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Relationship of Injection Drug Use, Antiretroviral Therapy Resistance, and Genetic Diversity in the HIV-1 *pol* Gene

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Abstract

Objectives—To determine if a history of injection drug use influences genotypic PI resistance to antiretroviral agents..

Methods—We assessed the presence of resistance mutations in PI-naïve injection drug users (IDUs) and non-IDUs participating in the Women’s Interagency HIV Study. Eighteen HIV-infected participants who reported injection drug use prior to study enrollment and 32 HIV-infected non-IDUs contributed a total of 34 and 65 person-visits, respectively to analyses.

Results—Based on data from multiple clones obtained from different time points from each individual we determined that primary PI resistance mutations were more frequent among person-visits contributed by IDUs (24%) than non-IDUs (8%, $p=0.05$). While neither reached statistical significance, diversity was higher within the protease region among study visits carrying PI resistant clones at both the nucleotide level (2.66 vs. 2.35; $p=0.08$) and at the amino acid level (1.60 vs. 1.32; $p=0.23$). Most of the primary resistance mutations could not be detected using the standard population sequencing employed in the clinical setting. Five of six individuals in whom clones encoding PI resistance mutations were identified failed PI-containing HAART within 12 months of therapy initiation.

Conclusions—Our findings indicate that more aggressive sampling for resistance mutations among viral clones prior to HAART initiation might permit selection of more effective treatment, particularly in IDUs.

Keywords

Injection drug use; antiretroviral therapy; genotypic resistance; HIV; clonal sequencing; population sequencing

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Introduction

Recent studies among carefully monitored, therapy-adherent HIV-1-infected drug users have indicated that the outcome of HAART in this group is worse than in non-drug abusing populations^{1,2}. For example, Gill *et al.* reported at the XVII International HIV Drug Resistance Workshop that injection drug users had a 70% higher risk of resistance than non-drug users¹. The basis for this divergence was not identified in these reports, but findings of a high incidence of early HAART failure have led to the suggestion that individuals should be tested for the presence of primary resistance mutations prior to the onset of therapy³.

The genotypic testing proposed and evaluated in these types of studies, termed population sequencing, involves direct sequencing from plasma of amplified regions of the viral *pol* gene, the products of which are targeted by ART⁴⁻⁶. Because the studied sequences are obtained from the uncloned PCR product from the plasma of individuals carrying many different viral haplotypes^{5,6}, the sequences are representative of the most common haplotypes present in the studied individuals. For individuals who have initially failed ART, such most-common-haplotype sequencing could be justified on the grounds that resistant clones would have already been selected by the ART. Yet, as a general rule, viral clones carrying resistance mutations are less replication competent than wild-type viruses⁷⁻⁹. Thus, in untreated individuals in whom virus faces no antiretroviral selection pressure, one would expect to find the minority clones carrying resistance mutations to be poorly sampled by the standard population genotyping techniques. Even in individuals in whom viral replication has been largely suppressed, the large preponderance of viruses replicating at low levels are archival clones from recently activated cells, rather than newly evolved variants¹⁰. Therefore, the emergence of resistance most frequently results from selection for pre-existing minority clones that become predominant only in the face of ART selection pressure.

In accordance with this analysis, studies using population sequencing to report pre-ART genotypic resistance patterns in ART-naïve individuals have shown a relatively low rate of resistance mutations to protease inhibitors (PI), a class of drugs unlikely to be used outside of the multi-drug therapy context. In a representative study, Gange *et al.* examined primary genotypic resistance to protease inhibitors in HAART naïve women from the Women's Interagency HIV Study (WIHS)¹¹. That study determined that *pol* mutations encoding primary resistance to PIs were detected in virus from 4% of the HAART-naïve women who were evaluated. However, using the same population genotyping technique, this group found that within approximately one year of HAART initiation, 21% of subjects "developed" PI resistance mutations, based on population sequences at the study visit at which viral relapse was first noted.

Because viral replication continues only at a very low level in the face of effective HAART, it is likely that this rapid emergence of resistance reflected the pre-existence of clones resistant to PI that were not detected in the original, pre-HAART sample. If this is the case, then more sensitive techniques for detecting pre-existing primary resistance might provide a more effective guide for selection of ART medication to include in the initial HAART regimen.

