

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Celum C, Wald A, Lingappa JR, et al. Acyclovir and transmission of HIV-1 from persons infected with HIV-1 and HSV-2. *N Engl J Med* 2010;362:427-39. DOI: 10.1056/NEJMoa0904849.

Web Appendix for Partners in Prevention HSV/HIV Transmission Study

Laboratory procedures

Dual rapid HIV-1 antibody tests were performed at the clinic and confirmed with HIV-1 EIA. HIV-1 serostatus at enrollment visits for all participants and at follow-up visits for HIV-1 seroconverters was evaluated using Western blot (Genetics Systems™ HIV-1, Bio-Rad laboratories, Hercules, California) at the University of Washington at the end of the study. HSV-2 serostatus for eligibility for HIV-1 infected partners was determined by HerpeSelect-2 EIA (Focus Technologies, Cypress CA), using an index value cut-off of 3.4 to improve test specificity.¹⁻³ HSV-2 serostatus for all participants was confirmed with HSV Western blots using enrollment sera at the University of Washington.⁴ Couples in which the HIV-1 infected partner did not have serologically-confirmed HIV-1 and HSV-2 infection at enrollment were excluded from all analyses.

CD4 quantification for HIV-1 infected participants was performed at screening and 6-month intervals using FACSCount or FACS Calibur Instrumentation (BD Biosciences, San Jose, USA). Plasma HIV-1 RNA was quantified in acid citrate dextrose (ACD) samples collected at the enrollment and month 3, 6, 12, and study exit visits using the 96-test COBAS AmpliPrep/COBAS TaqMan HIV-1 RNA assay, version 1.0, (Roche Diagnostics, Indianapolis, IN) at the University of Washington with a limit of detection of 240 copies/mL.

STI testing using samples collected at the enrollment visit included syphilis testing by rapid plasma reagin (RPR) at site laboratories (with MHA-TP confirmatory testing where available) and batch testing of endocervical swab (women) and urine (men) samples for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis* by nucleic acid amplification assays at the University of Washington (Gen-Probe, San Diego, CA).⁵ Swabs of genital ulcers were tested for HSV DNA at the University of Washington;^{6, 7} samples with ≥ 150 copies of HSV DNA/mL were considered positive.

Determination of HIV-1 transmission linkage among seroconverting couples

To differentiate valid study endpoints from seroconversions not attributable to transmissions within the study partnership, sequencing of HIV-1 strains from participants in all seroconverting pairs was performed (Campbell et al, manuscript in preparation). In summary, viral RNA was extracted from the blood plasma from

both the HIV-1 infected participants and their partners who seroconverted to HIV-1 during follow-up. cDNA was synthesized and a 514 base pair (bp) region of the *env* gene (C2-V3-C3 region) and a 1007 bp region of the *gag* gene (encoding the p17/p24 region) amplified by nested PCR. All partner pair viral sequences were first evaluated by consensus sequencing. Subsequently, viral sequences of 43 partner pairs not initially linked by consensus sequence were evaluated using cloned single molecule templates. Finally, single molecule templates without cloning (pyrosequencing) were used to evaluate 11 putative transmitting partners for rare variants linked to the transmitted virus. Laboratory technicians were blinded to study participants' identification numbers and randomization arm. To minimize risk of specimen mix-up and contamination, specimens from HIV-1 infected partners and seroconverting partners were processed by different technicians, with physical and temporal separation of laboratory work on partners' specimens.

