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Genital tenofovir concentrations correlate with protection against HIV infection in the CAPRISA 004 trial: Importance of adherence for microbicide effectiveness

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Abstract

Objective—The CAPRISA 004 trial showed that coitally-dosed tenofovir 1% gel reduced HIV acquisition by 39% overall and 54% when used consistently. The objective of this analysis was to ascertain its pharmacokinetic-pharmacodynamic relationship to protection against HIV acquisition.

Design—Genital and systemic tenofovir concentrations in 34 women who acquired HIV (cases) were compared to 302 randomly selected women who remained HIV uninfected (controls) during the CAPRISA 004 trial. In total, 336 cervicovaginal fluid (CVF), 55 plasma, and 23 paired cervical and vaginal tissue samples were assayed by validated methods for tenofovir and tenofovir diphosphate (tenofovir-DP) detection.

Results—Tenofovir was detected in the genital tract in 8(23.5%) cases and 119(39.4%) controls (p=0.076). Among those with detectable genital tract tenofovir, the median CVF concentrations were 97% lower in cases compared to controls, 476ng/ml versus 13821ng/ml (p=0.107). A total of

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14.7% (5/34) of cases and 32.8 % (99/302) of controls were found to have tenofovir CVF concentrations above 100ng/mL (Odds Ratio (OR): 0.35, p=0.037). At a higher threshold, 8.8 %(3/34) of cases and 26.2 % (79/302) of controls were found to have tenofovir CVF concentrations above 1000ng/mL (OR: 0.27, p=0.036). Plasma tenofovir concentrations were <1ng/mL in all women and were less frequently detected in cases (0%) than controls (16.7 %) (p=0.031). Returned used tenofovir gel applicators and CVF concentrations were correlated (Spearman r=0.22, p=0.001).

Conclusion—A tenofovir concentration of ≥100ng/mL in CVF was associated with 65% (CI: 6%; 87%) protection against HIV, while a ≥1000ng/mL concentration correlated with 76% (CI: 8%; 92%) protection against HIV infection.

Keywords

tenofovir; genital tract; HIV; protection; adherence

Introduction

The global HIV epidemic is driven by sexual transmission with an estimated 2.1 million individuals acquiring HIV in 2013[1]. Research efforts to prevent new infections focusing on drug targets, particularly antiretrovirals, are underway [2]. Recent successful trials testing antiretrovirals for prevention have demonstrated the efficacy of oral tenofovir containing products in preventing HIV infection [3-5]. In 2010, the Centre for the AIDS Program of Research in South Africa (CAPRISA) 004 trial assessed the safety and effectiveness of the 1% vaginal gel formulation of tenofovir. This study found a 39% reduction in risk for acquisition of HIV [6] and a 51% reduction in HSV-2 infection [7]. In 336 subjects in this study who were considered to be at least 80% adherent to tenofovir gel, the reduction in risk for HIV acquisition was 54%.

The concentration of tenofovir achieved in the vaginal tract in the CAPRISA 004 trial, 24 hours after coitally dependent dosing, was estimated to be $\approx 10^5$ng/mL [8]. Two previous animal studies of tenofovir 1% gel in pig-tailed macaques and humanized bone marrow liver thymic (BLT) mice have demonstrated 88-100% efficacy against viral acquisition[9,10]. However, the limited data on drug exposure in these animals does not allow direct human exposure comparisons. Therefore, it is unclear whether the more modest clinical trial results were due to intermittent adherence, dissimilar drug exposure, or both. Differentiating between these two etiologies are difficult using the traditional adherence measures of self-report or, as in the case of CAPRISA 004, returned used gel applicator counts, as none of these directly capture intervention-taking behavior.

Using a pharmacologic measure may allow better interpretation of adherence from samples stored during a clinical trial, and could eventually provide real-time information to guide behavioral interventions. Also, since standard Phase II drug development dose-ranging pharmacokinetic-pharmacodynamic studies used to generate clinical Emax models (non-linear model using dose-response analysis) of efficacy are not straight-forward for HIV prevention, pharmacologic data from placebo-controlled clinical trials may provide some

insight into the exposure of tenofovir required in the female genital tract to protect against infection.

The purpose of this study was to assess the relationship between local/systemic tenofovir concentrations and HIV acquisition in order to identify a pharmacokinetic marker of adherence as a correlate of protection in women.