In the current study, we have compared the relative ability of population sequencing and clonal sequencing to detect primary resistance in a population of women with or without a history of injection drug use. Injection drug users (IDUs) were selected because our previous work examining genetic diversity within the *env* region demonstrated that IDUs showed a significantly higher frequency of viral mutation than non-IDUs¹². We also examined the relative frequency of resistance to reverse transcriptase inhibitors (RTI), to which many of our subjects had been exposed, and resistance to protease inhibitors (PI), which none of our subjects had received.

Materials and Methods

Study population

The WIHS is a multicenter, prospective cohort study to investigate the impact of HIV-1 infection on women¹³. In 1994, 2,623 women (2,054 HIV-1 positive and 569 HIV-1 negative) were recruited by both institution and community based programs. Every six months the participants met with study personnel for an encounter termed a “visit”, during which WIHS participants were interviewed using a structured questionnaire and received a physical examination¹³. An institutional review board approved study protocols and consent forms, and each WIHS participant gave written informed consent.

Fifty-eight HIV-1 infected individuals contributing 123 study visits were selected for analysis. All samples were from visits that occurred prior to 2000. All participants met the following criteria: 1) defined IDU status prior to WIHS enrollment 2) a visit within 12 months prior to initiating HAART 3) HIV-1 RNA >10,000 copies/ml of plasma to avoid re-sampling the same virion¹⁴ and 4) CD4 cell count <200 cells/mm³ on the last pre-HAART visit as an indication of disease progression. Nineteen IDU (33%) met these criteria and from the non-IDU that met the criteria 39 (67%) were randomly selected for analysis.

Sequencing Technique, Clonal

A total of 1100 cloned sequences within the *pol* gene of HIV-1 were obtained¹⁵. Gene regions (protease or RT) from specific clones that were identified by sequence analysis as having stop codons were excluded, resulting in a total of 583 RT region clones and 755 protease clones that contributed to the final analysis. To obtain clonal sequences HIV-1 RNA was isolated from stored samples of plasma using the QIA amp viral RNA mini- kit (QIAGEN, Valencia, CA). The isolated RNA was subjected to RT-PCR (Life Technologies Superscript One-Step RT-PCR for long templates) using the primers pro-1 (TTGGAAATGTGGAAAGGAAGGAC) and RT-0 (CATATTGTGAGTCTGTTACTATGTTTAC) with cycles of 50°C 30 minutes, 94°C 2 minutes, and 35 cycles of 94°C 40 seconds, 50°C 40 seconds, 68°C 3 minutes, followed by one cycle of 72°C 10 minutes and then held at 4°C. A second round PCR was run using the Gene Amp XL PCR kit (Roche Applied Biosystems, Indianapolis IN), with the primers pro-3 (GAGCCAACAGCCCCACC) and RT-3 (GCTGCCCCATCTACATAGAA); with an amplification protocol of 94°C for 1 min, followed by 35 cycles of 94°C for 40 seconds, 52°C–56°C for 40 seconds, 68°C for 2 minutes, 30 seconds, followed by one cycle of 72°C for 10 minutes with the product held at 4°C until harvested for running the product on an 8% agarose gel. A band at the 1,617 base-pair size was extracted from the gel using the QIA Quik Gel Extraction Kit (Qiagen, Valencia, CA), and the obtained DNA was ligated into the TOPO 2.1 vector and transformed into TOPO 10 competent cells (Qiagen, Valencia, CA), according to the manufacturer's instructions. The transformed cells were plated on LB agar plates containing 50µg/ml Ampicillin and 40µl of 40mg/ml X-gal. Confirmed transformants were grown overnight and plasmid DNA was extracted for sequencing, using an ABI prism 3700 DNA Analyzer (Perkin Elmer Biosystems, Boston, MA). The cloned sequences were obtained in nucleotide format and translated into amino acids using MegAlign software by DNASTar (DNASTAR Inc., Madison, WI). The entire protease (PR) region (297 nucleotides) and partial reverse transcriptase (pRT) region (674 nucleotides, including all known sites of resistance mutations) were available from each of the 123 study visits¹⁶.