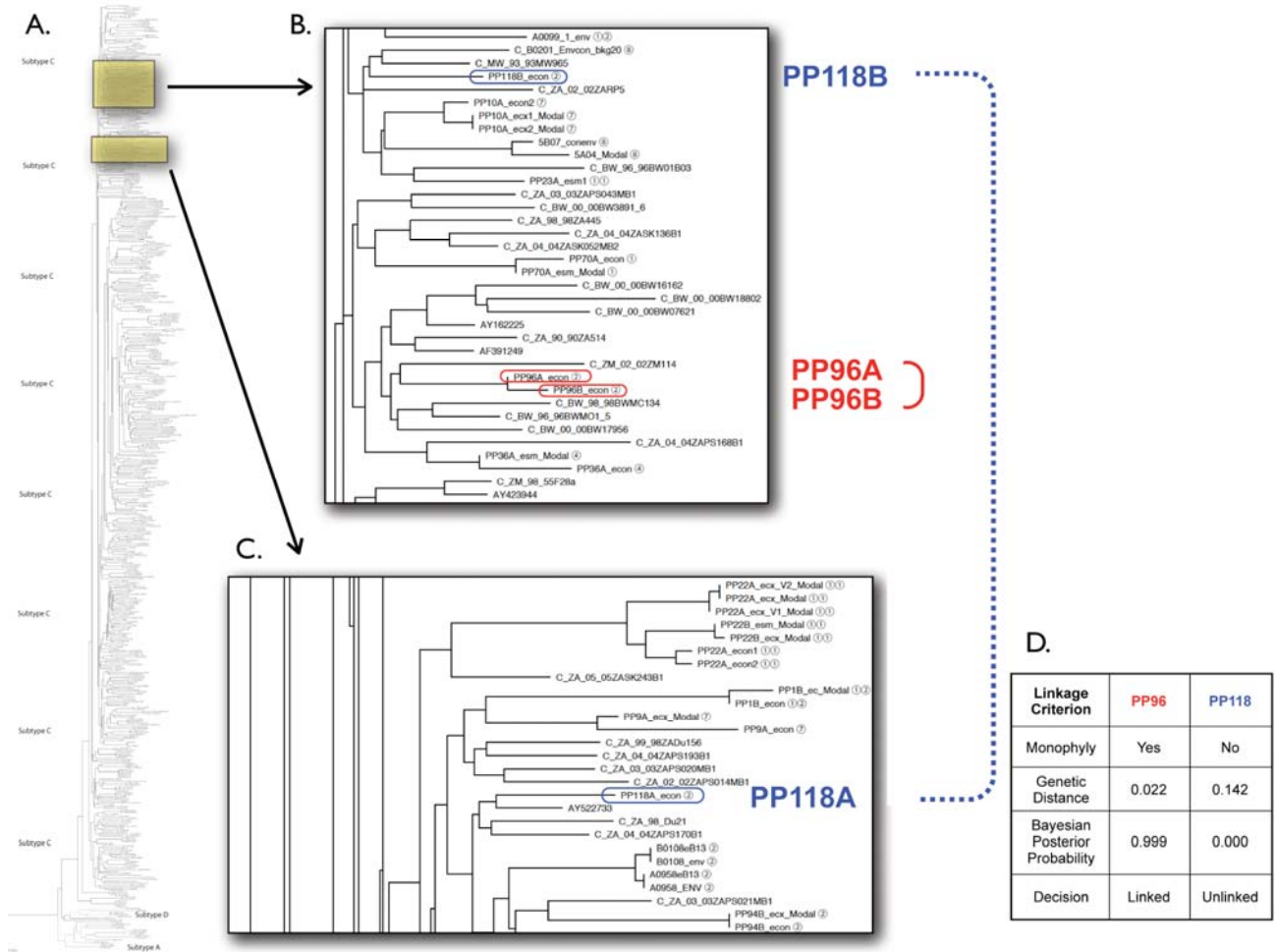
Viral subtypes were determined using REGA 2.0 (<http://dbpartners.stanford.edu/RegaSubtyping>) or the NCBI subtyping tool (<http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>). Separate alignments were created for subtypes A, C, and D for *env* and *gag* sequences, and maximum likelihood phylogenetic trees and pairwise distances were determined with DIVER (<http://indra.mullins.microbiol.washington.edu/cgi-bin/DIVER/diver.cgi>) using a generalized time reversible model of evolution.

In order to ascertain the linkage status of sequences from HIV-1 infected participants and their seroconverting partners, we determined linkage of each couple's sequences based on the phylogeny (yes/no) and the distribution of genetic distances in epidemiologically linked and unlinked sequence pairs from the literature. We included the most closely related sequences from the Los Alamos database⁸ to our sequences for the first 59 pairs, and ≥ 10 local control sequences from each community from which the transmitting pairs were derived. We developed a Bayesian algorithm to include an estimate of the probability of linkage from pairwise genetic distance data. Using the distributions of genetic distances from the reference datasets in known transmission pairs and in epidemiologically unlinked individuals, we determined the conditional probabilities that each pair was either linked or unlinked. In this manner, we used our knowledge about the probability of linkage between sequence pairs with a given distance to derive a posterior probability of linkage between a pair of sequences in our cohort based on the genetic distance data generated in our laboratory.

