**Supplemental Methods.**

The FEM-PrEP trial and procedures.

FEM-PrEP was a randomized, blinded, placebo-controlled trial of daily oral FTC/TDF among women in four sites in South Africa, Kenya, and Tanzania. The primary objective was to assess the effectiveness and safety of FTC/TDF in preventing HIV acquisition. The secondary objectives include assessing the impact of FTC/TDF on CD4+ T-cell count and plasma HIV RNA-level (viral load), and FTC and TFV resistance. The trial and its primary results have been reported elsewhere.[[1](#_ENREF_1)]

Blood was processed within four hours of sample collection. The plasma was obtained by double centrifugation. In brief, a first centrifugation step was conducted for 10 minutes at 800 x g, the plasma was transferred to a conical 15mL sterile tube and a second centrifugation step performed for 30 minutes at 1200 x g. Plasma specimens were aliquoted in cryovials and stored at -70° C until shipped to the Institute of Tropical Medicine (ITM; Antwerp, Belgium). Approximately 1mL of the upper layer packed cells (ULPC) obtained after the first centrifugation step was transferred into a cryo-vial and stored at -20°C until shipment to ITM. Specimens were shipped using a temperature controlled dry shipper.

An in-house HIV DNA PCR was performed according to previously published primers and testing algorithm.[[2](#_ENREF_2), [3](#_ENREF_3)] In order to quantify the cell input in the HIV DNA PCR, an endogenous retrovirus-3 real time PCR was performed as previously described.[[4](#_ENREF_4)] Overall, DNA from 150,000 peripheral nuclear cells was analyzed in each of the two independent nested PCR assays, targeting the *pol* andthe *env* region of HIV-1, respectively. A specimen was considered positive for HIV-1 DNA when at least one amplification assay was positive. To further investigate whether HIV infection was present at enrollment, the DNA PCR was repeated 10 separate times using blood cell specimens from women who seroconverted within three months of enrollment, using two HIV primer sets, allowing analysis of at least three million blood cells at enrollment. As the ULPC is typically comprised of neutrophils (50-70%), eosinophils (2-5%), basophils (<1%), lymphocytes (20-40%) and monocytes (3-6%), we estimate that approximately one third of the cells tested with HIV DNA PCR were lymphocytes and approximately 50% of them are CD4+ T lymphocytes. In summary, approximately 300,000 to 600,000 CD4+ T lymphocytes were analyzed for the presence of HIV-1 at enrollment.

HIV-1 subtypes were assigned from population sequences of HIV-1 protease codons 10-99 and reverse transcriptase codons 40-247 using the Recombinant Identification Program (RIP, <http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html>) with consensus alignment, 300 nucleotide window size and 90% confidence threshold using regions of significant match between query and consensus sequence.

Phenotypic drug resistance assays were interpreted in a standard manner. Phenotypic drug susceptibility is expressed as fold change in drug concentration required for 50% inhibition of viral replication (FC-IC50) relative to wild-type controls.[[5](#_ENREF_5)] The lower clinical cutoff for drug resistance (the cutoff associated with any impairment in virological response) is 3.5 for FTC and 1.4 for TDF; FC-IC50 of < 0.4 is considered hypersusceptible.[[6](#_ENREF_6)]

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[[7](#_ENREF_7)] and by deep sequencing using the 454 platform. Subtype C viruses were amplified using conditions for high fidelity in homopolymer regions that may influence target sequence assessment at the K65R codon (manuscript in preparation). The biological cutoff (BCO) representing the natural frequency of minor variant drug resistance in the absence of drug selection was determined at each drug resistance site for each primer set by measuring panels of subtype C and non-subtype C clinical isolates from subjects infected before the availability of antiretroviral compounds that select for FTC or TFV resistance. Cutoffs were established by using the highest frequency result plus 3SD, or 0.5%, whichever was higher. Cutoff values ranged from 0.5% to 0.7% for ASPCR and from 0.5% to 1.7% for deep sequencing. Study samples were considered positive for minor variant drug resistance if frequencies were above the BCO for the specified subtype and assay primer set. Prior virological studies suggested that RT K65R mutations (that confer resistance to TFV) occur as minor sequence variants more frequently in subtype C infections.[[8](#_ENREF_8)] The genetic background of subtype C eliminates a pause site for reverse transcriptase that increases the rates of base misincorportion.[[9](#_ENREF_9)] We found that use of high fidelity enzymes lowered the biological cutoff for specific detection of RT K65R mutants in subtype C, although these cutoffs remain higher than for other assays and subtypes.

Drug concentration analyses were done by the University of North Carolina (UNC) Center for AIDS Research Clinical Pharmacology and Analytical Chemistry Core at the Eshelman School of Pharmacy, UNC, Chapel Hill, USA. Tenofovir (TFV) and FTC were measured in plasma samples using protein precipitation and LC-MS/MS detection with deuterated internal standards as previously described.[[10](#_ENREF_10)] The lower limit of quantification was 0.25ng/ml. Intra- and inter-assay accuracy ranged from 96-112%, intra- and inter-assay precision ranged from 5-13%. 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.[[11](#_ENREF_11)] The dynamic range of the assay was 1– 2500 ng/mL ULPC lysate. All calibrators and quality control samples were within 15% of the nominal value for both within-day and between-day runs. Within-day and between-day precision was <15%. Recoveries of all analytes were 99-100%.