Sequencing Technique, Population

Among the 18 person-visits with PI-resistance mutations detected by clonal analysis, as determined by the Stanford website (see Genotypic Resistance section), 13 specimens were available for population sequencing. Specifically, population sequencing was done on codons 1–99 of the protease gene using the Trugene HIV-1 Genotyping Kit (Bayer Diagnostics,

Emeryville, CA) ^{5, 6}. These studies were performed at the The AIDS Research Institute at the University of California, San Francisco Laboratory of Clinical Virology. Because sequences obtained by population sequencing reflect the most replication competent virions, none of the sequences determined by that method contained stop codons and therefore none were excluded from analysis.

Genetic Diversity

Genetic diversity among clones obtained at a single visit was estimated by a heterogeneity measure ^{15, 17} that combines information among clones by averaging a genetic distance defined at each study visit among all distinct pairs of clones obtained from an individual. Larger values of this measure are associated with greater heterogeneity among clones. Genetic diversity was measured using both the amino acid (aa) and nucleotide (nucleic acid, na) representation of the *pol* genes to identify the presence of both synonymous and non-synonymous mutations and their association with injection drug use history.

Exclusions

To ensure that differences observed between study visits contributed by IDUs and non-IDUs, with respect to clonal diversity and ART resistance, would be attributable to lifetime drug use, rather than increased exposure to multiple viruses we excluded *pol* clones for which a phylogenetic analysis suggested the possibility of multiple infections. To exclude subjects at risk for multiple infections, we first constructed a single neighbor-joining tree using PAUP* ¹⁸ of all the *pol* sequence data from all subjects and all study visits. A participant was regarded as having only a single source infection if the *pol* sequences defined a single monophyletic cluster in the multi-subject neighbor-joining tree. Additional analyses were performed if the *pol* sequences from a participant defined two or more disjoint clusters (polyphyly) within the multi-subject neighbor-joining tree. When polyphyly was detected, a tree was constructed that forced all the sequences from a single participant to be monophyletic, and the Templeton test option in PAUP* ¹⁸ was used to test the null hypothesis that the polyphyletic tree was not significantly different from the monophyletic tree. Every case of polyphyly was highly significant. Many of these cases involved a single sequence that was an evolutionary outlier from most of the other sequences from the same participant. These sequences were discarded from all subsequent analyses as either representing a possible contamination or a possible multiple infection in which only one source of infection dominated.

Sometimes two distinct evolutionary clusters existed such that both contained two or more haplotypes. These cases were regarded as multiple infections. Even though both clades had more than one sequence representative in these cases, one of the clades was numerically dominant. In this analysis, the clade with the lesser number of sequences in it was discarded so as not to confound the diversity that evolved within a subject versus diversity brought in by possible multiple infections. Overall, a total of 51 haplotypes (from 20 individuals) were excluded from the entire database.

Genotypic Resistance

The determination of each sequence from the cloned (non-stop codon) sequences as whether resistant or not to PI or reverse transcriptase inhibitors (RTI), including non-nucleoside RTIs (NNRTI) and nucleoside RTIs (NRTI), was made by comparing sequences to those in the Stanford HIV Drug Resistance Database (<http://hivdb.stanford.edu/>, version 03/2006). A viral clone was categorized as PI resistant or RTI resistant, if the level of resistance, as defined by the algorithms of the Stanford HIV drug resistance database, indicated the presence of mutations associated with resistance to a particular drug. Each of the 99 study visits from the Protease region and 83 study visits from the RT region were defined as resistant to a class of antiretroviral agents if any amplified clone showed evidence of genotypic resistance to an agent

within that drug class. The resistance status from sequences obtained by the population method of genotyping was done in a similar manner.

Statistical Analyses

Wilcoxon rank sum tests and χ^2 tests were used to test for differences in baseline characteristics between IDUs and non-IDUs. The relationships between injection drug use, ART resistance, and genetic diversity were assessed using generalized linear models with generalized estimating equations for estimating parameters and standard errors, assuming an exchangeable correlation structure for each outcome from the same individual over available study visits 19, 20.

Results

Description of the Study population

A detailed characterization of the study population is shown in Table 1. Because some of the sequenced gene regions contained stop codons and were therefore not included in the analysis, there are differences in the number of subjects and visits included in the analyses of PI resistance (Table 1A: n=50; 18 IDUs and 32 non-IDUs) and RTI resistance (Table 1B: n=44; 15 IDUs and 29 non-IDUs). There was considerable overlap with 44 women (15 IDUs and 29 non-IDUs) contributing a total of 82 person-visits to both analyses.