Patients and Methods

Patients and Study Design

The CAPRISA 004 trial was conducted between May 2007 and March 2010 in KwaZulu-Natal, South Africa at the CAPRISA Vulindlela Clinical research site in Vulindlela, a rural community 150 km west of Durban (rural) and at the CAPRISA eThekwini Clinical research site in the Durban city centre (urban). The source population for this cohort has been described elsewhere [11].

Participant screening, enrolment and randomization procedures have been described in detail previously[6]. Briefly, volunteers were provided with study information in English and/or *isiZulu* and those agreeing to continue with study participation were eligible if they were 18 to 40 years of age, willing to provide written informed consent for screening, agreed to provide adequate locator information for study retention purposes, agreed to adhere to study visit schedule, were sexually active (defined as having had vaginal intercourse at least twice within the last 30 days prior to screening), HIV negative, not pregnant, agreeable to be on a non-barrier form of contraception, creatinine clearance of >50 ml/min using the Cockcroft and Gault method [12] and no evidence deep epithelial disruption on pelvic examination. Volunteers were excluded if they had an untreated sexually transmitted infection. Within 30 days of the first screening visit, returning eligible volunteers were randomly assigned to receive either 1% tenofovir gel or the hydroxyethylcellulose (HEC) placebo in a 1:1 ratio. Participants received an assigned study gel in quantities guided by the frequency of coital activity.

From May 2007 to January 2009, 2160 women were screened and 1085 were enrolled, of whom 889 were included in the primary analysis. Women followed a dosing strategy referred to a “BAT24”: insert one dose of gel within 12 hours before sex and a second dose of gel as soon as possible within 12 hours after sex and no more than two doses of gel in a 24-hour period. Participants had monthly follow-up visits for 30 months. Participants were requested to return their used (from October 2007 onward) and unused applicators at every visit. Each month, the applicators returned by women as used and unused were counted, reconciled against the number dispensed, and thereafter discarded. At months 3, 12, 24 and at exit, blood plasma and cervicovaginal fluid aspirates were collected for pharmacokinetic analysis. Upon detection of HIV seroconversion, vaginal and cervical tissue biopsies were also obtained.

Sample Processing and Analyses

Tenofovir was quantified in blood plasma and cervicovaginal fluid (CVF). Tenofovir and tenofovir diphosphate was quantified in vaginal and cervical tissue. For each blood plasma

sample, 4mL of blood was collected in a tube containing K-EDTA. Within 30 minutes of collection, whole blood was centrifuged at 800g for 10min at 4°C. Plasma was pipetted into cryovials and stored at -80°C until analysis. Specialty collection syringes (UNC CFAR Vaginal Specimen Aspirators) were used to obtain directly-aspirated, undiluted CVF samples. After collection, samples were expelled into a cryovial and stored at -70°C until analysis. To prepare the mucosal tissue for biopsy, the areas of the planned biopsies were gently wiped with a small cotton swab moistened with warm saline followed by a small cotton swab with betadine. Topical gel containing lidocaine 25mg/prilocaine 25mg/g was applied to biopsy area for anesthesia. A medium-Tischler biopsy forceps was used to obtain one 5 × 3 mm biopsy from the vaginal wall and one 5 × 3 mm biopsy from the ectocervix. Each biopsy was placed in a pre-weighed individual vial and weighed, snap frozen in liquid nitrogen, and stored at -80°C until transport.

At the end of the study, samples were shipped on dry ice to the UNC Center for AIDS Research Clinical Pharmacology and Analytical Chemistry Laboratory at the University of North Carolina at Chapel Hill.

Sample Analysis

Analysis of all samples was performed using an LC-MS/MS method that quantified tenofovir and tenofovir diphosphate simultaneously, as described previously [13]. For blood plasma, the calibration curve for tenofovir ranged from 0.25-200 ng/mL. Across 3 quality control concentrations, intra-day accuracy and precision was >99% and <12%, respectively, and inter-day accuracy and precision was >90% and <13%, respectively. For CVF, the calibration curve for tenofovir ranged from 2-1000 ng/mL. Intra-day accuracy and precision was >93% and <8%., and inter-day accuracy and precision was >97% and <5%. For vaginal and cervical tissue, a calibration curve with a range of 2-2,000 ng/mL for tenofovir, and 10-10,000 ng/mL for tenofovir-DP was prepared. Intra-day accuracy and precision for tenofovir was >90% and <10%, respectively: for tenofovir-DP it was >94% and <9%, respectively. Inter-day accuracy and precision for tenofovir was >93% and <5%, respectively: for tenofovir-DP it was >98% and <6% respectively.

