Supplementary Information

# HIV-1 infections with multiple founders are associated with higher viral loads



Our goal was to assess whether individuals who become HIV-1 infected with multiple founder variants have markers of worse disease outcomes than individuals infected with a single founder variant. Our study compared data from subjects infected with single vs multiple founder variants. The different founder variants are phylogenetically-linked and the distance between variants corresponds to what could be seen between sequences sampled from a chronically-infected subject. Infections with multiple founder variants are different from dual infections (co- or super-infections) in which the different HIV-1 strains are phylogenetically distinct (the distance between strains corresponds to what can be observed between two unlinked HIV-infected individuals).

## 1. Dataset

We tested our hypothesis using sequencing data from HIV-1 breakthrough infections diagnosed in the Step and RV144 vaccine efficacy trials [1,2]. HIV-1 near full-length genome sequences from these two cohorts had previously been deposited in GenBank under the accession numbers JF320002-JF320643 (Step) and JX446645-JX448316 (RV144).

The Step Study tested three vaccinations with the Merck Adenovirus 5 (MRK-Ad5) HIV-1 subtype B gag/pol/nef vaccine in 3,000 individuals between January 2005 and October 2007. The vaccine showed no effect in preventing HIV-1 infections or decreasing viral load (VL) setpoint (defined in this study as the average of the measurements at week 8 and 12 after diagnosis) upon infection [3]. Eighty-two infections had been diagnosed when immunizations were halted and HIV-1 sequences were obtained from 68 subjects residing in the United States, Canada or Peru. All but one Step subject were MSM and all but one were infected with HIV-1 subtype B. In the present study, we excluded the only female in the sequenced

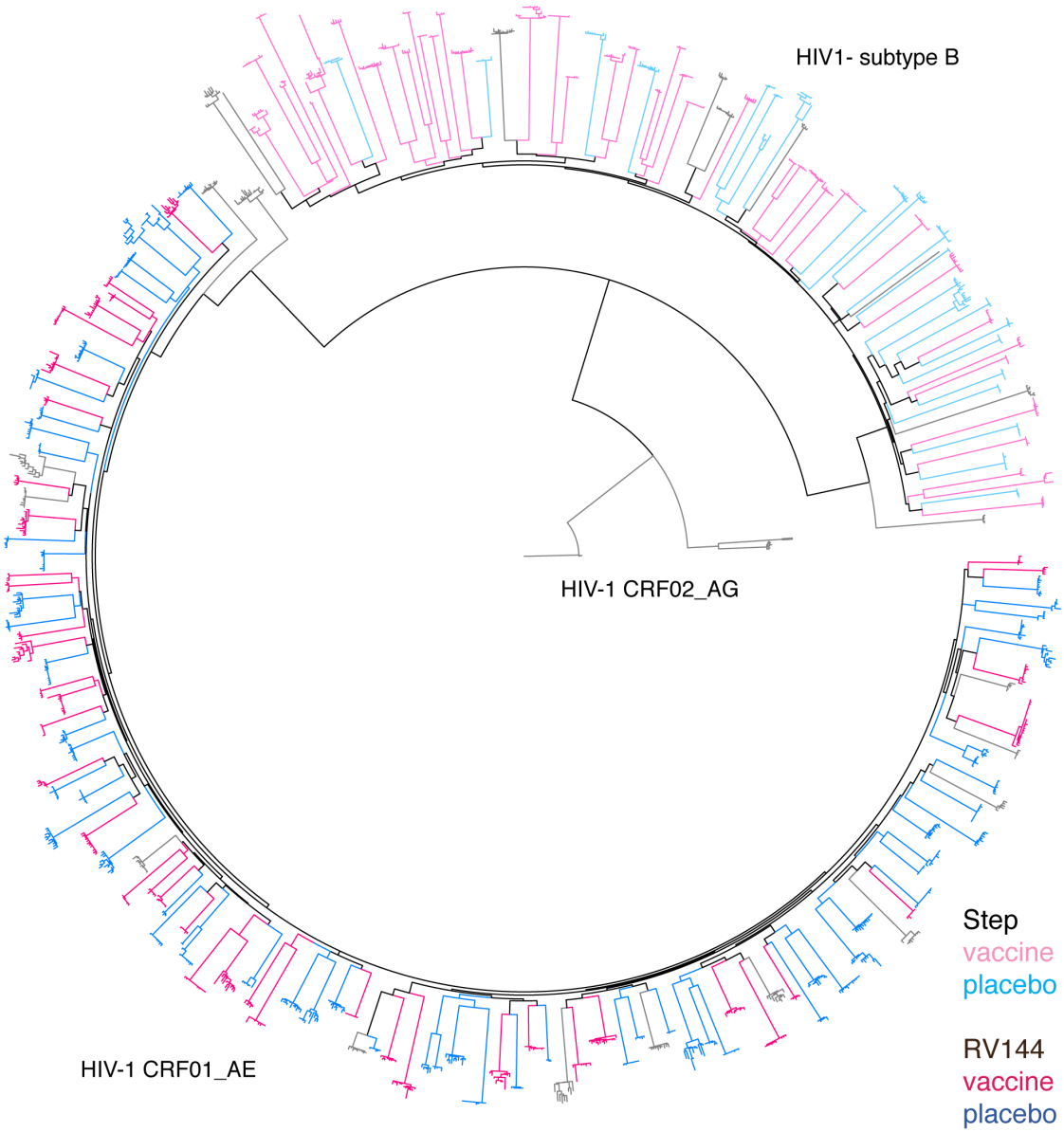
dataset, the only subject with a non-subtype B infection, a subject who was on ART at diagnosis and two subjects with only one sequence available.

