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# Evaluation of HIV-1 Ambiguous Nucleotide Frequency during Antiretroviral Treatment Interruption

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## Abstract

Nucleotide mixtures in HIV-1 population sequences reflect sequence diversity. We evaluated *gag* and *pol* ambiguous nucleotide frequencies during an analytic treatment interruption (ATI) in an HIV-1 therapeutic vaccine study. The proportion of ambiguous nucleotides was significantly higher at ATI week 16 than at either the time of first detectable viremia (P<0.001 *gag* and P=0.03 RT) or pre-antiretroviral therapy (P=0.007 *gag*). No significant differences were observed in the proportion of ambiguous nucleotides between those receiving vaccine and placebo. Increased HIV diversity during the ATI may represent a potentially higher barrier to success for a therapeutic as compared to a preventative vaccine targeting cell-mediated immunity.

## Keywords

diversity; ambiguous nucleotides; treatment interruption; therapeutic vaccination

## INTRODUCTION

Nucleotide mixtures are commonly detected in sequences of HIV-1 populations by the presence of multiple fluorescent peaks at one position on the chromatogram. These ambiguous nucleotides represent a mixture of HIV variants with differing nucleotides at that position. The proportion of such ambiguous nucleotides serves as a reflection of HIV diversity and has been shown to correlate with the length of HIV-1 infection.[1] However, relatively little is known about the effect of prolonged antiretroviral therapy (ART) on diversity of the latent reservoir and the extent of viral diversity seen when ART is stopped. Here, we evaluate changes in the frequency of ambiguous nucleotides in HIV-1 from participants undergoing an analytic treatment interruption (ATI) as part of the AIDS Clinical Trials Group (ACTG) protocol A5197 study. That study was a randomized, placebo-controlled phase 2 trial to test the safety, immunogenicity, and antiviral activity of a

recombinant adenovirus serotype 5 (rAd5) HIV-1 *gag* therapeutic vaccine on plasma viral load (pVL) in subjects undergoing an ATI.[2] Although immunogenic, this vaccine did not achieve a significant alteration in HIV-1 viral load during the ATI as compared to those receiving placebo. The goals of this analysis were to 1) assess whether the proportion of ambiguous nucleotides can serve as a surrogate marker for the time since treatment interruption; 2) determine the effect of the rAd5 HIV *gag* vaccine on viral diversity; and 3) compare ambiguous nucleotide frequencies during chronic infection with that seen during the ATI.

## METHODS

Study design and patient inclusion criteria for ACTG A5197 have been described previously in detail.[2] Eligible participants were on ART with CD4+ cell counts 500/mm$^3$, plasma HIV-1 RNA levels of 50 copies/mL at screening with a history of pVL 500 copies/mL for 24 months prior to enrollment. Participants received a rAd5 vaccine containing an HIV-1 *gag* insert or placebo at three time points during the first 26 weeks. Starting at week 39, 110 participants (N = 73 vaccine, N = 37 placebo) underwent a 16-week ATI. Most participants had plasma viral sequences analyzed at two time points: the time of initial detectable viremia (tp1, ATI weeks 2–8) and ATI week 16 (tp2). Pre-antiretroviral therapy (pre-ART) plasma was available for 19 participants. The *gag* and *pol* (reverse transcriptase, RT) coding sequences were amplified from plasma HIV-1 RNA by nested RT-PCR using gene-specific primers (Gag codons 1–501 and RT codons 1–400). Population ("bulk") sequencing was performed on an ABI 3730 automated DNA sequencer. Chromatograms were analyzed using Sequencher (Genecodes) and ambiguous nucleotide positions were detected by the presence of multiple fluorescent peaks at one position on the chromatogram (secondary peaks 25% of primary peak). Changes in ambiguous nucleotide frequencies over time were analyzed using the Wilcoxon matched-pairs signed rank test. For the time-averaged area-under-the-curve (TAAUC) analysis, viral rebound kinetics for each study participant were estimated by the linear trapezoidal method, with the use of the first, last, and intervening plasma $\log_{10}$ HIV-1 RNA levels observed during the first 16 weeks of the ATI to calculate the AUC, and by dividing the AUC by the number of days between the first and last observations. A multiple linear regression model was used to derive independent correlates of ambiguous nucleotide frequency. Each participant's time on ART was calculated as the interval between the date of first ART initiation and date of treatment interruption. Only participants initiating ART within the previous ten years (in the era of combination ART) were included in the analysis. All of the p-values are exact 2-sided p-values. No adjustments were performed for multiple comparisons.

