdot}2 \times 10^4$–$9{\cdot}6 \times 10^4$) in the seven who developed ulcers compared with $1{\cdot}1 \times 10^5$ ng/mL (IQR $1{\cdot}1 \times 10^5$–$1{\cdot}2 \times 10^5$) in the two who did not (p=0·22). (B) Ectocervical tissue; obtained at indicated timepoints and assayed for tissue tenofovir diphosphate. Each circle is a different participant; horizontal black lines denote participants who developed an ulcer during the study period.

IL-17 were significantly increased on day 14 or day 28, or both, compared with concentrations at enrolment in the participants randomised to the tenofovir disoproxil fumarate ring group (including the two participants who completed 3 months of ring use, 902 and 903), but not the placebo group (appendix p 20). Most of the mediators returned to baseline by day 84, although IL-8, IL-1β, CXCL9, and RANTES remained significantly higher than they were at enrolment.

RNA sequencing of available ectocervical tissue included tissue from the following timepoints in the tenofovir disoproxil fumarate ring group: 1 h (n=1), 4 h (n=2), 24 h (n=1), day 8 (n=1), day 14 (n=2), days 20–21 (n=4), days 26–30 (n=5); days 56–58 (n=3, including participants 902 and 903); and days 84–86 (n=11), which corresponds to about 2 months after ring removal in nine women and a few days after ring removal in participants 902 and 903 who did not develop ulcers. Tissue was available at the following timepoints from participants in the placebo ring group: day 28 (n=5), day 56 (n=5), days 84–85 (n=3), and for one participant each at 1 h, 24 h, day 15, and day 21. Per protocol, ectocervical tissue was intended for pharmacokinetic analysis and tissue was not collected before initial ring insertion (baseline) or from ulcerative lesions.

Because differences in cervicovaginal fluid cytokines were most pronounced at about day 28 and because of the small samples size at earlier timepoints, we did a cross-sectional analysis of the RNA-Seq data comparing samples from days 26–30 between the tenofovir disoproxil fumarate ring group and the placebo group. 457 genes were significantly differentially expressed between tenofovir disoproxil fumarate ring and placebo recipients at these timepoints (232 higher and 225 lower; appendix pp 4, 21). However, when the transcriptome was ranked for GSEA, significantly enriched pathways were only identified containing genes expressed at higher levels in the tenofovir disoproxil fumarate ring group than the placebo group. This included genes associated with inflammation, interferon responses and T lymphocyte activation and proliferation (appendix pp 17, 21). Inflammatory and T-cell enrichment gene expression peaked on day 28, were increased in women who did (908 and 920) and did not (902, 903, and 919) develop ulcers, and generally returned to concentrations recorded before day 28 levels by days 84–85, except in subject 903 who maintained ring use until the final biopsy was done (appendix p 21). Genes driving enrichment of inflammatory pathways (leading edge genes) included those encoding cytokines such as IL-6 and IL-15 and chemokines such as CXCL10, CCL5, CCL7, and CCL17 (appendix p 21). Genes driving enrichment of T-cell activation included T-cell receptor components and signalling molecules (CD3D, CD3G, CD3E, TRAC, TRBC1, LCK, ZAP70, ITK, and SLAMF1), costimulatory molecules (ICOS and CD28), transcription factors (EOMES), T-cell effector molecules, chemokines, and cytokines (PRF1, GZMB, GZMK, GZMA, and XCL1; appendix p 21).

Several genes associated with the NLRP2 inflammasome were found in the leading edge of significantly enriched pathways, including *NLRP2, IL1B, CASP1,* and *CASP4.* These were expressed at higher levels in tenofovir disoproxil fumarate ring than placebo recipients at days 26–30, and *NLRP2, CASP1,* and *CASP4* also showed trends of increased expression at timepoints preceding days 26–30 (appendix p 17). Although *IL1B* gene expression was not obviously increased before days 26–36, IL-1β cytokine concentrations were increased in cervicovaginal fluid at day 14. These data suggest that the observed inflammatory response might be mediated by the NLRP2 inflammasome and activated before days 26–30.