IDUs and non-IDUs were of similar ethnic background and had similar baseline CD4 and HIV RNA levels. IDUs were older and more likely to be positive for the hepatitis C virus. None of the participants in either analysis had reported prior exposure to PIs. Among the 44 women that were evaluated for the presence of resistance mutations to RTI, 38% of the non-IDUs and 20% of the IDUs (P=0.31) reported taking RTIs at their initial study visit and 67% of the IDUs and 55% of the non-IDUs (P=0.46) had reported a history of exposure to RTI prior to the initial visit.

For the analysis of PI resistance, among the 18 women in the IDU group, 277 protease region clones were analyzed from 34 person-visits (mean of 8.1 clones per person-visit) while among the 32 women in the non-IDU group, 478 distinct viral clones were analyzed from 65 person-visits (mean of 7.4 clones per person-visit). For the analysis of RTI resistance, among the 15 women in the IDU group, 202 clones were analyzed from 27 person-visits (mean of 7.45 clones per person-visit) while among the 29 women in the non-IDU group, 381 clones were analyzed from 56 person-visits (mean of 6.8 clones per person-visit).

Frequency of Resistance Mutations by IDU status

Because our study population contained individuals with a relatively high rate of previous exposure to NRTIs and no exposure to PIs, we examined the relative frequency of resistance mutations to these two classes of ART between IDU and non-IDU subject-visits (Table 2). The frequency of PI resistance mutations was three fold greater among IDUs (24% of study visits) than among non-IDUs (8% of study visits, p=0.05). In both groups the presence of resistance mutations to RTI was strikingly high, though not significantly different (p=0.98); 54% of non-IDU and 56% of IDU person-visits contained viral clones carrying such resistance mutations.

Both the non-IDU and IDU subjects had a high prevalence of individuals who were previously exposed to RTIs, either prior to or at the baseline visit. Due to its temporal proximity to the sampling point, we focused on the RTI use at the baseline visit and assessed whether there was a correlation between RTI use at that visit and the appearance of RTI resistance mutations at that visit or any of the subsequent subject-visits. Compared to those not using an RTI at baseline, the risk of carrying an RTI resistance mutation at any subject-visit was 5.28 times

greater among those using RTI at the baseline visit (95% C.I. 1.74, 16.01, $p<0.01$) (data not shown).

Association of IDU status and genetic diversity among within-subject-visit viral clones

Since neither the IDU nor non-IDU subjects had reported a history of PI use prior to baseline, we sought alternative explanations for the higher frequency of PI resistance mutations among IDUs. Previous studies from this laboratory demonstrated an association between frequency of injection drug use and genetic diversity among viral clones circulating within IDUs at a given time point¹². Increased diversity among drug users might be expected to give rise, simply on a chance basis, to a higher frequency of resistance mutations. However, in the protease region there was no statistically significant association at either the nucleotide or amino acid level between within-visit genetic diversity and IDU status (Table 3).

Given this finding we assessed if, independent of IDU status, there was an association within the protease region between diversity and resistance (Table 4). While neither reached statistical significance, diversity was higher within the protease region among study visits carrying PI resistant clones at both the nucleotide level (2.66 vs. 2.35; $p=0.08$) and at the amino acid level (1.60 vs. 1.32; $p=0.23$).

Relative Sensitivity Of Clonal and Population Genotyping For Detection Of Primary PI Resistance Mutations In IDU

Because the primary resistance frequency to PI among PI-naïve IDU was higher than in any previous reports, we ascertained whether this level could be attributed to the increased sensitivity of the clonal sequencing method compared to the population sequencing method that was employed in earlier studies (Table 5). We were able to compare the relative sensitivity of the two sequencing methods in 10 of the 13 person-visits at which PI resistance mutations were detected. Only 2 of the 10 subject-visits containing clones carrying PI resistance mutations could be identified as carrying resistance mutations using the population sequencing method. In one of the subject-visits detected by population sequencing, only a minor resistance mutation (A71T) was identified, while in the clonal analysis the V82A mutation was identified which was sufficient to encode drug resistance.

Because the genotypic resistance patterns were not available at the time of initiation of HAART, six of the subjects identified by clonal analysis as carrying PI resistance mutations were subsequently placed on HAART that included a PI. Five of those six had viral relapse within one year of initiating this therapy.

