Drug resistance and plasma viral RNA level after ineffective use of oral pre-exposure prophylaxis in women

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Background: Pre-exposure prophylaxis (PrEP) with daily oral emtricitabine (FTC)/tenofovir disoproxil fumarate may select for drug resistance if there is low adherence.

Methods: Plasma viral HIV-1 RNA level, $CD4^+$ T-cell counts, and drug resistance were evaluated among seroconverting women in the FEM-PrEP trial (clinicaltrials.gov NCT00625404) using standard clinical tests, allele-specific PCR (ASPCR), and by deep sequencing. Tenofovir, FTC, and their intracellular metabolites were measured in plasma and cells.

Results: There was no difference in plasma HIV-1 RNA level or $CD4^+$ cell count among seroconverters in the active arm versus those receiving placebo. Tenofovir resistance was not observed. FTC resistance was detected using clinical assays in five seroconverters (four in the active arm and one in the placebo arm); two in the active arm occurred among women having moderate concentrations of PrEP drugs in the blood. The first evidence of infection occurred at the first postenrollment visit in three of the four with FTC resistance, although none had detectable viral nucleic acids at enrollment. FTC-resistant minor variants were detected in an additional four seroconverters (one in the active arm and three in the placebo arm).

Conclusions: Drug resistance detected during ineffective PrEP use had characteristics suggesting transmitted infection or incubating infection prior to starting PrEP.

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Introduction

Emtricitabine (FTC) or tenofovir (TFV) resistance was not observed among pre-exposure prophylaxis (PrEP) users with incident HIV infections in PrEP trials that demonstrated efficacy [1–4]. Resistance may occur more frequently when adherence to daily PrEP is ineffective, as occurred in the FEM-PrEP trial [5]. Such resistance could undermine the activity of first-line therapies, which frequently include these agents. Concerns that

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suboptimal PrEP use could engender drug resistance among PrEP users and their partners have inspired caution in recommending PrEP.

Standardized clinical resistance testing methods may underestimate the true incidence of drug resistance. HIV-1 populations within people are diverse, and clinical assays characterize only variants that comprise the majority of the virus population. Nonetheless, minor variants are known to occur frequently in some clinical settings, such as people who have recently stopped therapy [6], have transmitted resistance [7], or mothers exposed to nevirapine monotherapy for prevention of vertical transmission [8]. Such minor variants have been associated with impaired virological responses even while they are not detectable with standard assays [9]. More sensitive detection systems have been developed based on allele-specific PCR (ASPCR) and deep sequencing.

Possible virological consequences of ineffective PrEP use in FEM-PrEP were evaluated including plasma HIV-1 RNA level, time from RNA appearance to antibody detection, and drug resistance occurrence using standardized and sensitive assays for minor viral variants. Drug concentrations near the time of infection were measured directly to help distinguish primary resistance from resistance that may have been selected for during PrEP use.

Methods

FEM-PrEP was a randomized, blinded, placebo-controlled trial of daily oral FTC/tenofovir disoproxil fumarate (TDF) among women in four sites in South Africa, Kenya, and Tanzania as previously described [5]. All seroconverters detected during the randomized treatment phase were included in this analysis. All research participants provided written informed consent in accordance with protocols that were approved by all applicable regulatory agencies [5].

Viral nucleic acid testing was done with the Cobas AmpliPrep/Cobas TaqMan HIV-1 v2.0 having a lower limit of quantitation of 20 RNA copies/ml (Roche Molecular Systems, Branchburg, New Jersey, USA). Quantitative HIV RNA PCR was performed on samples from the seroconversion visit and weeks 4, 8, 12, 16, 24, 36, and 52 after seroconversion. Plasma specimens collected at visits prior to the seroconversion visit were tested retrospectively until the last HIV RNA PCR-negative plasma specimen was detected. The upper layer packed cells (ULPCs) of plasma specimens testing negative using the HIV RNA PCR were analyzed for the detection of HIV-1 DNA (see Supplement, http://links.lww.com/QAD/A621). The infection window period was defined by the last visit with no detectable HIV-1 infection by nucleic acid testing and the first visit with any laboratory evidence of infection, whether it be antibody or nucleic acids or both. $CD4^+$ absolute cell count was performed locally using Trucount tubes on BD Facscalibur.