## Discussion

Despite extensive preclinical safety data, a 3-month trial was electively terminated early after enrolling 17 of a planned 80 participants because eight of 12 women in

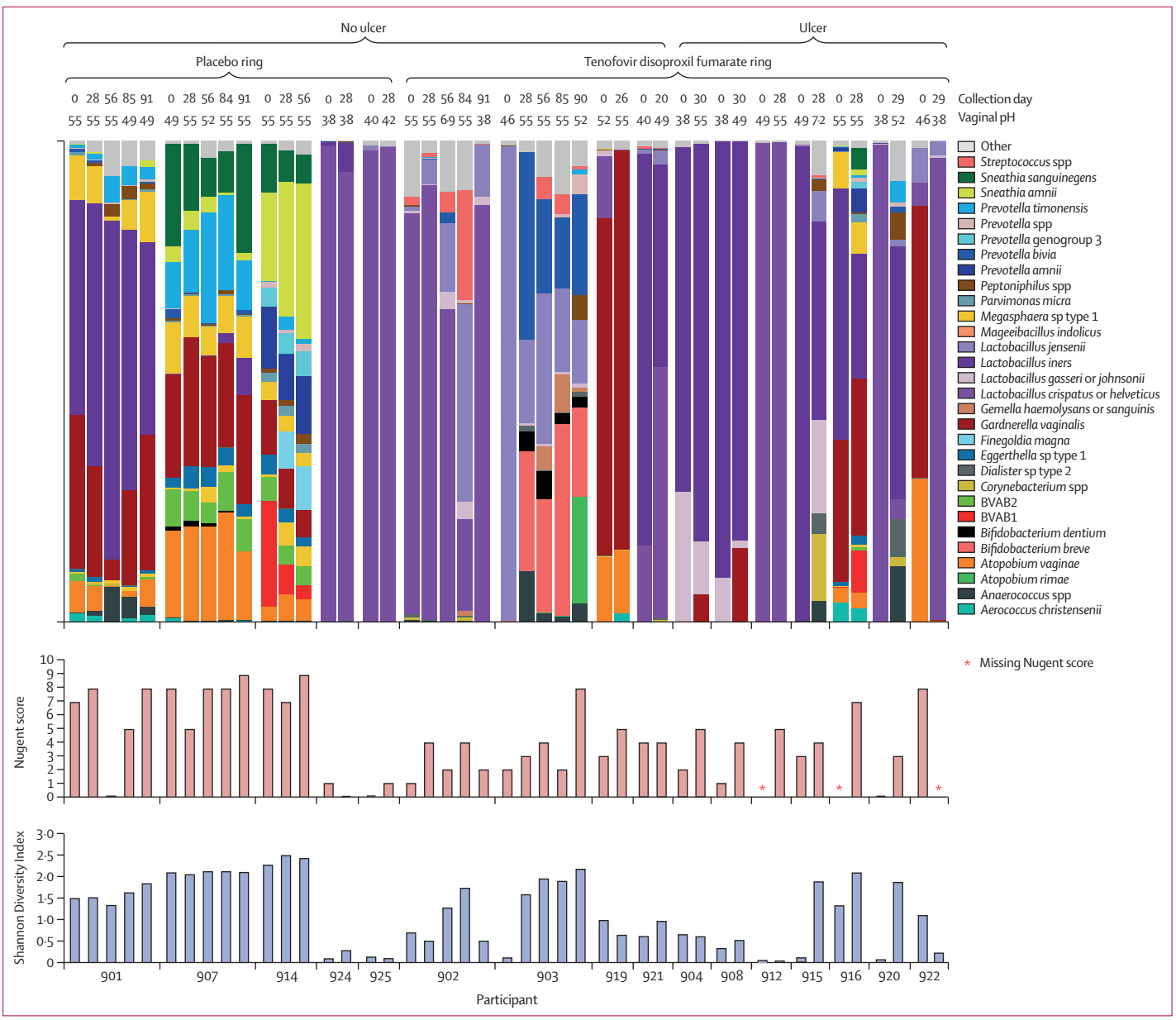


***Figure 3*: Relative abundance of bacteria detected in vaginal swabs**

Bars show the top 30 most abundant bacteria across all samples in each participant at various timepoints. Nugent scores and α diversity are indicated below the heat map and presence or absence of vaginal ulcer are indicated above the heat map. *Prevotella* spp indicates any *Prevotella* species not otherwise specified in the legend. BVAB=bacterial vaginosis-associated bacterium.