Statistical Analyses

The demographic characteristics at baseline were summarized using basic descriptive statistics. Fisher's exact test was used for the analysis of categorical data, and unpaired t-tests or the Wilcoxon rank sum tests for the analysis of continuous data.

A nested case-control study, post-trial, was conducted in order to determine a correlate of protection related to drug concentration. For the cases, post-infection samples of the HIV seroconverters on the active arm were selected. Controls were selected randomly from those who did not acquire HIV. Only visits with tenofovir concentrations measured were considered. Detectable concentrations below the limit of quantification (BLQ) were imputed to be one-half of the lower limit of quantification (the lowest point on the standard curve) and values below the limit of detection (BLD) were set to 0 ng/mL. Tenofovir threshold concentration cut points were selected as follows: (1) Tenofovir = 0 ng/mL: indicates that tenofovir concentration was BLD (2) Tenofovir ≥ 1 ng/ml: indicates that any tenofovir was

present, (3) Tenofovir ≥ 100ng/ml: tenofovir concentration expected in plasma and cervicovaginal fluid following oral dosing and (4) Tenofovir ≥ 1000ng/ml: tenofovir at concentrations at or above the surrogate level of protection identified previously [8]. Logistic regression was performed comparing the different tenofovir thresholds in cases and controls. An adjusted model was fitted controlling for possible confounders: age, site, marital status, education, condom at last sex act and time on study at sample collection.

Spearman correlation was used to assess the relationship between tenofovir plasma and CVF concentrations, as well as between tenofovir and tenofovir-DP concentrations within both vaginal and cervical tissue. Wilcoxon signed rank test was used to compare both tenofovir and tenofovir-DP concentrations between vaginal and cervical tissue. Spearman correlation was also used to compare CVF tenofovir concentrations to the number of returned used applicators brought back at monthly study visits. All analyses were performed using two-sided tests. SAS software version 9.3 (SAS Institute Inc., Cary) was used for analysis while graphs were created using GraphPad Prism version 5.

Regulatory considerations

The protocol for the primary study, informed consent forms and study related materials were reviewed and approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee, Ref: E111/06, the Protection of Human Subject Committee in the Office of International Research Ethics at FHI360 Ref: 9946, and the South African Medicines Control Council (MCC), Ref: 20060835. The trial was registered with ClinicalTrials.gov, number NCT 00441298.

Results

Tenofovir concentrations in cases and controls

Of the 889 women enrolled in CAPRISA 004, 445 were randomized to the tenofovir gel arm and of these, 340 had at least one tenofovir CVF concentration measured. Of the 340 women with tenofovir concentration data, post infection samples were available for 34 of the 38 HIV seroconverters (cases). The remaining four seroconverters only had pre-infection samples available and were therefore excluded. Control samples were selected at a random visit from the remaining 302 women who remained HIV negative. No statistically significant differences in demographic characteristics were seen between HIV cases and controls (Table 1). Median age in both cases and controls were 23 years, with approximately 60% of women representing the rural site. The 336 samples included in this case control analysis were obtained at a median of 9 days (IQR 3 – 27) after the last reported gel use (Figure 1).

Tenofovir was detected in the genital tract in 23.5% (8/34) cases and 39.4% (119/302) controls (p=0.076). Among those with detectable tenofovir in the genital tract, the median CVF concentrations were 97% lower in cases compared to controls, 476ng/ml versus 13821ng/ml (p=0.107) (Figure 2). With 1000ng/mL as a correlate of protection, we found 8.8% (3/34) of cases and 26.2% (79/302) of controls had tenofovir CVF concentrations above this threshold (OR: 0.27, p=0.036). After adjustment for multiple covariates, a

concentration of 1000ng/mL in the CVF was associated with 76% protection against HIV (adjusted p=0.024) (Table 2). At lower tenofovir concentrations, protection against HIV was concomitantly lower, with efficacy of 53% (p=0.076) and 65% (p=0.037) when tenofovir CVF concentrations were ≥1 ng/mL and ≥100ng/mL, respectively. For the latter tenofovir cut-off concentrations, the efficacy against HIV was 53% (adjusted p=0.089) and 67% (adjusted p=0.034, after adjusting for age, site, marital status, education, condom at last sex act and time on study at sample collection.