Discussion

This study had two purposes: first, to explore whether the use of the clonal sequencing technique within the HIV-1 *pol* gene would provide greater sensitivity for detecting resistance mutations than the population sequencing technique currently in clinical practice and secondly, whether, using this more sensitive technique, IDU could be shown to have a greater risk of carrying resistance mutations to a class of antiretroviral agents to which neither IDU nor non-injection drug-using subjects had been exposed. This latter hypothesis arose from previous observations that genetic diversity within the HIV-1 *env* gene was strongly associated with frequency of injection drug use¹².

We found, using the more sensitive cloning technique to identify resistance mutations, that both IDU and non-injection drug using subjects had a high prevalence of visits (54–56%) in which a minimum of one clone was resistant to at least one RTI. The similarity in resistance proportions was observed despite the fact that among the study-visits evaluated for RTI

resistance a greater proportion of non-injection drug using subjects were taking RTI at their first study visit than was the case for IDU, and taking RTI at the time of study enrollment was, as might be expected, a significant risk factor for carrying viral clones with RTI resistance mutations ($p=0.002$). However, the proportion of subjects among the two cohorts distinguished by IDU history, that had any history of RTI exposure was similar, suggesting that even more temporally distant exposure to RTI contributed to the presence of RTI resistance mutations. Because one of the requirements for entry into the study was an HIV-1 viral load greater than 10,000 copies/ml., all of the enrollees would have been considered to have failed ART whether or not they were actively taking RTI at the time of enrollment. Of particular interest, however, was the marked difference in the prevalence of study-visits in which clones carrying PI resistance mutations were detected among IDU compared to non-injection drug using subjects ($p=0.05$). Among the subject-visits studied for PI resistance, viral clones from 24% of the person-visits from this cohort of PI-naïve HIV-1-infected women with a history of IDU carried mutations encoding resistance to PI. Virions from only 8% of person-visits of non-injection drug using women from the same cohort carried viral clones encoding such mutations.

None of the subjects were exposed to PI prior to or during the observation period. Thus, in the case of PI the differences could not reflect a selection phenomenon related to previous exposure. It should be noted that the frequency of NRTI resistance mutations was much greater than PI resistance mutations independent of IDU classification and the high frequency of such resistance mutations have been reported in another study of the WIHS cohort ¹¹, which used only population sequencing to study the prevalence of genotypic resistance. This frequency of resistance mutations likely reflects the prior widespread use of these agents among these women initiating HAART in the timeframe of this study when this class of agents were used as monotherapy or as part of a dual therapy regimen. Because the prevalence of resistance to these agents is so high in the non-drug using population, any additional effects of drug abuse on NRTI resistance might be difficult to demonstrate. Alternatively, the effects of drug use on the emergence of NRTI resistance mutations may be insignificant compared to the effects of many years of selection under drug pressure.

It is less clear why NNRTI resistance mutations should be less frequent than PI resistance mutations, especially since some of the subjects had been previously exposed to NNRTI. The reasons for this are likely complex and related not only to the issue of selection pressure, but also to the replicative efficiency of viruses carrying PI and NNRTI resistance mutations in the absence of any antiretroviral therapy. There is an extensive literature addressing the issue of replication competence and resistance mutations, e.g., ^{21–25}, particularly as it relates to specific mutations. Most of this work is based on *in vitro* studies of replication competence and may not integrate the complex set of host factors that will influence replication competence in a given host environment. One feature that may favor greater persistence of PI resistance mutations is the ability to restore replicative capacity through further mutation even in the absence of selective pressure ²⁶. Such adaptation would increase the likelihood that these genotypically resistant clones could be detected with the limited clonal sequencing that we undertook in the current study.

Our previous studies¹² have provided some basis for understanding why IDU that had not been exposed to PI might have a higher frequency of resistant clones. Those studies showed that diversity within the HIV-1 *env* gene was significantly correlated with intensity of injection drug use. In the current study we did not demonstrate an association between diversity within the protease region and the presence of resistance mutations and did not show increased genetic diversity in the protease region among IDU compared to non-injection drug using subjects.

One difference between the previous study and the current study is the time frame in which drug abuse occurred. In the previous study of the effect of injection drug use patterns on within-

person-visit diversity within the HIV-1 *env* gene we demonstrated that the concomitant intensity of injection drug use by an HIV-1-infected individuals who were actively injecting was strongly associated with genetic diversity¹². In the current study of women participating in the WIHS, women reported a history of injection drug use but did not report actively injecting. Thus the immunologic and drug-attributable^{27–29} stimuli to viral replication, and its associated enhancement of diversity, were not concurrently present at the time of sampling of the *pol* gene for resistance mutations and genetic diversity. Previous studies have indicated that immunologic stimuli give rise to bursts of viral replication that diminish when the stimulus is removed^{30–33}.