## RESULTS AND DISCUSSION

The patient characteristics of those enrolling in ACTG A5197 have previously been reported[2]. HIV-1 *gag* and RT were successfully sequenced from plasma collected at the time of initial detectable viremia (ATI weeks 2–8) for 104 participants, at ATI week 16 for 101 participants, and pre-ART for 19 participants. There was no significant difference in the proportion of ambiguous nucleotides in HIV-1 *gag* at ATI week 16 between those receiving vaccine and placebo (1.13% vs. 1.23%, P=0.82). For both *gag* and RT, the proportion of ambiguous nucleotides was significantly higher at ATI week 16 than at the first detectable viremia (median 1.20% vs. 0.46% for *gag*, median [IQR] difference 0.27% [−0.06%, 1.01%], Wilcoxon matched-pairs signed rank P<0.001; 0.92% vs. 0.67% for RT, median [IQR] difference 0.09% [−0.17%, 0.58%], P=0.03, N=98, Figure 1). However, there were wide ranges in the distribution of ambiguous nucleotide frequencies at each time point and no obvious cut-offs could be discerned to differentiate the early versus late ATI time points in an accurate manner. No significant correlations were detected between the time on ART

and the frequencies of ambiguous HIV-1 *gag* and RT nucleotides at ATI week 16 (Spearman ρ=−0.004, P=0.97, N=83 for HIV-1 *gag* and Spearman ρ=−0.07, P=0.54, N=83 for RT). To evaluate the relationship between cumulative viral replication and the change in viral diversity over time, we evaluated the correlation between the viral load TA-AUC during the ATI with the change in the proportion of ambiguous *gag* and RT nucleotides between the first detectable viremia and at ATI week 16. A marginally significant association was detected between the pVL TA-AUC and the change in the proportion of ambiguous HIV-1 RT nucleotides (Spearman ρ=0.21, P=0.05, N=90 for HIV-1 RT and Spearman ρ=0.15, P=0.16, N=90 for *gag*).

Pre-ART plasma was available from 19 participants. These samples were considered to represent a time point during chronic infection as the pre-ART sample was collected more than one year after initial HIV diagnosis in 7 of the 8 participants with documented date of first HIV+ test; the median time from first HIV-1 diagnosis to the pre-ART sample was 690 days. The median time between ART initiation and the start of the ATI was 6.1 years. There was a significant correlation between the proportion of ambiguous *gag* nucleotides detected in the pre-ART and ATI week 16 time points (Spearman's rho = 0.51, P=0.03). The proportion of ambiguous nucleotides in HIV-1 *gag* was significantly higher at ATI week 16 than pre-ART (0.86% vs. 0.33% for *gag*, median [IQR] difference 0.27% [0.13%, 0.80%], P=0.007; 0.75% vs. 0.58% for RT, median [IQR] difference 0.17% [−0.09%, 0.59%], P=0.21, N=19, Figure 2).

Multiple linear regression analysis was performed to examine factors associated with the proportion of ambiguous HIV-1 *gag* nucleotides at ATI week 16. ATI week 16 viral load (B coefficient = 0.38% per $\log_{10}$ RNA copies/mL, P=0.02), but not treatment arm (P=0.85) or the number of HIV-1 *gag*-specific interferon-γ-producing CD4+ cells (P=0.24), was significantly correlated with the proportion of ambiguous nucleotides in *gag* at ATI week 16.

The detection of ambiguous nucleotides in HIV bulk sequencing is a potential marker of time since infection[1]. In this study, we evaluated whether this method could also be used to estimate time since treatment interruption. Although the proportion of ambiguous nucleotides in HIV-1 *gag* and RT increased over the first 16 weeks of the ATI, the wide distribution of values at each time point makes it unlikely that ambiguous nucleotide frequency could serve as a sensitive clinical marker of time since treatment interruption. A possible explanation for the wide range of ambiguous nucleotide frequencies observed during the ATI could be that reactivation reflects the inter-patient variation in the size and diversity of the latent reservoir, which is seeded on an ongoing basis during acute and chronic infection. In contrast, the majority of acute infection events are a result of the productive infection from a single variant.[3, 4] Just as the extent of viral rebound during a treatment interruption is correlated with the size of the latent reservoir[5], so too are the characteristics of this reservoir (e.g. size and degree of HIV diversity) likely to be important factors in determining the rapidity with which HIV diversifies during the ATI. We hypothesized that a longer time on ART may be associated with a smaller viral reservoir and diminished viral diversity during the ATI as reflected in the proportion of ambiguous nucleotide frequencies. However, we did not detect a significant correlation between time on ART and ambiguous nucleotide frequency at the end of the 16 week treatment interruption.