There was a correlation, though not very strong, between CVF tenofovir concentrations and number of returned used applicators (Spearman r=0.22; p<0.001) in women (331/336) with applicator data available.

Blood plasma samples

Plasma tenofovir concentrations were measured in 55 women randomized to tenofovir gel. Of these, 93% (51/55) were BLD or BLQ. In all subjects, plasma concentrations were less than 1ng/mL, but nonetheless were detected less frequently in cases than in controls. Tenofovir was detected in plasma in none (0/31) of the HIV positive women and in 16.7% (4/24) of the HIV negative women. Of the 55 plasma tenofovir concentrations, 54 had a tenofovir CVF concentration on the same collection date. Plasma and CVF tenofovir concentrations were moderately correlated with a Spearman correlation coefficient of 0.46 (p=<0.001). In the four women with detectable tenofovir in plasma, their median tenofovir concentration in CVF was 312 482 ng/ml (IQR 121 298 – 510 512). The four women with detectable concentrations reported gel use at a median of 5 days prior to sampling (IQR 3 – 18), while the remaining undetectable plasma concentrations were in visits where women reportedly used the gel at a median of 10 days ago (IQR 4 – 83) (p=0.313).

Cervical and vaginal biopsies in seroconverters

Cervical and vaginal biopsies were collected in 23 of 38 HIV seroconverters at a median of 37 days post-infection (IQR 29 – 67) and a median of 43 (IQR 20-93) days after the last reported gel insertion. In total, 6 women had detectable tenofovir and 6 women had detectable tenofovir-DP from the 23 cervical and 23 vaginal biopsies collected. Tenofovir-DP concentrations correlated with tenofovir concentrations in both vaginal and cervical tissue (rho=0.61, p=0.002 and rho=0.53, p=0.009, respectively). The median tenofovir concentration was 10ng/g (IQR 7.1-16.7) and 10ng/g (IQR 8.3-25.0) in cervical and vaginal tissue, respectively. Among those with detectable tenofovir- DP, median (IQR) Tenofovir-DP concentrations in vaginal and cervical biopsies were 79 fmol/mg (IQR 0-186) and 0 fmol/mg (IQR 0 – 93), respectively.

Discussion

Following topical application, the tenofovir concentration in the genital compartment provides a potential clinical correlate of protection against HIV infection, with 76% effectiveness demonstrated when genital concentrations are in excess of 1000ng/mL. In women with no detectable tenofovir in the genital tract, protection against HIV was not observed while the detection of any genital tenofovir (>1ng/mL) was associated with 53%

effectiveness. The increasing levels of effectiveness associated with log increases in tenofovir concentrations highlights the importance of adherence to attain high levels of protection against HIV. This is further supported by the recent outcome of the VOICE trial that failed to demonstrate the protective efficacy of tenofovir gel due to low adherence to the prescribed daily gel application schedule [14].

Tenofovir was seldom detected systemically in the CAPRISA 004 trial, confirming that intermittent topical application achieves limited and short-lived systemic tenofovir exposure. With intermittent topical application, there was no observed correlation between random systemic tenofovir concentrations and protection against HIV. Since the median serum tenofovir C_{max} is 3.0 ng/mL at 2.1 hours after gel insertion and was <1ng/mL at 24 hours after insertion [15], a coital dosing strategy would not produce consistently detectable tenofovir systemically. Further, the lack of detectable tenofovir in blood plasma in this analysis supports the findings of limited drug-related systemic adverse effects [16] and may explain why no tenofovir resistance was observed in plasma viruses, even in women who continued applying tenofovir gel, for approximately 3 -4 weeks, after HIV infection in the CAPRISA 004 trial [6].

The assays on the vaginal and cervical tissue from the genital biopsies showed that concentrations of the active form of tenofovir, tenofovir-DP, correlated with concentrations of the tenofovir pro-drug, noting that the gel contains only the tenofovir pro-drug. Despite the biopsies being sampled a median of 43 days since last gel insertion, tissue concentrations were detected in 30.4 % of women sampled.

The specified concentration of genital tract tenofovir, or intracellular tenofovir-DP required to protect women from HIV infection is unknown. Protective concentrations modulated by additional acquisition risk factors such as genital inflammation and presence of sexually transmitted infection/s are yet to be described. Two animal studies have previously demonstrated >80% efficacy of tenofovir 1% gel. In 2009, 100% protection from $SHIV_{SF162P3}$ acquisition was found in 6 pig-tailed macaques given 3mL of a tenofovir 1% vaginal gel 30min prior to viral challenge twice weekly for 10 weeks [9]. In 2011, 88% protection from HIV-$1_{JR\text{-}CSF}$ acquisition was demonstrated in 8 humanized BLT mice given 20μL of a tenofovir gel 4 hours prior to, and 4 hours after, a single viral challenge[10].