As the active stimulus to viral replication declines, the continuous emergence of new variants and the active population of replicating virus would be reduced, with an associated reduction in diversity detected by limited sampling of viral clones. However, a more complete spectrum of the diversity generated during the periods of high replication would likely be maintained in the viral archive and accessible to more aggressive sampling techniques. Thus, while our limited analysis of viral clones might not demonstrate the full range of the diversity within the viral archive generated by previous intravenous exposure to illicit drugs, we might still be able to detect a higher frequency of drug resistance mutations within that archive.

Our study clearly demonstrates that sequencing more deeply into the viral genome of infected individuals allows the detection of resistance mutations that are not detected by population sequencing. Palmer et al.³⁴ have shown that, even in individuals in whom ART-resistant clones might be selected by ART, mutations exist within the viral archive that are not detected by the population genotyping that is used in the clinical setting to guide therapy for individuals who have failed an initial course of HAART. That relevant mutations might exist at high frequency in individuals who have never received a particular class of agents, as we have found, suggests that appropriate screening would also be useful for therapy naïve individuals.

The previously cited study by Gange *et al.*¹¹ demonstrating rapid emergence of resistance among individuals who had no evidence of such resistance by pre-treatment population sequencing, supports the need for more aggressive pre-treatment resistance analysis. Tobin *et al.* have shown that even in individuals in whom viral replication has been largely suppressed by ART, the large preponderance of viruses replicating at low level are archival clones from recently activated cells, rather than newly evolved variants¹⁰.

The current findings also suggest that the development of ART-resistance may be a particular problem for IDU. While many studies have documented that ART-treated IDU have less favorable treatment outcomes than non-injection drug using subjects^{2, 35–38}, treatment failure has been attributed primarily to non-adherence to prescribed regimens and to continued illicit drug use^{36–38}. Wood *et al.* provided data indicating that there was no difference within a cohort from Vancouver in the failure rate between IDU and non-injection drug using subjects³⁸. However, the overall failure rate in this cohort was only 25% over a 30-month period. An earlier study of this Vancouver cohort had revealed no higher incidence of primary PI resistance among recently infected IDU³⁹, compared to non-injection drug using subjects, but in that study only the population sequencing method to detect such resistance was used. In the WIHS study by Gange *et al.*, which did not distinguish between IDU and non-injection drug using subjects, the failure rate among HAART-naïve individuals was 54% over a 12-month period. The reason for the profound difference in treatment outcomes between these two cohorts is unclear.

In a study providing one of the few indications that poorer outcomes in IDU might not be solely related to poor adherence to therapy, Moore *et al.*² found that the relative incidence of AIDS-defining illnesses (ADI) among IDU in Baltimore, MD, with access to treatment increased

approximately 50% compared to non-injection drug using subjects during the period between 1998 and 2002. In both groups the incidence of ADI fell during the study period, but combination antiretroviral therapy was much more effective in altering disease progression in non-injection drug using subjects than in IDU. As indicated, a more recent report on an injection drug-using Canadian cohort has found a similar divergence in outcomes when compared to non-injection drug users¹. In the latter study adherence to HAART was carefully documented and was not responsible for the divergent outcomes.

Although we have speculated that greater diversity within the viral archive attributable to past replicative bursts accounts for the higher frequency of PI resistance in IDU person-visits, another possible explanation is that viral clones circulating among IDU are distinct from those among non-injection drug using subjects, and that IDU are carrying more PI-resistance mutations as a result of exposure to PI in index populations. Given the historically lower use of HAART or PIs among IDU 40-41, this possibility seems rather unlikely. Furthermore, phylogenetic analysis of clones obtained from the subjects in this study did not reveal segregation by IDU status (data not shown).

It should also be noted that in most of the subjects in whom PI resistance mutations were noted by clonal sequencing, only 1 or 2 clones were carrying the identified resistance mutations. This can lead, as in the case of Subject 5 from Table 3, to discrepancies between the mutations found at two different pre-HAART visits. This finding suggests that in examining 10 clones from an individual person-visit, we may still be at the lower level of sensitivity necessary to detect the full range of primary resistance mutations in HAART-naïve IDU.