We also found that the proportion of ambiguous *gag* nucleotides was greater at week 16 of the ATI as compared to pre-ART. The implication of this finding is that the greater diversity of HIV encountered during treatment interruption may reflect a higher barrier to success for therapeutic as compared to preventative HIV vaccines targeting the cell-mediated immune system. This finding is in contrast to that of a report based on the Swiss Spanish Intermittent

Treatment Trial, which found that viral diversity is restricted during treatment interruption as compared to pre-ART.[6] There are, however, a number of uncertainties that could explain the discrepancy in results. The participants of the Swiss Spanish Intermittent Treatment Trial underwent four cycles of 2 week treatment interruptions followed by 8 weeks of ART prior to a final, extended treatment interruption. It is unknown whether these repeated cycles of short treatment interruptions may have constricted viral diversity at the final treatment interruption. Viral diversity increases during acute and early HIV infection, but then stabilizes and may become restricted as chronic infection progresses, most likely due to the selection of highly fit variants adapted to the host environment.[7–9] Therefore, the comparison of diversity between the ATI and pre-ART time point is not only dependent on the diversity seen during the treatment interruption, but also on when the virus is sampled during chronic infection. Further studies of participants from other treatment interruption cohorts are needed to help clarify this issue. The proportion of ambiguous RT nucleotides was also higher at ATI week 16 than at the pre-ART time point, but this difference was not statistically significant. The statistical significance seen for the *gag*, but not RT may be due to the limited sample size and/or a reflection of greater immune-driven selective pressure on HIV-1 *gag* as compared to *pol*.[10]

This analysis has several potential limitations. First, two-thirds of participants received an HIV-1 gag therapeutic vaccine, which could have altered viral replication kinetics due to enhanced virus-specific host immune responses. However, we found no significant differences in the ambiguous nucleotide frequencies between those receiving the vaccine and placebo, and treatment arm assignment was not found to be a significant predictor of ambiguous nucleotide frequency on multivariable linear regression analysis. Another limitation is that the chronicity of HIV infection was unknown for a subset of participants with available pre-ART samples. However, for those who did have a documented date of initial HIV diagnosis, almost all of the pre-ART samples were collected more than a year after the date of diagnosis, supporting our designation of these samples as representative of chronic infection. Finally, ambiguous nucleotide proportion is one approximation of viral diversity and further studies with more intensive methodologies (e.g. single genome sequencing, deep sequencing) would likely provide a more detailed picture of HIV diversity during treatment interruption.

In summary, we found that the proportion of ambiguous nucleotides within HIV-1 *gag* and RT increased over the first 16 weeks of treatment interruption, but that this is unlikely to be a useful clinical marker for time since medication non-adherence. The increased ambiguous nucleotide frequency during the ATI, as compared to chronic infection, may represent greater viral diversity and thus a higher barrier to success for a therapeutic vaccine as compared to a preventive vaccine targeting the cell-mediated immune system. Ambiguous nucleotide frequency is an easy, but imperfect measure of viral diversity, and further work is needed to explore these results.

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**Clinical Trials Registration.** NCT00080106.

## References

1. Kouyos RD, von Wyl V, Yerly S, et al. Ambiguous nucleotide calls from population-based sequencing of HIV-1 are a marker for viral diversity and the age of infection. Clin Infect Dis. 2011; 52:532–9. [PubMed: 21220770]

2. Schooley RT, Spritzler J, Wang H, et al. AIDS clinical trials group 5197: a placebo-controlled trial of immunization of HIV-1-infected persons with a replication-deficient adenovirus type 5 vaccine expressing the HIV-1 core protein. J Infect Dis. 2010; 202:705–16. [PubMed: 20662716]

3. Kearney M, Maldarelli F, Shao W, et al. Human immunodeficiency virus type 1 population genetics and adaptation in newly infected individuals. J Virol. 2009; 83:2715–27. [PubMed: 19116249]