Clinical resistance testing was done in a manner blinded to randomization group using the TRUGENE genotyping kit (Siemens Healthcare Diagnostics, Inc. Tarrytown, New York, USA) and the PhenoSense Assay (Monogram Bioscience, South San Francisco, San Francisco, USA; see supplement for interpretation). The PhenoSense assay also provides estimates of viral replication capacity, expressed as the percentage of replication relative to a drug-susceptible control in the absence of drug [6]. If resistance to FTC or TDF was detected, subsequent specimens were tested through the end of the study. HIV-1 subtypes were assigned from the population sequences (see Supplement, http://links.lww.com/QAD/A621). Drug-resistant mutants present in a minority of the virus population were evaluated using an ASPCR designed to control for sequence heterogeneity in the primer target regions and by deep sequencing using the 454 platform [10] (see Supplement, http://links.lww.com/QAD/A621).

Tenofovir and FTC were measured in plasma samples using protein precipitation and LC-MS/MS detection as previously described [11]. The lower limit of quantification was 0.25 ng/ml. Intra and interassay accuracy ranged from 96 to 112%, and intra and interassay precision ranged from 5 to 13%. The intracellular active metabolites tenofovir diphosphate (TFV-DP) and emtricitabine triphosphate (FTC-TP) were measured in ULPC samples using an LC-MS/MS method as previously described [12]. Concentrations from these two matrices were utilized to develop an adherence algorithm to PrEP based on previously published data of TFV and TFV-DP concentrations in plasma and red blood cells (see Supplement, http://links.lww.com/QAD/A621 and [11,12]).

Results

Participants and seroconversion

As reported previously [5], we randomized 2120 women, having a mean age of 24 years. Thirty-three infections occurred in the TDF/FTC arm (incidence density 4.7/100 person-years) and 35 in the placebo arm (incidence density 5.0/100 person-years), leading to an estimated hazard ratio of 0.94 [95% confidence interval (CI) 0.59–1.52, $P = 0.81$). The percentage of seroconverters in whom nucleic acids were first detected prior to seroconversion visit was 61% in the active arm and 54% in the placebo arm, and the median time from HIV-1 RNA detection to seroconversion was 4 weeks in both groups.

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Table 1. Emtricitabine-resistant infections.

Assay type/ Fig Ref	Infection window (study week)	FTC before and after window (plasma ng/ml ULPC fmol/ml)	Estimated FTC/TDF PrEP use	FTC resistance mutations (% by ASPCR/% by deep sequencing)	Other NRTI mutations	NNRTI resistance mutations	PI resistance mutations	Resistance interpretation
				Active arm				
Clinical 1a	44–48	Stopped PrEP at week 4	None	M184V (>99.5% /98.5%)	None	K103N	None	Primary
Clinical 1b	0–8	ND–BLQ ND – BLQ	None	M184V (98.6%/96.9%)	None	None	None	Likely primary
Clinical 1c	0–4	ND – 363 ND – 445 000	Sporadic	M184I (99.3%/>99.5%)	None	None	None	Secondary
Clinical 1d	0–4	ND – 1419 ND – 507 000	Sporadic	M184V (99.5%/99.3%)	None	None (K103N appeared 4 weeks later)	None	Possibly secondary
Minor variant	12–26	BLQ – BLQ BLQ – BLQ	None	M184I (0.7%/<BCO)	None	None	None	Primary
				Placebo arm				
Clinical	16–20	n/a	None	M184V (>99.5%/98.8%)	T215Y, M41L	K103N	None	Primary
Minor variant	17–20	n/a	None	M184V (<BCO/1.4%)	None	None	None	Primary
Minor variant	20–24	n/a	None	M184I (<BCO/1.9%)	None	None	None	Primary
Minor variant	13–17	n/a	None	M184V (0.7%/<BCO)	None	None	None	Primary