Further studies, under conditions rigorously controlled for adherence to therapy, are required to determine if the primary ART resistance patterns found in the more intensive analysis provided by examination of multiple clones from infected IDU is, in fact, predictive of the ultimate clinical course. However, the finding that 83% of those individuals shown by clonal analysis to be carrying PI resistance mutations rapidly failed therapy when subsequently treated with PI containing HAART supports the view that more sensitive sequencing techniques to detect resistance mutations prior to initiation of HAART may well be appropriate, especially in IDU. Larger studies to address the predictive value of such pre-treatment analyses are indicated.

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Table 1A

Baseline Characteristics by reported IDU history prior to study enrollment of 50 Women's Interagency HIV Study (WIHS) participants included in an analysis of protease inhibitor resistance.

Characteristic	Study Participants		<i>p</i>^a
	IDU (n=18)	Non-IDU (n=32)	
Median (IQR) CD4+ cell count	222 (85, 479)	294 (119, 572)	0.43
Median (IQR) log ₁₀ HIV RNA ^b	5.10 (4.41, 5.34)	4.67 (4.15, 5.26)	0.19
Median age (IQR)	39 (36, 44)	35 (30, 40)	0.05
No. (%) HCV antibody positive ^c	18 (100%)	3 (10%)	<0.01
Race			
No. (%) African-American	12 (67%)	22 (69%)	0.88
No. (%) non African-American	6 (33%)	10 (31%)	
History of antiretroviral therapy prior to baseline			
No. (%) no therapy	7 (39%)	15 (47%)	0.59
No. (%) monotherapy or combination therapy ^d	11 (61%)	17 (53%)	
Antiretroviral therapy usage at initial visit			
No. (%) no therapy	15 (83%)	20 (63%)	0.12
No. (%) monotherapy or combination therapy ^e	3 (17%)	12 (38%)	

IQR, inter-quartile range

^a comparisons of categorical characteristics between IDU and Non-IDU participants were based on χ^2 test (or χ^2 exact test if any expected cell counts <5); comparisons of continuous characteristics between IDU and Non-IDU groups were based on Wilcoxon rank sum test

^b 1 Non-IDU was missing HIV RNA data

^c 2 Non-IDUs were missing data on Hepatitis C antibodies

^d 6 of 11 IDUs reported at most monotherapy, 9 of 17 non-IDUs reported at most monotherapy

^e 1 of 3 IDUs reported monotherapy, 10 of 12 non-IDUs reported monotherapy

Table 1B

Baseline Characteristics by reported IDU history prior to study enrollment of 44 Women's Interagency HIV Study (WIHS) participants included in an analysis of reverse transcriptase inhibitor resistance.

Characteristic	Study Participants		<i>p</i>^a
	IDU (n=15)	Non-IDU (n=29)	
Median (IQR) CD4+ cell count	243 (85, 479)	276 (133, 509)	0.68
Median (IQR) log ₁₀ HIV RNA ^b	5.26 (4.40, 5.36)	4.74 (4.16, 5.27)	0.22
Median age (IQR)	40 (36, 45)	35 (31, 41)	0.05
No. (%) HCV antibody positive ^c	15 (100%)	3 (11%)	<0.01
Race			
No. (%) African-American	11 (73%)	20 (69%)	1.00
No. (%) non African-American	4 (27%)	9 (31%)	
History of antiretroviral therapy prior to baseline			
No. (%) no therapy	5 (33%)	13 (45%)	0.46
No. (%) monotherapy or combination therapy ^d	10 (67%)	16 (55%)	
Antiretroviral therapy usage at initial visit			
No. (%) no therapy	12 (80%)	18 (62%)	0.31
No. (%) monotherapy or combination therapy ^e	3 (20%)	11 (38%)	

IQR, inter-quartile range

^a comparisons of categorical characteristics between IDU and Non-IDU participants were based on χ^2 test (or χ^2 exact test if any expected cell counts <5); comparisons of continuous characteristics between IDU and Non-IDU groups were based on Wilcoxon rank sum test

^b 1 Non-IDU was missing HIV RNA data

^c 2 Non-IDU participants were missing data on Hepatitis C antibodies

^d 6 of 10 IDUs reported at most monotherapy, 8 of 16 non-IDUs reported at most monotherapy

^e 1 of 3 IDUs reported monotherapy, 9 of 11 non-IDUs reported monotherapy

Table 2

Percentage of study visits with indicated antiretroviral therapy resistance by IDU status.