4. Keele BF, Giorgi EE, Salazar-Gonzalez JF, et al. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc Natl Acad Sci U S A. 2008; 105:7552–7. [PubMed: 18490657]

5. Yerly S, Gunthard HF, Fagard C, et al. Proviral HIV-DNA predicts viral rebound and viral setpoint after structured treatment interruptions. AIDS. 2004; 18:1951–3. [PubMed: 15353981]

6. Joos B, Fischer M, Kuster H, et al. HIV rebounds from latently infected cells, rather than from continuing low-level replication. Proc Natl Acad Sci U S A. 2008; 105:16725–30. [PubMed: 18936487]

7. Shankarappa R, Margolick JB, Gange SJ, et al. Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. J Virol. 1999; 73:10489–502. [PubMed: 10559367]

8. Delwart EL, Pan H, Sheppard HW, et al. Slower evolution of human immunodeficiency virus type 1 quasispecies during progression to AIDS. J Virol. 1997; 71:7498–508. [PubMed: 9311829]

9. Arien KK, Vanham G, Arts EJ. Is HIV-1 evolving to a less virulent form in humans? Nat Rev Microbiol. 2007; 5:141–51. [PubMed: 17203103]

10. Brumme ZL, John M, Carlson JM, et al. HLA-associated immune escape pathways in HIV-1 subtype B Gag, Pol and Nef proteins. PLoS ONE. 2009; 4:e6687. [PubMed: 19690614]
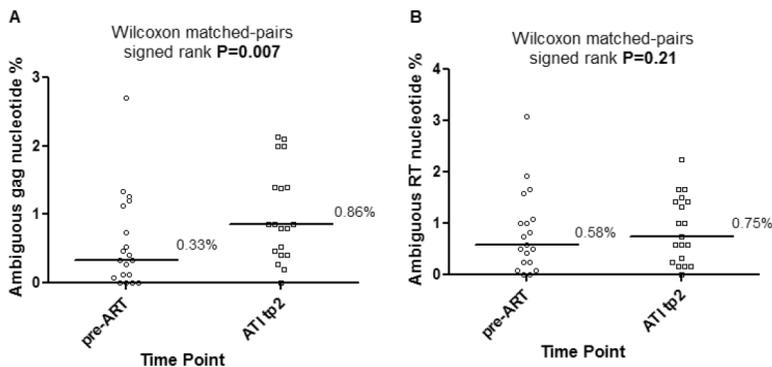
**Figure 1.**
Comparison of ambiguous nucleotide frequency between HIV sequenced during initial viremia (tp1, ATI wk 2–8) and the end of the ATI (tp2, ATI week 16). A, Median and Wilcoxon matched-pairs signed rank test P-values for the percentage of ambiguous nucleotides within *gag*. B, Median and Wilcoxon matched-pairs signed rank test P-values for the percentage of ambiguous nucleotides within reverse transcriptase (RT). ATI, analytic treatment interruption.
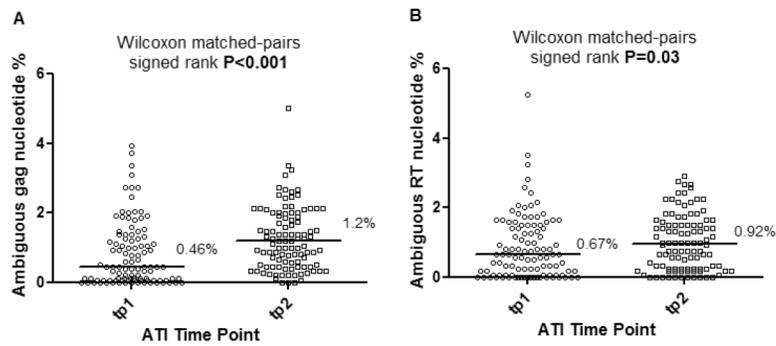
**Figure 2.**
Comparison of ambiguous nucleotide frequency between HIV sequenced from samples collected pre-ART versus at the end of the ATI (ATI tp2, ATI week 16). A, Median and Wilcoxon matched-pairs signed rank test P-values for the percentage of ambiguous nucleotides within *gag*. B, Median and Wilcoxon matched-pairs signed rank test P-values for the percentage of ambiguous nucleotides within reverse transcriptase (RT). ATI, analytic treatment interruption; ART, antiretroviral therapy.