Infection windows, PrEP drug concentrations and, estimated adherence, all resistance mutations, and interpretation. BLQ, below limit of quantitation; n/a, not applicable; ND, not done; NRTI, nucleoside or nucleotide reverse transcriptase inhibitor; PI, protease inhibitor; PrEP, pre-exposure prophylaxis; ULPC, upper layer packed cell.

Viral load and CD4$^+$ cell counts

At the time of first detection of infection, the mean viral load was 4.9 ($N=33$, SD 1.8) and 5.1 ($N=35$, SD 1.7) $\log_{10}$ RNA copies/ml in the active and placebo groups, respectively ($P=0.77$), and did not differ in the subsequent 52 weeks. Among women in the active arm at the earliest time of HIV detection, either by nucleic acid testing or antibody testing or both, there was a trend toward less plasma HIV-1 RNA level among those with concentrations of drug commensurate with use of four or five tablets per week versus those with detectable drug at lower concentrations and those with no detectable drug in the active arm and those in the placebo arm (mean viral loads 3.5 versus 4.8 versus 5.2 $\log_{10}$ HIV-1 RNA copies/ml, respectively; $P=0.19$). FTC/TDF was discontinued at the time of seroconversion in all participants and plasma RNA levels were similar at subsequent visits. At the seroconversion visit, CD4$^+$ T-lymphocyte counts were 561 ($N=31$, SD 243) versus 614 ($N=31$, SD 288) in the active and placebo arms, respectively ($P=0.59$).

Clinical drug resistance

Tenofovir resistance was not detected in genotypic or phenotypic assays. FTC-associated resistance mutations (RT M184 V/I) were detected in viruses from five study participants at the time of the seroconversion visit (Table 1): one in the placebo arm and four in the FTC/TDF arm, as reported previously [5]. Phenotypic resistance to FTC and lamivudine (3TC) was confirmed in viruses from four of these participants, and the assay was indeterminate in one.

Phenotypic tenofovir hypersusceptibility and viral subtypes

Two of the four FTC-resistant viruses were hypersusceptible to TFV (fold change in $IC_{50} \leq 0.4$), and the other two tended to have greater susceptibility to TFV (fold change in $IC_{50} > 0.4$ and < 1.0; Table 2); three of three with valid results had reduced replication capacity (Table 2). The HIV-1 subtype of the PR-RT sequence was C in 41 (60%), A1 in 20 (29%), D in 3 (4%), and AC or AD mosaics in 4 (6%).

Minor variant drug resistance

Allele-specific PCR and ultradeep sequencing yielded highly concordant results (Fig. 1). The resistant mutants were predominant in the virus population at the time when HIV-1 infection was first detected, either by nucleic acids or antibodies, and waned to levels below the biological cutoff (BCO) of the virus population after FTC/TDF PrEP was discontinued. Among seroconverters without evidence of FTC resistance in clinical resistance assays, analysis of resistance using minor variant assays detected the RT M184I mutation (associated with FTC resistance) at the seroconversion visit for one participant randomized to the FTC/TDF arm at a frequency just above the BCO (0.66% by PCR, <BCO by 454): FTC, TFV, and their intracellular metabolites were undetectable at this and two previous visits. Three participants in the placebo arm had virus showing minor variant FTC resistance by a single assay, one with M184I (1.9% by 454, <BCO by PCR) and two showing M184V (1.4% by 454, <BCO by PCR; <BCO by 454, and 0.7% by PCR).

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Table 2. Resistance cases.