the tenofovir disoproxil fumarate ring group developed grade 1 vaginal ulcers. The tenofovir disoproxil fumarate ring, but not the placebo ring was associated with increases in inflammatory cytokines or chemokines in vaginal swabs and inflammatory gene expression in tissue in women with and without ulcerations. On average, there was 5–15-times increase in most cytokines or chemokines detected in the cervicovaginal fluid samples in women assigned to the tenofovir disoproxil fumarate ring. These findings were not predicted by the preclinical macaque studies in which no ulcerations and no consistent changes in cytokine or chemokine patterns in genital tract secretions were observed over a 6-month period of ring use, although gene expression was not assessed.[26] The findings were also not predicted by a previous 14-day study in sexually abstinent women who used this same tenofovir disoproxil fumarate ring with no safety concerns, although neither cervicovaginal fluid cytokines nor genital tissue gene expression were measured.

Precisely why the tenofovir disoproxil fumarate ring, but not the placebo was associated with the development

of vaginal ulcers remains uncertain, but the findings suggest several possibilities. Unlike most phase 1 studies, this study intentionally recruited sexually active women (minimum of four vaginal sex acts per month). The first-in-human 14-day study was conducted in sexually abstinent participants and the preclinical macaque studies were in the absence of simulated sex.[15,26] Thus, one possibility is that microabrasions associated with sex and the physical presence of the ring combined with the drug or one of its metabolites to inhibit healing or promote an inflammatory response.

The cervicovaginal fluid tenofovir concentrations were similar on days 14 and 28 and similar to those observed in the previous 14-day tenofovir disoproxil fumarate ring study in sexually abstinent women. Moreover, the tissue tenofovir diphosphate concentrations on day 28 in this study were similar to those detected on day 14 in the previous study.[15] Thus, it seems unlikely that excessively high drug concentrations contributed to the observed toxicity. Additionally, the cervicovaginal fluid tenofovir disoproxil fumarate concentrations were similar to those in the previous study on day 14 and lower on day 28. The reason for this decrease in cervicovaginal fluid tenofovir disoproxil fumarate concentrations on day 28 is unclear because most other pharmacokinetics measures did not change to this degree in parallel. The drop might reflect a reduction in drug release towards the end of the 30-day period but would not have contributed to the observed toxicity. Tenofovir disoproxil fumarate contains equimolar amounts of tenofovir disoproxil and fumaric acid and generates two molecules of formaldehyde. The possibility that fumaric acid or formaldehyde, which is rapidly converted to formic acid, contributed to the adverse outcomes cannot be excluded and assays to quantify these molecules are not available. However, one might have expected a decrease in vaginal pH if there was substantial accumulation of these acids.

Several lines of evidence suggest that uninterrupted intracellular exposure to the drugs, particularly intracellular tenofovir diphosphate, might have contributed to the development of vaginal ulcers. This notion is supported by in vitro studies and clinical experiences with related products, which suggest that sustained intracellular concentrations of tenofovir diphosphate might induce inflammatory responses or interfere with epithelial cell repair. For example, one study demonstrated that in vitro exposure of primary genital epithelial cells and fibroblasts to tenofovir or tenofovir alafenamide (at doses designed to yield similar concentrations of intracellular tenofovir diphosphate) resulted in significant impairment of wound healing.[27] Moreover, changes in gene expression similar to those reported in this study were observed in a previous clinical study of global gene expression by oligonucleotide microarray analysis in rectal biopsy samples obtained after seven daily doses of rectally applied 1% tenofovir gel.[28] Although the timepoints and tissues analysed in that study differed from this study, genes upregulated in response to tenofovir gel substantially overlapped with those up-regulated in response to the tenofovir disoproxil fumarate ring (appendix p 17). These genes included cytokine and chemokine genes (CCL2 and CCL19) associated with inflammation, interferon response genes (IFI27, IFIT1, MX1, and GBP2) associated with T-cell activation and maturation (CD3D, GZMB, SELL, and CD7), as well as genes associated with tissue remodelling and wound repair (MMP9 and MMP12). The authors also observed similar differences in vaginal cells treated in vitro with tenofovir.[28] The notion that the toxicity observed in this study might be mediated by tenofovir diphosphate is further supported by studies with a related nucleotide analogue, cidofovir. In a clinical trial of 1% topical cidofovir cream for human papillomavirus disease, 13 of 33 participants developed mild to moderate ulcerations.[29]