An adjudication committee consisting of independent experts who were HIV-1 virologists evaluated the data from each seroconverter pair. The adjudicators had not participated in the design of the protocol and

remained blinded to the treatment assignments of participants. A final adjudication meeting took place prior to study unblinding, in which the adjudicators conducted a comprehensive review of the dataset and made final linkage assignments by concurrence. An illustrative phylogenetic tree and linkage information for a linked and unlinked transmission is shown in Figure 1.

Figure 1: Illustrative figure of linked and unlinked transmission pairs



Legend for Figure 1: Determination of HIV-1 sequence linkage. HIV-1 sequences (C2-V3-C3 region of envelope) for trial participants and epidemiologically unlinked individuals are shown in the phylogenetic tree (A). Sections C of the tree are magnified (B and C) to highlight sequences from partner pair (PP) 96 whose sequences were determined to be 'linked' and PP118 whose sequences were determined to be 'unlinked'. Linked sequences from PP96 are circled in red and the two unlinked sequences from each partner in PP118 are circled in blue. Table D shows the criteria used for linkage determination: monophyly (i.e. originating from the same terminal node on the tree), genetic distance (proportion), and Bayesian posterior probability (proportion) (web Appendix).

Retention of study participants by treatment arm and visit

Follow-up of the HIV-infected and uninfected partners by treatment group and study visit is summarized in Table 1. Of those eligible for 24 months of follow-up, 84% and 92% of HIV-1 negative and HIV-1 positive partners, respectively, remained in the study for 24 months

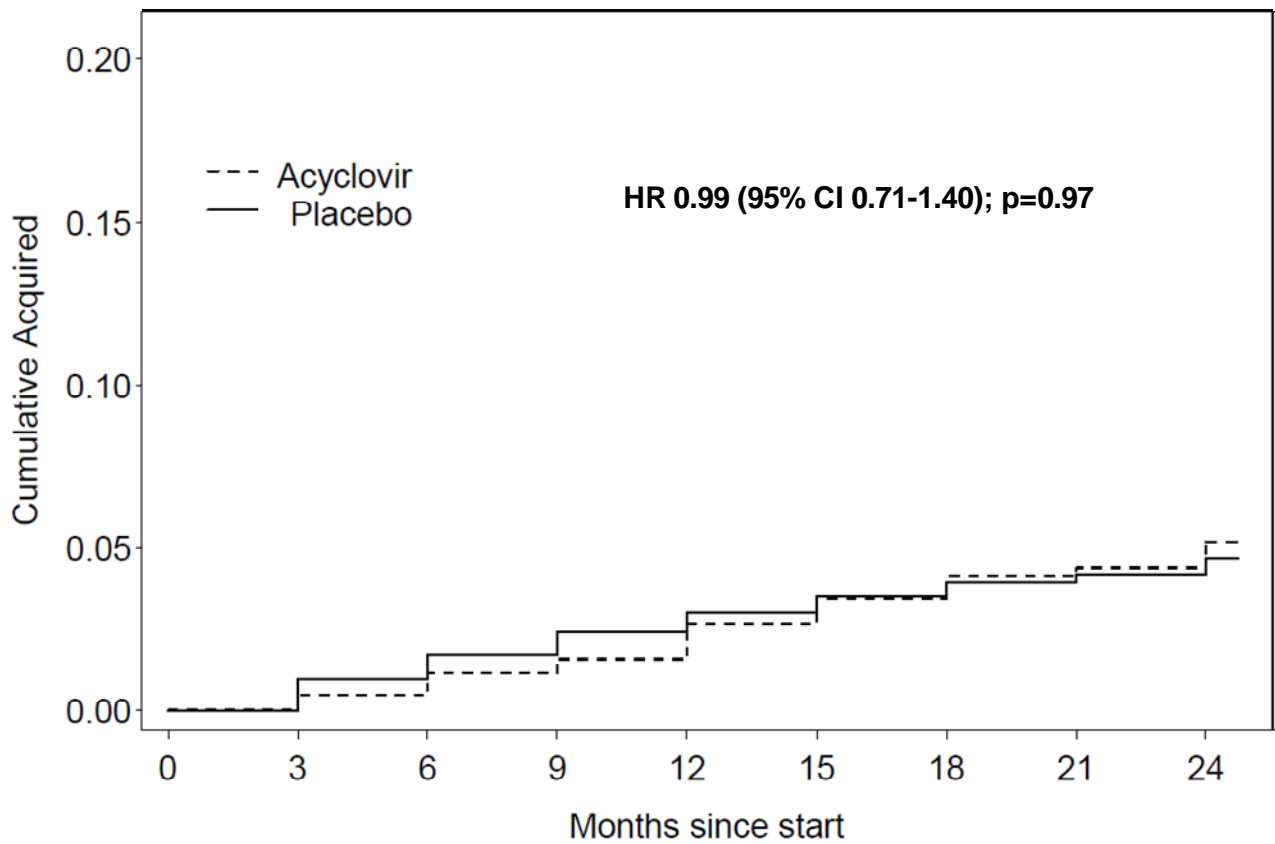
Table 1: Retention by treatment arm and study visit