Case	Replication capacity % (95% CI)	ZDV	3TC	FTC	TFV	d4T	NVP	LPR
Active arm								
Clinical 1a	19 (12–30)	0.59	>300	>100	0.58	0.77	>20	0.51
Clinical 1b	Assay failed	**0.17**	>300	>100	**0.29**	0.63	0.65	0.91
Clinical 1c	32 (20–50)	**0.39**	>300	>100	0.56	0.90	0.64	1.06
Clinical 1d	41 (26–64)	0.59	>300	>100	0.60	0.65	3.98	0.74
Minor variant	Assay failed	0.73	1.02	0.92	0.84	0.80	0.45	0.54
Placebo arm								
Clinical	Assay failed				Assay failed			
Minor variant	33 (21–52)	1.17	1.00	1.34	1.16	1.00	1.37	0.65
Minor variant	Assay failed				Assay failed			
Minor variant	Assay failed				Assay failed			

Viral replication capacity (% of wild type) and antiviral susceptibility (fold changes in IC_{50}). Bold values represent hypersusceptibility. 3TC, lamivudine; CI, confidence interval; d4T, stavudine; FTC, emtricitabine; LPR, lopinavir; NVP, nevirapine; TFV, tenofovir; ZDV, zidovudine.

Extracellular and intracellular drug concentrations

Drug concentration analysis was performed on plasma and ULPC at all active arm visits prior to seroconversion. Of the 33 seroconverters in the active arm at the end of the infection window, 21 (64%) had drug concentrations that were negligible or not detected (including three whose infection window period started with enrollment), four (12%) had drug concentrations commensurate with use of 1–2 tablets per week, eight (24%) had concentrations