In the macaque model, Dobard et al [17] analyzed tenofovir-DP concentrations in isolated vaginal lymphocytes at necropsy after dosing with 3mL of tenofovir 1% gel. The authors demonstrated high concentrations (median 1810, range 1312–2684 fmol/10^6 cells) 4h post-dose, where tenofovir 1% gel is 100% efficacious in macaques. At 72h after a dose of tenofovir gel, when protective efficacy in macaques is 67%, Tenofovir-DP concentrations are 86% lower (252 (196-295) fmol/10^6 cells). These data can't be directly compared to women using tenofovir gel, as isolated vaginal lymphocytes cannot be obtained in sufficient quantities by standard biopsy techniques. However, they do demonstrate that a tissue concentration-effect relationship exists for tenofovir gel and should be defined in human tissue.

In the CAPRISA 004 study, as expected, tenofovir CVF exposures were highly variable 0-3 days post-dose. For those women who reported using the gel within 24h of their visit, CVF concentrations were ~ 100,000 ng/mL. These concentrations are similar to a recent Phase I investigation of tenofovir 1% gel by Schwartz et al [18]. However, one to 3 days post-dose, CVF concentrations in the women in the CAPRISA 004 study were up to 100-fold lower (~1000ng/mL). This is not unexpected due to the phase I investigation being conducted under rigorous restricted conditions while the CAPRISA 004 study participants were exposed to sexual activity and accompanying practices. Yet this concentration of 1000 ng/mL was useful in predicting efficacy in this case-control analysis. Based on the previously-established ~10:1 relationship between CVF and vaginal tissue concentrations, and the 3% molar conversion rate between tenofovir and tenofovir-DP in vaginal tissue [18], we estimate the CVF concentration of 1000ng/mL should yield 500 fmol/mg of tissue of tenofovir-DP. Whether this concentration will be directly predictive of efficacy is yet to be determined.

These case-control dose-response data are consistent with preliminary analysis of the CAPRISA 004 CVF concentrations [8] and recently published data in PrEP trials testing oral tenofovir-containing products. In the Partners PrEP trial, 81% of blood samples had detectable tenofovir and HIV efficacy was 75% in that trial [3]. Similarly, in the TDF2 [19], iPREX [5] and FEM-PrEP [20] trials, 79%, 51% and 26% of blood samples had detectable tenofovir with a corresponding HIV protection point estimate of 62%, 44% and 6% respectively. Most recently, the MTN003 trial failed to show efficacy of oral TDF, oral Truvada, and topical Tenofovir 1% gel with tenofovir detected in only 28%, 29%, and 23% of blood samples, respectively.[14]

Finally, objective measures of adherence are critical to the interpretation of clinical trial data. Measurement of adherence in HIV prevention trials using self-reports or pill counts have been shown to overestimate adherence [21,22]. Utilizing used applicator counts, the CAPRISA 004 investigators determined that tenofovir 1% gel could confer 52% protection against HIV acquisition in those who were >80% adherent. However in this analysis, cervicovaginal fluid concentrations taken at a single visit only weakly correlated with the number of gel applicators used the previous month. Self -reports of gel insertion compared to concentrations and model simulated data indicate that self-report of gel use are prone to bias. Based on a PK model developed for tenofovir gel dosing[23], 62.5% of participants on active product could be considered adherent based on their tenofovir CVF concentrations (data not shown).

Limitations of this analysis include the inability to determine a threshold correlate of protection from tissue concentrations as genital tissue biopsies were not permitted in HIV uninfected women, furthermore tissue biopsies from seroconverters were performed on average more than 6 weeks after last gel insertion. It is also acknowledged that the time of last gel insertion was collected from participant self-report and is subject to recall bias. There was poor correlation between time of last dose and drug level in this trial. This was not unexpected as gel use was intermittent (coitally dependent), unscheduled, and not associated with clinic visits when PK samples were collected or seroconversion was detected.