	Acyclovir		Placebo	
	HIV+	HIV-	HIV+	HIV-
3 months	1626/1680 (97%)	1618/1681 (96%)	1616/1672 (97%)	1593/1675 (95%)
6 months	1610/1678 (96%)	1568/1671 (94%)	1588/1664 (95%)	1541/1658 (93%)
9 months	1555/1653 (94%)	1525/1658 (92%)	1538/1642 (94%)	1496/1643 (91%)
12 months	1446/1535 (94%)	1439/1597 (90%)	1409/1520 (93%)	1393/1571 (89%)
15 months	1253/1354 (92%)	1221/1369 (89%)	1220/1317 (93%)	1178/1348 (87%)
18 months	1030/1124 (92%)	1049/1211 (87%)	1011/1100 (92%)	1027/1185 (87%)
24 months	621/684 (91%)	618/736 (84%)	636/684 (93%)	617/733 (84%)

Kaplan-Meier curve for Intent-to-Treat Analysis

Analysis of time to HIV-1 transmission among the 131 post-randomization transmissions, after excluding the one incorrect study drug dispensation, regardless of linkage by viral sequencing, is depicted in the Kaplan-Meier curve (Figure 2).

Figure 2: Kaplan-Meier curve for Intent-To-Treat analysis



No. at risk	0	3	6	9	12	15	18	21	24
Placebo	1657	1657	1622	1580	1480	1259	1073	814	618
Acyclovir	1645	1645	1600	1551	1440	1225	1053	811	617

Legend for Figure 2: Dotted line represents cumulative probability of genetically-linked HIV-1 transmission for the acyclovir arm, solid line for the placebo arm

Serious Adverse Events

Given the extensive worldwide experience with acyclovir suppressive therapy in the past two decades, only serious adverse events (defined as new onset seizures, hospitalizations, and deaths) were reported. As shown in Table 2 below, SAEs was balanced by study arm. Only one SAE was possibly related to study drug, which was an intentional overdose by a participant who had been randomized to placebo with hospitalization for nausea and vomiting for 24 hours in a participant who had been randomized to placebo.

Table 2: Incidence of serious adverse events, overall and by study arm

	Total	Acyclovir	Placebo
Injury	43	27	16
Pneumonia and other respiratory illness	40	16	24
Malaria	54	27	27
Tuberculosis	7	4	3
Gastrointestinal infections and other disorders	29	14	15
Gynecologic conditions (Pregnancy complications, reproductive system, and breast)	53	31	22
Other infections	17	6	11
Psychiatric	10	2	8
Other	51	21	30
Death	77	35	42
Total	381	183	198

Acyclovir content of study product under field conditions

We performed testing of acyclovir content in study product using an independent certified pharmacologic laboratory (ASI Inc, Durham, NC). The analytical testing was performed to GMP standards on over 20 lots of active acyclovir and placebo tablets returned from the study sites (thus, after storage at field conditions for 12-24 months). The blinded testing confirmed that acyclovir tablets contained 90-100% of indicated label strength.

Acyclovir levels to assess adherence to study drug:

Serum samples from HIV-1 infected study participants on acyclovir or placebo (N=183 and N=10, respectively) were analyzed for acyclovir by liquid chromatography tandem mass-spectrometry (NMS Labs, Willow Grove, PA); the limit of acyclovir quantitation was 20ng/ml. Specimens for testing were randomly selected with representation of sites that obtained time of last dose of study drug, collected at the final study visit from individuals reporting having taken their last study drug dose either ≤ 12 hours (N=114 for ACV arm and N=7 for placebo) or 12-24 hours (N=69 for ACV arm and N=3 for placebo) prior to their study visit.

Of those who reported taking a dose of study drug within the 12 hours prior to blood sampling, 82% (93/114) had detectable acyclovir, compared to 49% (34/69) of those whose most recent dose was >12 hours

prior. The median amount of acyclovir detected correlated with time of last dose (data not shown). None of 10 randomly selected participants on the placebo arm had detectable acyclovir.

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