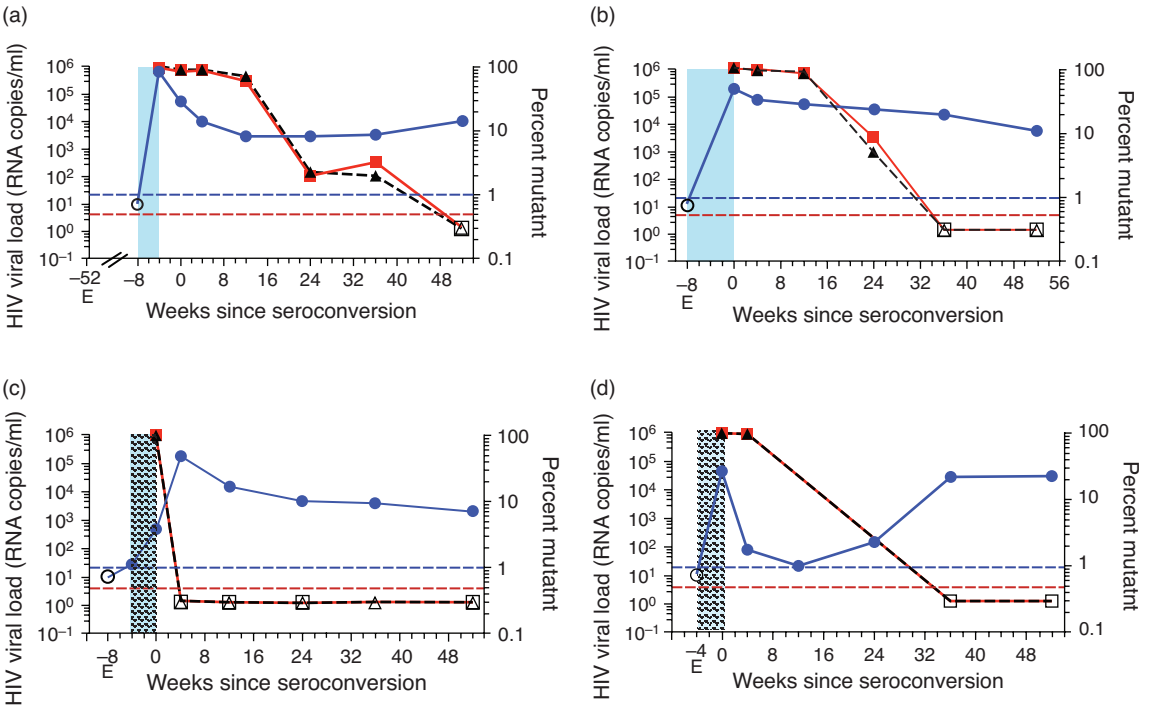

Fig. 1. Longitudinal measurements of HIV RNA and FTC-associated drug resistance. The HIV-1 RNA plasma viral load (left Y-axis, circle) and relative proportion of FTC-associated drug resistance mutations (right Y-axis) measured by ASPCR (PCR, square) and deep sequencing (454, triangle) are shown for the four seroconverters with undetectable plasma HIV-1 RNA at study entry, randomized to the FTC/TDF arm, and with FTC resistance ((a) to (d)). Viruses from participants in (a), (b), and (d) showed drug resistance mutation M184V, while M184I was exclusively and transiently detected in plasma virus from the participant shown in (c). Measurements below the relevant assay cutoff values are represented by open symbols with horizontal blue dotted lines marking the HIV-1 viral load cutoff at 20 copies/mL and red dotted lines marking the background cutoff (BCO) for both PCR and 454 sequencing at 0.5% mutant (V or I). Study entry timepoints are designated below the X-axis by the letter E. The estimated window of infection is marked by the cyan shaded area, defined by the last visit timepoint with undetectable plasma HIV RNA (< 20 copies/ml), and the first visit timepoint with detectable plasma RNA. Plasma and blood cells from either or both visit timepoints bracketing the infection window showing detectable FTC/TFV and FTC-TP/TFV-DP are designated by stippling in (c) and (d). FTC, emtricitabine; FTC-TP, emtricitabine triphosphate; TDF, tenofovir disoproxil fumarate; TFV-DP, tenofovir diphosphate.

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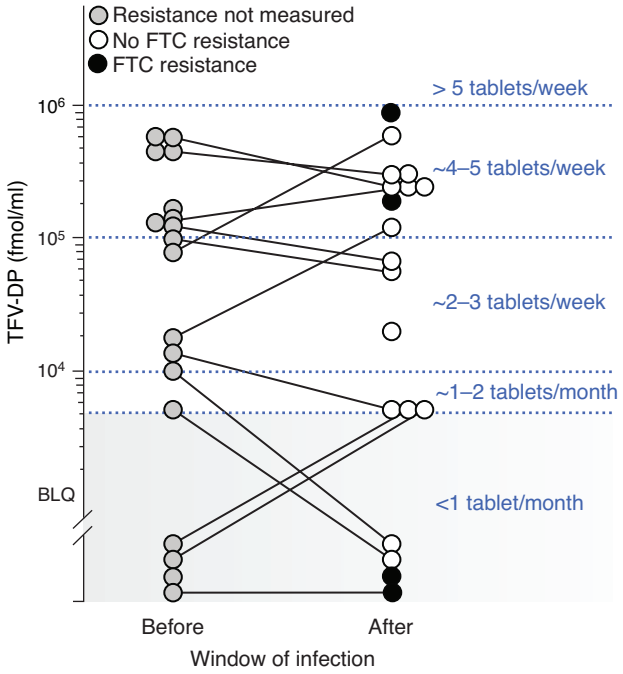

Fig. 2. Intracellular TFV-DP concentrations and clinical drug resistance. Values from active arm seroconverters with drug detected before and/or after the infection window period are plotted, and from all seroconverters with evidence of FTC resistance. Information from seroconverters with no detectable drug or drug resistance is not shown. There was no TFV resistance detected. Values below the limit of detection (5000 fmol) are plotted in the grey area. Black dots represent cases in which FTC resistance was detected using clinical assays. White dots are cases with no evidence of resistance. Resistance could not be measured before the infection window because there was no detectable virus at that visit. FTC, emtricitabine; TDF, tenofovir disoproxil fumarate; TFV-DP, tenofovir diphosphate.