The RV144 trial was conducted in Thailand and tested two priming injections of a recombinant canarypox vector (ALVAC-HIV), followed by two booster injections of ALVAC-HIV in combination with a recombinant gp120 subunit vaccine (AIDSVAX B/E) in 16,402 individuals between October 2003 and September 2009. The RV144 vaccine regimen showed an estimated vaccine efficacy of 31% (*P* value = 0.04)[4]. There were 125 infections in the mITT cohort and HIV-1 sequences were obtained from 121 subjects. One hundred and ten subjects were infected with CRF01_AE viruses. The present study included 100 subjects who were infected with CRF01_AE and who were enrolled in the post-infection follow up study.

HIV-1 sequencing was performed via endpoint-dilution PCR (single genome amplification: SGA) on plasma samples collected at the time of HIV-1 diagnosis (except for one Step Study subject sampled 28 days after diagnosis and 6 RV144 subjects sampled on average 26 days later). For both trials, sequencing was performed using similar methods by the same sequencing team. While our depth of SGA-sequencing for HIV genomes was limited, our conclusions on the multiplicity of founders were confirmed for 48 of 63 subjects whose samples were subjected to 454-pyrosequencing of three HIV-1 regions (gag, gp120, gp41/nef) (Iyer and Mullins, personal communication) (samples were selected on the basis of sample availability).

We note that infections were diagnosed earlier in the Step Study: 22 of 68 Step subjects, but only 6 of 125 RV144 subjects, were seronegative at diagnosis, due to shorter times since the last negative visit (105 vs. 181 days) [5].

In sum, the cohort corresponds to 100 Thai individuals (men and women) infected with CRF01_AE and 63 MSM infected with HIV-1 subtype B in the US, Canada and Peru. Due to the differences between the two study groups (different variables among subjects and follow-up schedules), the statistical analyses were performed separately for each cohort.




Phylogenetic tree based on *env-gp120* nucleotide sequences from 67 Step and 121 RV144 participants. Sequences in grey correspond to subjects who were excluded from the analysis (for lack of follow up data or features that violated the treatment/ HIV-1 subtype/gender assumptions for each group).

## 2. Additional analyses

### a. Step Study

We also analyzed "setpoint" VL, using the definition of setpoint used in the Step Study: the average $\log_{10}$ VL values from weeks 8 and 12 post-infection diagnosis. Only 31 of the 63 subjects had both week 8 and 12 VL measurements prior to ART initiation; the remaining 32 had setpoint VL filled in using a multiple imputation procedure [6]. When subjects were divided between homogeneous or heterogeneous founder viral populations, we found that mean setpoint VL was significantly higher for subjects who presented heterogeneous founder viral populations (*P* value = 0.015, adjusted for baseline subject characteristics). Correspondingly, setpoint VL increased significantly with the *env* diversity measured at diagnosis (*P* value = 0.003, Supplementary Table 1).

We assessed longitudinal VL in two additional ways: focusing on measurements within the first 3 months post-infection diagnosis, to look for an early effect of sequence diversity; and focusing on measurements within the first two years post-infection diagnosis, to see if the effect of sequence diversity was still apparent. Focusing on the first three months, subjects with heterogeneous founder viral populations showed significantly higher mean VL than subjects with homogeneous founder viral populations: the estimated difference was 0.44 $\log_{10}$ VL (*P* value = 0.013). Similarly to the categorical data, mean VL over the first three months was found to increase with increasing *env* diversity of the founder population (*P* value = 0.002, Supplementary Table 1). When all VL measurements obtained up to two years post-diagnosis were included, the size of the effects was smaller but still significant with the continuous diversity measure ($p < 0.001$) or trending with the categorical measure (estimated difference = 0.26 $\log_{10}$ VL, *P* value = 0.058) and. There was a significant interaction between *env* diversity and time since infection diagnosis (*P* value = 0.002 and *Q* value = 0.076), with the diversity effect waning over time since infection diagnosis. We also note that the number of data points decreased sharply over time (mostly due to HAART-uptake): 208 VL measurements within 3 months, 276 (an additional 33%) within 1 year, 315 (an additional 14%) within 2 years; and 144 CD4 measurements within 3 months, 216 (an additional 50%) within 1 year, 255 (an additional 18%) within 2 years.