Concentrations from LC-MS/MS analysis 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.[[10](#_ENREF_10), [11](#_ENREF_11)] There are currently no investigations of ULPC exposure with different dosing patterns. However, recent data in isolated red blood cells (RBCs)[[12](#_ENREF_12)] suggests a linear relationship between TDF dose and TFV-DP RBC concentration such that each dose per week contributes approximately 19 fmol of TFV-DP per 106 isolated red blood cells. Since the number of RBCs in a ULPC sample is approximately 1010, daily dosing would equate to a ULPC concentration of TFV-DP of > 1,000,000 fmol/mL. Based on this information, the algorithm defined 6 dosing patterns: TFV in plasma not detected and TFV-DP < 10,000 fmol/mL (essentially no drug use in interval), TFV in plasma detectable but TFV-DP < 10,000 fmol/mL (probably only a couple of doses taken in the past 4 weeks), TFV-DP 10,000-100,000 fmol/mL regardless of TFV in plasma (perhaps 1-2 doses per week), TFV in plasma < 10 ng/mL and TFV-DP > 100,000 fmol/mL (consistent with adherence early in the interval followed by a stop in the week or two before the visit), TFV in plasma > 10 ng/mL and TFV-DP 100,000-1,000,000 fmol/mL (perhaps 4-5 doses per week), TFV in plasma > 10 ng/ml and TFV-DP > 1,000,000 fmol/mL (perhaps 6-7 doses per week).

**Case Descriptions of participants with FTC resistant viruses detected on clinical testing in the active arm of the study.**

Case 1a (Fig 1A): Seroconversion occurred 48 weeks after FTC/TDF was discontinued due to toxicity at week 4 of the study. At week 4 visit, she was taken permanently off product due to a toxicity (hypophosphatemia and low bicarbonate) and thus had no access to her study drug afterward. The participant never missed a study visit. She was HIV antibody (Ab)-positive at week 52 with an estimated infection window between weeks 44-48. Her week 4 plasma TFV concentration was 63 ng/ml and TFV-DP concentration was 5000 fmol/mL, indicating low adherence. At her week 4 visit, she returned 5 pills. She was infected with subtype A1, and had also NNRTI resistance mutations. The M184V mutation disappeared 24 weeks post seroconversion. There were no symptoms that are characteristic for an acute infection syndrome. At week 12 after her seroconversion, she said her partner was also HIV infected. This infection likely reflects primary FTC resistance transmitted from a partner.

Case Ib (Fig 1B): Seroconversion was detected on week 8 after a missed visit at week 4 due to an upper respiratory tract infection. At the week 8 exam, she still had signs of pharyngitis and swollen submandibular lymph nodes. The estimated infection window is weeks 0-8. She had no detectable drug in plasma or UPLC at week 8. The M184V mutation disappeared at week 24 post seroconversion visit. She had a subtype C virus with an increased susceptibility to TDF and ZDV. She did not use any adherence tool and did not return any study medication tablets at week 8. Given no detected drug at seroconversion, this infection likely represents primary or transmitted resistance to FTC, although some drug exposure early in the infection window cannot be ruled out. The viral syndrome at week 4 reported by the patient likely reflected an acute HIV infection, suggesting that infection may have been incubating at enrollment.

Case 1c (Fig 1C): HIV-1 RNA was first detected at week 4 and seroconversion was detected at week 8 The estimated infection window was between weeks 0-4. Drug concentrations at week 4: TFV: 51 ng/ml; TFV-DP UPLC 196000 fmol/ml; and at week 8 TFV: 65 ng/ml in plasma and ULPC was not tested. These concentrations indicate sporadic adherence, at least until her week 4 visit. The FTC resistance mutation RT M184I was detected at the week 8 visit; the viral load was too low to analyze at week 4. The mutation disappeared on the week 12 sample (4 weeks after the seroconversion visit). She was infected with subtype C. The virus strain showed no other resistance mutations and was hypersusceptible to AZT. She reported almost always using the pills and used the calendar, cell phone alarms and pill boxes as adherence aids. She returned four pills at week 4 and five at week 8. The RT M184I mutation is an uncommon mutation that is typically observed early during drug selection and is quickly overgrown by RT M184V with continued treatment.[[13](#_ENREF_13)] The moderate concentrations of drug present throughout the infection window, the viral mutational pattern, and the course of infection suggests, but does not prove, selection during PrEP rather than transmission of the resistant variant in this case. The first viral detection at week 4 suggests that HIV infection may have been incubating at enrollment.

Case 1d (Fig 1D): Seroconversion was detected at week 4 giving an estimated infection window from weeks 0 to 4. At week 4, the drug concentrations were 467 ng/ml for TFV in plasma and 896,000 fmol/mL for TFV-DP in ULPC. This is indicative of use of four to five tablets per week. The M184V mutation had disappeared 36 weeks after the seroconversion visit, there are no results for 12 and 24 weeks after seroconversion. An additional mutation indicative of NNRTI resistance (the RT K103N) appeared 4 weeks after seroconversion. She was infected with subtype C and the virus had mutations indicating resistance to NNRTI. She used a pill box. She returned 11 pills at week 4. The moderate concentrations of drug and the initial appearance of an FTC-resistance mutation suggest that the FTC resistance may have been selected during PrEP. The seroconversion detected on week 4 suggests that HIV infection may have been incubating at enrollment.

**Supplemental Citations:**

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