commensurate with use of 4–5 tablets per week, and none had drug concentrations commensurate with use of more than 5 tablets per week (Fig. 2). At the beginning of the infection window period, 20 (61%) had drug concentrations that were negligible or not detected (including 6 that were not tested because their enrollment visit was the beginning of the window period), six (18%) had drug concentrations suggesting use of 1–2 tablets per week, four (12%) had drug concentrations suggesting use of 4–5 tablets per week, and none had drug concentrations commensurate with use of more than 5 tablets per week. Three of the 33 (9%) seroconverters had cellular drug concentrations suggesting average use of 4 to 5 tablets per week at the beginning and end of the window period, of which two had no detectable TFV in plasma at the end of the infection period, indicating no recent use of PrEP.

Correlates of drug-resistant cases in the active arm

Clinical FTC resistance occurred in two of eight (25%) women with indicators of average use of four to five tablets per week at the end of the window period, in none of 4 with detectable but lower concentrations of drug, and in 2 of 21 (9%) of women with no drug detected. Of the six participants in the active arm whose infection window period started with enrollment (the visit of PrEP initiation), three (50%) had clinical FTC resistance. Two infections with FTC-resistant viruses had concomitant resistance to drugs other than TFV and FTC, suggesting primary or transmitted resistance: one infection in the placebo arm had mutations indicating resistance to zidovudine (ZDV) (RT M41L, T215DY), FTC (RT M184V), and non-nucleoside RT inhibitors (NNRTIs; RT K103N; Table 1). In two additional infections, viruses had mutations associated with primary NNRTI resistance and no evidence of FTC or TFV resistance.

Case histories

The time course of clinical resistance detection is given in Fig. 1, and detailed case descriptions are provided in supplemental materials [http://links.lww.com/QAD/A621 (see supplement, http://links.lww.com/QAD/A621)].

Discussion

There were five cases (7%) of FTC-resistant infections among 68 seroconverters in the FEM-PrEP study detected using clinical assays, representing an overall prevalence that is similar to the prevalence of primary or transmitted resistance in Africa (1 to 12%) [13]. Analysis using highly sensitive assays identified one more case in the active arm, and three additional cases in the placebo arm. As with trials that showed evidence of oral FTC/TDF PrEP efficacy [1–4], this trial reveals no excess in the rate of occurrence of FTC resistance even when more sensitive assays are used [10].

Drug resistance can be transmitted between people or selected within a person by antiretroviral medications used for treatment or prophylaxis. One of the clinical FTC-resistant infections had several characteristics associated with recent drug selection: RT M184I was present and typically indicates recent selection by FTC or 3TC, the drug levels were among the highest observed in seroconverters, and the resistant mutant waned quickly in blood plasma (within 4 weeks) after PrEP was stopped. A second FTC-resistant infection also had moderate concentrations of drug and resistance isolated only to FTC, although the month-long delay in the waning of her RT M184V mutant is more typical of primary or transmitted resistance. The two other infections with clinical FTC resistance occurred with no recent drug exposure at the time of seroconversion, although one woman had missed her visit prior to seroconversion, so drug exposure during the infection window could not be ruled out. One infection had evidence of transmitted

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drug resistance to NNRTIs, indicating that these mutants were likely to have been transmitted rather than selected. There were no cases of TFV resistance.

Other PrEP trials indicated that the risk of drug resistance was limited to those in the viral RNA-positive/antibody-negative window period before starting PrEP [1–3]. In the FEM-PrEP study, both FTC-resistant infections that occurred in the presence of detectable PrEP drug concentrations had detectable infection by week 4 of PrEP use. In these two participants, the very rapid appearance of systemic infection in the face of detectable concentrations of PrEP drugs suggests that infection had been incubating at the time that PrEP was started. In contrast to other studies, retrospective use of sensitive standard and investigational assays for viral RNA and DNA in blood specimens did not identify incubating infection in these cases. The sensitivity of such assays may have been impaired by processing, cryopreservation, and shipment required for remote testing. Alternatively, the infections may have been in the eclipse phase between viral exposure and the first expression of virus in the blood, thought to last for 4 to 11 days [14–17].