### b. RV144 Trial

We also analyzed longitudinal VL focusing only on the subset of measurements obtained within three months of diagnosis. Subjects with heterogeneous founder viral populations showed significantly higher mean VL than subjects with homogeneous founder viral populations: the estimated difference was 0.30 $\log_{10}$ VL (*P* value = 0.04) (Supplementary Table 1). Similarly to the categorical data, mean VL over the first three months was found to increase with increasing *env*

diversity of the founder population (*P* value = 0.02). Including all data within two years of diagnosis, the difference in mean VL between subjects with homogeneous and heterogeneous founding viral populations was still significant (estimated difference = 0.27 $\log_{10}$ VL, *P* value = 0.04), and trended toward significance when the diversity predictor was considered (*P* value = 0.06). There was a drop off over time in the number of pre-HAART measurements we could include in our analyses: there were 247 VL measurements within 3 months, 485 (an additional 96%) within 1 year, 643 (an additional 33%) within 2 years; and 164 CD4 measurements within 3 months, 402 (an additional 145%) within 1 year, 560 (an additional 39%) within 2 years.

## 3. Tests of interaction

Wald tests of interaction were used to test for statistical interactions between each predictor and the vaccine/placebo assignment. *Q* values were calculated to account for multiplicity across endpoints; we considered $Q < 0.20$ to be significant, implying that up to 20% of the "significant" results could be false positives.
For the analysis of VL in Step, tests of interaction between vaccine/placebo assignment and either homogeneous/heterogeneous founding viral population or *env* diversity were not statistically significant (*P* value = 0.61, *Q* value = 0.86 and *P* value = 0.93, *Q* value = 0.98, respectively).
Regarding the analysis of VL from the RV144 trial during the first year of follow up, tests of interaction between treatment assignment and either homogeneous/heterogeneous founding viral population or *env* diversity were not statistically significant (*P* value = 0.30, *Q* value = 0.61 and *P* value = 0.05, *Q* value = 0.36, respectively).
For RV144 longitudinal $CD4^+$ T cell counts over the first year of infection, tests of interaction between treatment assignment and either homogeneous/heterogeneous founding viral population or *env* diversity were not statistically significant (*P* value = 0.08, *Q* value = 0.36 and *P* value = 0.06, *Q* value = 0.36, respectively).

## 4. Sensitivity analyses

To address caveats about potential confounders, sensitivity analyses were performed. First, the viral sequencing protocol was designed to initially sequence five viruses, with five additional sequences generated if the first five sequences showed some diversity. Since VL at the time of sequencing may affect the ability to obtain sequences, this induces potential confounding. Thus, we performed sensitivity analyses using the initial dataset of five sequences. Results were generally consistent with those for the primary analysis (Supplementary Table

2). Second, the time since HIV-1 infection is a potential confounder: subjects more recently infected are expected to have higher VL and lower genetic diversity. As in most HIV-1-1 cohorts, we lacked data on the precise time of infection, and the small number of subjects diagnosed pre-seroconversion precluded formal analysis of that subset. In RV144, we performed sensitivity analyses omitting 15 participants with sequence data that suggested an infection older than two months: estimated effects of viral diversity were generally smaller and not statistically significant (Supplementary Table 3). Importantly, not accounting for time since HIV-1 infection would be expected to attenuate, not magnify, the estimated associations between viral diversity and measures of disease progression. Indeed, if older infections were mis-categorized as heterogeneous, we would see lower VL in the heterogeneous group as viral load decreases over the first six months of infection (When low viral loads occurred in conjunction with ELISA and Western Blot reactivity, samples were considered to not be samples from the VL upslope but to be samples from later in infection).

## 5. Variance between groups for different endpoints

We compared the variance calculated for subjects presenting either homogeneous or heterogeneous founder HIV-1 populations. There was some heterogeneity between subjects with homogeneous vs. heterogeneous founder viral populations especially in the Step study, but overall the variance was not considered dissimilar between the groups.

| Endpoint | HIV-1 founder population | Step | RV144 |
|---|---|---|---|
| $Log_{10}$ VL at diagnosis | Homogeneous | 0.88 | 0.59 |
| | Heterogeneous | 0.58 | 0.52 |
| $Log_{10}$ setpoint VL | Homogeneous | 0.66 | n.a. |
| | Heterogeneous | 0.24 | n.a. |
| $Log_{10}$ VL over year 1 | Homogeneous | 0.64 | 0.46 |
| | Heterogeneous | 0.46 | 0.38 |
| Square root CD4 over year 1 | Homogeneous | 48.7 | 14.4 |
| | Heterogeneous | 18.3 | 22.1 |