Antiretroviral Therapy Resistance Category	% of study visits		Odds ratio (95% C.I.)	P ^c
	IDU (n=34)	Non-IDU (n=65)		
Protease Inhibitor	24%	8%	3.52 (0.98, 12.71)	0.05
	% of study visits			
	IDU (n=27)	Non-IDU (n=56)		
Reverse Transcriptase Inhibitor ^a	56%	54%	1.01 (0.40, 2.57)	0.98
NRTI	48%	45%	1.00 (0.35, 2.85)	0.99
NNRTI	7%	13%	0.59 (0.11, 3.10)	0.53
NRTI & NNRTI	0%	4%	Undefined	NA

^a RTI resistance category includes NRTIs or NNRTIs^b Odds of resistance for IDUs relative to Non-IDUs^c significance of difference between IDUs and Non-IDUs was determined from logistic regression model with generalized estimating equations

Table 3

A Comparison of Mean (standard error) clonal diversity estimates within HIV-1 protease gene region representation among 18 injection drug users and 32 non-injection drug users.

HIV-1 pol gene region (representation)	Injection Drug Use		Mean difference (95% C.I.)	p ^a
	IDU (n=34)	Non-IDU (n=65)		
PR (amino acid)	1.40 (0.14)	1.33 (0.08)	0.07 (−0.24, 0.38)	0.65
PR (nucleotide)	2.55 (0.16)	2.31 (0.13)	0.23 (−0.17, 0.64)	0.26

^a significance of difference between IDUs and Non-IDUs was determined from linear regression model with generalized estimating equations

Table 4

A Comparison of Mean (standard error) clonal diversity estimates within HIV-1 protease gene region representation.

HIV-1 protease region representation	Antiretroviral Therapy Resistance		Mean difference (95% C.I.)	p ^a
	PI Resistant (n=13)	Non-PI Resistant (n=86)		
PR (amino acid)	1.60 (0.22)	1.32 (0.07)	0.28 (−0.17, 0.73)	0.23
PR (nucleotide)	2.66 (0.16)	2.35 (0.11)	0.31 (−0.04, 0.66)	0.08

^a significance of difference between PI resistance and Non-PI resistant groups was determined from linear regression model with generalized estimating equations

Table 5

PI resistance profiles by genotyping method (population, clonal).

Subject No.	Visit No.	Mutations		Drug Resistance	
		Population Sequencing Major ^a Minor	Clonal Sequencing Major Minor (No. plasmids with mutation(s)/No. plasmids) ^b	Population Sequencing	Clonal Sequencing
1	1	None	D30Y, M46V (2/10)	No Resistance	ATV, FPV, IDV, LPV, NFV
1	10	D30N, M46I, N88D L10F	D30N, M46I, N88D (2/2), L10F (2/2)	ATV, FPV, IDV, LPV, NFV, SQV, TPV	ATV, FPV, IDV, LPV, NFV, SQV, TPV
2	1	None	N88S (1/10) L10F (1/10)	No Resistance	ATV, IDV, NFV, SQV
4	1	None	I54T (1/9)	No Resistance	ATV, FPV, IDV, LPV, NFV, SQV
5	2	None	I50V, I54T, V82A (2/10)	No Resistance	ATV, DRV, FPV, IDV, LPV, NFV, SQV
5	3	None	N88S (1/10)	No Resistance	ATV, IDV, NFV
7	3	None	M46I (1/10)	No Resistance	ATV, FPV, IDV, LPV, NFV
9	4	None	I47V (2/10)	No Resistance	DRV, FPV, LPV, TPV
10	1	None	D30N, V82A, (1/7)	No Resistance	ATV, FPV, IDV, LPV, NFV, SQV, TPV
20	4	A71T	V82A (1/10) A71T (10/10)	No Resistance	ATV, FPV, IDV, LPV, NFV, SQV, TPV

^aThe particular designation of mutations as major or minor are in accordance with the Stanford HIV drug resistance database.

^b mutation detection rate estimate (the no. of plasmids with mutation(s) reflects the number of plasmids with any listed mutation).