The clinical implications of the observed FTC resistance are not yet known. The RT M184V/I mutations primarily affect 3TC and FTC while increasing susceptibility to ZDV and TFV, and have no direct impact on the activity of protease inhibitors, NNRTIs, integrase inhibitors, entry inhibitors, or fusion inhibitors. The FTC-resistant viral mutants had diminished replication capacity, which has been associated with better long-term immunological responses to therapy [18] and decreased rates of vertical transmission [19]. The FTC-resistant mutations are present in untreated infections, as discerned by their rapid out-growth after starting 3TC [20] or by deep sequencing that revealed their prevalence between 0.07 and 0.09% [21]. Selective enrichment of these pre-existing mutants by PrEP, even if for a few months, may increase their eventual fitness and their prevalence in the virus population after PrEP is stopped. If so, the prior circulation of M184V/I mutants in these women, whether they were acquired by transmission or enriched by PrEP, could require use of additional active agents to assure the best chance for a treatment response. Use of such second-line regimens could increase costs of treatment and side effects for the patient.

The low frequency of drug resistance in the setting of PrEP failure contrasts with the experience from nevirapine (NVP) monotherapy to prevent mother to child transmission, in which resistance is detected in 25% of women using standard clinical assays that characterize the majority viral variant [22], and in nearly 90% of infants when sensitive assays for minor resistant variants are used [23]. The relatively low occurrence of drug resistance in the setting of oral FTC/TDF PrEP reflects its use in HIV-uninfected people, its high efficacy when used consistently, and the high fitness barrier to TFV and FTC resistance. Seroconversions among those offered PrEP are associated with no or low level of exposure to the drugs [1,2,24]; such low drug concentrations appear to be insufficient to select for FTC or TFV resistance in most people. Unlike NNRTI resistance which preserves viral replication capacity and persists after therapy is stopped [25,26], selected resistance to FTC impairs viral replication capacity and wanes after drug exposure ends, although likely it remains archived in tissue reservoirs [25,27].

The concentrations of drug in this trial were not sufficient to select for TFV resistance; TFV resistance was also not observed in nonhuman primate models [28,29]. This suggests that the concentration of drug required to select for TFV resistance is higher than the level required for prevention of infection. In contrast, the trend toward more frequent appearance of clinical FTC resistance among women with moderate drug concentrations suggests that risk of clinical FTC resistance occurs primarily with higher levels of oral FTC/TDF use, especially if infection is incubating when PrEP is started (as previously observed [1–3]). This finding is also consistent with nonhuman primate studies showing M184V mutations appearing when in some animals dosed daily with FTC, but not in animals receiving two doses per week [28].

Drug resistance was rare in the context of PrEP trials, even when adherence was low and sporadic, as in this study. In practice settings where follow-up and HIV testing may be less well controlled, drug resistance may occur more frequently. Ultimately, the risks of FTC resistance need to be weighed against the benefits of HIV prevention and engagement in care, and monitored during clinical practice.

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Authors' contributions: L.V.D., J.D., T.L., and R.G. conceived and designed the study; W.A., K.A., L.V.D., J.D., and P.D. performed the clinical study and managed specimens; A.K., M.A., G.B., K.F., I.B., and T.C. designed or conducted laboratory testing; D.T. and

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T.L. analyzed the data; R.G. wrote the paper; all authors critically reviewed, revised, and approved the final manuscript.

Conflicts of interest

R.M.G. receives consulting fees from Siemens, the manufacturer of the clinical genotypic test used in this report. Other authors did not report conflicts of interest.

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