In conclusion, an analysis of tenofovir concentrations in the CAPRISA 004 trial demonstrated a potential correlation between genital tract concentrations of the drug and its effectiveness, with CVF concentrations >1000ng/mL being associated with 76% protection against HIV infection. Tenofovir 1% vaginal gel is still under clinical trial evaluation for safety and effectiveness in the current Follow-on African Consortium for Tenofovir Studies (FACTS) 001 trial, which is required for its registration for use as a topical PrEP agent. Based on this analysis, the attainment of this concentration during sexual exposure consistently may be necessary to achieve high levels of protection against HIV. Achieving high levels of gel adherence is therefore going to be essential for PrEP trials, without which, an efficacious product may be found spuriously to be ineffective. Importantly. maintaining high adherence in HIV prevention trials is critical in light of the urgent need for the prevention of sexually transmitted HIV in young women in Africa.

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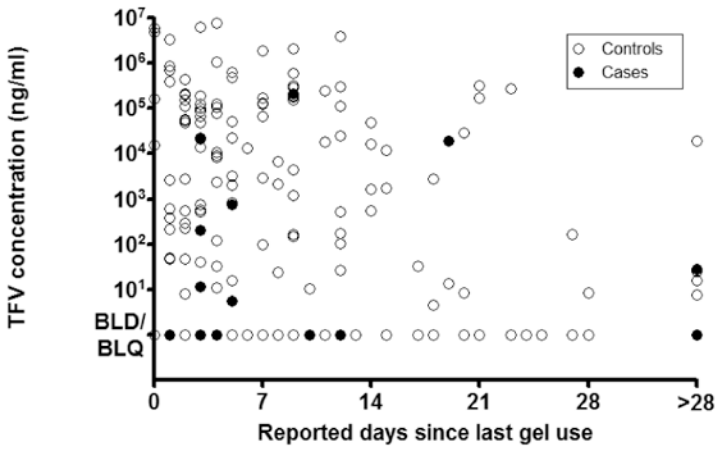
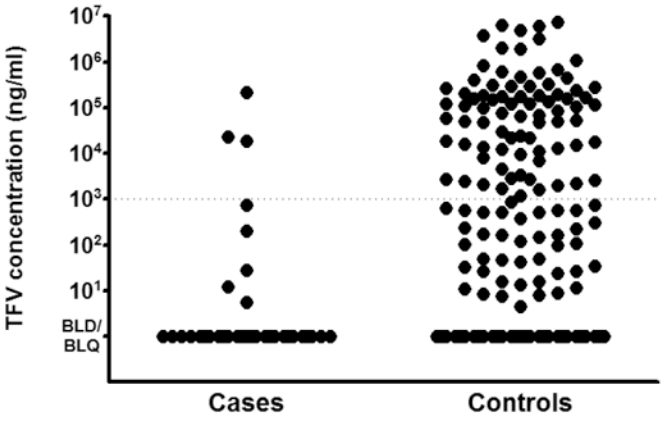


Figure 1. Cervicovaginal Fluid Concentrations of Tenofovir versus reported days since last gel use in cases and controls

TFV=Tenofovir, BLD=below the limit of detection, BLQ=below the limit of quantitation



	Cases		Controls
N	34		302
% detected (n)	23.5% (8)	*p=0.076*	39.4% (119)
Median (IQR) TFV (ng/ml) among detectable	476 (20-20674)	*p=0.107*	13821 (300-171920)

Figure 2. Cervicovaginal tenofovir concentrations for HIV cases and controls

Table 1
Baseline characteristics of HIV cases and controls

Variable	All (N=336)	Cases (n=34)	Controls (n=302)	p-value
Median age (IQR)	23 (20 – 27)	23 (20 – 26)	23 (20 – 27)	0.478
Rural % (n)	66.4% (223)	64.7% (22)	66.6% (201)	0.849
Completed high school % (n)	40.2% (135)	32.4% (11)	41.1% (124)	0.361
Married % (n)	5.4% (18)	0.0% (0)	6.0% (18)	0.235
Stable partner % (n)	92.6% (311)	100% (34)	91.7% (277)	0.092
Casual partner % (n)	8.3% (28)	8.8% (3)	8.3% (25)	1.000
Lives with regular partner % (n)	13.0% (43)	8.8% (3)	13.5% (40)	0.595
Income per month (R=South African Rands)				
None or <R1000	91.4% (307)	88.2% (30)	91.7% (277)	0.597
R1001-R5000	8.0% (27)	11.8% (4)	7.6% (23)	
>R5001	0.6% (2)	0.0% (0)	0.7% (2)	