Although these findings suggest that the toxic effects observed might be linked to sustained intracellular concentrations of tenofovir diphosphate, other possibilities cannot be excluded. The polymer was probably not directly toxic because the placebo ring, which is made of the same material, was not associated with ulcerations. However, an interaction between the polyurethane and tenofovir disoproxil fumarate or one of its metabolites that was not detected in preclinical studies, including a rabbit vaginal irritation study, could have contributed to inflammation and ulceration. Toxic effects did not seem to be linked to changes in the vaginal microbiota because no consistent changes in microbiota were observed.

Our data suggest that caution with other sustained-release formulations of tenofovir or its prodrugs (tenofovir disoproxil fumarate and tenofovir alafenamide) is warranted.[30] Results also indicate the need for direct comparisons of different tenofovir-based sustained-delivery products to establish the cause of the unanticipated and disappointing outcome with this tenofovir disoproxil fumarate ring. Further, our data also suggest that although the non-human primate model provides insights into potential efficacy, it might not be optimal for predicting safety. Indeed, the tenofovir disoproxil fumarate ring was highly protective in rigorous macaque challenge studies and prevented HSV in murine models of co-infection with HIV and HSV.[13,14,31] Moreover, safety results obtained in short-term studies with abstinent women might not predict results with more prolonged drug exposure in sexually active women.

#### Contributors

MJK, BCH, and JM designed the study. LLR, DNF, CWH, HMLS, and NM contributed to study design. MJK, LLR, LE, and JMA supervised protocol implementation. BF manufactured the rings. CWH, MAM, PLA, and BF performed and interpreted drug concentration assessments. SSi, JG, SSr, CL, LW, and JMB performed the laboratory experiments. MJK, BCH, SEB, JMB, APM, SSr, and DNF analysed the data. All authors reviewed and approved the manuscript.

#### Declaration of interests

MJK reports grants from National Institutes of Health (NIH) and non-financial support from Gilead during the conduct of the study. PLA reports grants from Gilead Sciences outside the submitted work. CWH reports grants from NIH, Viiv, and GlaxoSmithKline outside the

submitted work. CWH has a patent, United States Patent Application 15/120,852 for hypotonic microbicidal formulations and methods of use. BCH, JM, MAM, DNF, and SSr report grants from NIH during the conduct of the study.

#### Data sharing

The study protocol, informed consent document, and deidentified participant data will be made available to others upon request to marla.keller@einstein.yu.edu after publication. RNA-Seq reads and the normalised expression table were deposited in the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (NCBI; accession number GEO GSE122702). Sequencing reads for 16S rRNA gene sequences have been deposited to the NCBI short read archive (accession number PRJNA529191).

#### Acknowledgments

This work was supported by grants from the National Institute of Allergy and Infectious Diseases and the National Center for Advancing Translational Sciences at the National Institutes of Health (U19 AI03461, P30 AI124414, and UL1 TR002256). The Yerkes Non-Human Primates Genomics Core is supported in part by ORIP/OD P51OD011132. We acknowledge Gilead Sciences for providing tenofovir disoproxil fumarate and regulatory assistance. We thank Jeanna Piper, Cherlynn Mathias, and Jenny Stanwix for assisting with protocol development and study operations, Jason McConnell for helping with the manufacture of rings, and Michael Zinaman and Susan Cu-Uvin for serving as members of the Data Safety Monitoring Committee. We also acknowledge the site staff and study participants.

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