## References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3. Buchbinder, S.P., et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet 372, 1881-1893 (2008).
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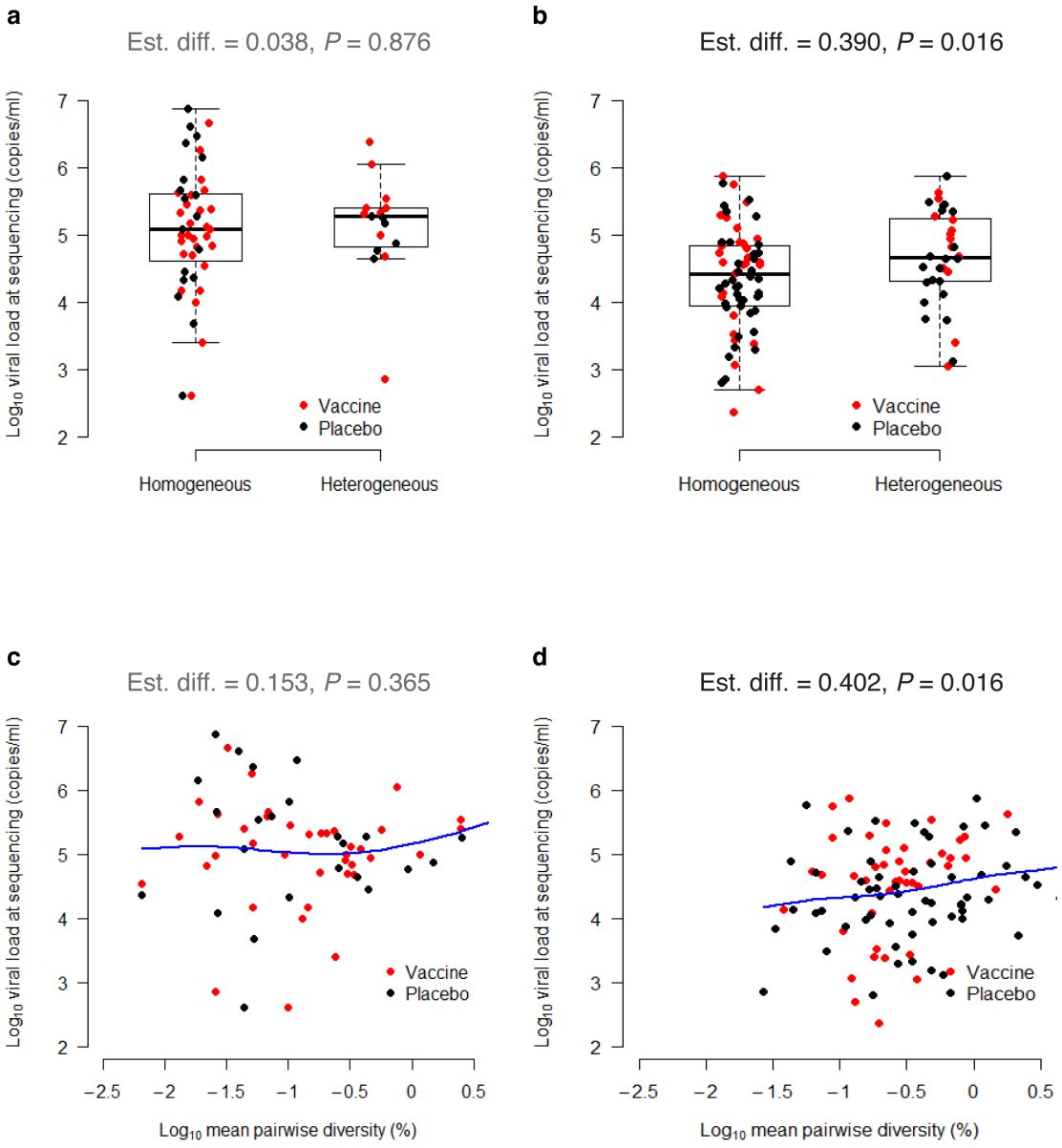


**Supplementary Figure 1:** Relationship between HIV-1 diversity and viral loads at HIV-1 diagnosis. Both HIV-1 diversity and viral loads were measured at the time of HIV-1 diagnosis, when viruses were sequenced. Boxplots show the distribution of $\log_{10}$ VL values ($25^{th}$, $50^{th}$, and $75^{th}$ percentiles) by homogeneous and heterogeneous viral population in the Step (**a**) and RV144 (**b**) vaccine trials. Scatterplots show the association between $\log_{10}$ VL and *env* diversity in the Step (**c**) and RV144 (**d**) vaccine trials. Smooth curves are overlaid.