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Transmitted Drug Resistance in the CFAR Network of Integrated Clinical Systems Cohort: Prevalence and Effects on Pre-Therapy CD4 and Viral Load


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Abstract

Human immunodeficiency virus type 1 (HIV-1) genomes often carry one or more mutations associated with drug resistance upon transmission into a therapy-naïve individual. We assessed the prevalence and clinical significance of transmitted drug resistance (TDR) in chronically-infected therapy-naïve patients enrolled in a multi-center cohort in North America. Pre-therapy clinical significance was quantified by plasma viral load (pVL) and CD4+ cell count (CD4) at baseline. Naïve bulk sequences of HIV-1 protease and reverse transcriptase (RT) were screened for resistance mutations as defined by the World Health Organization surveillance list. The overall prevalence of TDR was 14.2%. We used a Bayesian network to identify co-transmission of TDR mutations in clusters associated with specific drugs or drug classes. Aggregate effects of mutations by drug class were estimated by fitting linear models of pVL and CD4 on weighted sums over TDR mutations according to the Stanford HIV Database algorithm. Transmitted resistance to both classes of reverse transcriptase inhibitors was significantly associated with lower CD4, but had opposing effects on pVL. In contrast, position-specific analyses of TDR mutations revealed substantial effects on CD4 and pVL at several residue positions that were being masked in the aggregate analyses, and significant interaction effects as well. Residue positions in RT with predominant effects on CD4 or pVL (D67 and M184) were re-evaluated in causal models using an inverse probability-weighting scheme to address the problem of confounding by other mutations and demographic or risk factors. We found that causal effect estimates of mutations M184V/I (−1.7 log₁₀ pVL) and D67N/G (−2.1 √CD4 and +0.4 log₁₀ pVL) were compensated by K103N/S and K219Q/E/N/R. As TDR becomes an increasing dilemma in this modern era of highly-active antiretroviral therapy, these results have immediate significance for the clinical management of HIV-1 infections and our understanding of the ongoing adaptation of HIV-1 to human populations.


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Introduction

A substantial fraction (5–20%) of human immunodeficiency virus type 1 (HIV-1) variants that have been transmitted and establish infections in individuals who have never been exposed to antiretroviral drugs (i.e., ARV-naïve) already carry one or more mutations that are associated with resistance to ARVs [1,2]. Resistance mutations are generally slow to revert in ARV-naïve hosts [3,4] and can persist at low frequencies in treatment-experienced hosts as well [5]. The pre-existence of resistance mutations can significantly diminish effectiveness of subsequent ARV therapy [6,7]; for example, transmitted K103N mutations are associated with increased regimen failure and poorer outcomes on non-nucleoside reverse transcriptase inhibitors (NNRTIs) [8]. On the other hand, resistance mutations can also incur a measurable cost with respect to virus replicative capacity in vivo [9–11] and competitive growth in vivo [12]. Within therapy-naïve hosts, the fitness costs of transmitted drug resistance (TDR) mutations may influence the subsequent evolution and population dynamics of HIV-1. With the exception of limiting the choice of initial ARV regimens, however, the direct clinical consequences of TDR mutations in therapy-naïve hosts remain inconclusive [13–17]. Our central hypothesis in this study is that the presence of TDR mutations in therapy-naïve hosts has a measurable effect on not only the subsequent rate of growth of the virus population within the host, but also on the rate of depletion of CD4+ T-lymphocytes.
that are targeted by HIV-1. To evaluate this hypothesis, we assess
the relationship between TDR mutations and prognostic clinical
markers – CD4+ T-cell count (CD4) and plasma HIV-1 RNA load
(pVL) – at baseline using data from the Centers for AIDS
Research (CFAR) Network of Integrated Clinical Systems
(CNICS), an observational cohort created from a data-sharing
collaboration between multiple clinical sites in North America
[18]. ‘Baseline’ refers to the measurements of clinical markers
upon HIV diagnosis and/or entry into clinical care. It does not
correspond to a consistent time point after infection and many
patients will have already progressed into the chronic phase of
infection. Nevertheless, these data can provide useful insight into
the clinical implications of TDR in therapy-naïve hosts.

First, we quantify the effects of TDR mutations at both
aggregate and position-specific levels. An aggregate approach
sums across mutations occupying different positions in HIV-1
protease and reverse transcriptase, implicitly assuming that every
mutation has a similar effect (in both direction and magnitude) on
baseline CD4 or pVL. In contrast, a position-specific analysis
recognizes the possibility that some TDR mutations will have a
disproportionately large effects while others have negligible effects.
Furthermore, it enables us to identify compensatory interactions,
such that the effect of a specific mutation on CD4 or pVL depends
on the presence or absence of other mutations; such interactions
are an important aspect of the evolution of drug resistance in HIV-
1 [11]. Second, we re-evaluate statistically significant associations
of individual TDR mutations in a causal modeling framework, i.e.,
inverse probability-of-treatment weighting [19]. A statistical
association between a treatment (a TDR mutation) and an
outcome (baseline CD4 or pVL) in an observational study may be
biased if one or more variables, such as other co-transmitted
resistance mutations, are not only correlated with the treatment
but also affect the outcome [19]. In the presence of such
confounding factors, the significant association could be wrongly
attributed to a causal effect of the treatment on the outcome.
Using statistical procedures that can adjust for confounding by
weighting observational data has become an important compo-
nent of understanding the consequences of drug resistance in HIV
[20].

Methods
Ethics statement
All patients in the CNICS database have provided informed
written consent, as reviewed and approved by the institutional
review boards of all participating sites – University of Alabama,
Birmingham (UAB); University of California, San Diego (UCSD);
University of California, San Francisco (UCSF); University of
Washington (UW); Case Western Reserve University (CWRU);
and Harvard University (FENWAY) – to have their clinical
information used for the purposes of research. Patients were
informed on the minimum information being collected, alterna-
tives to participation, and possible uses of the data. In addition,
CNICS has received a Certificate of Confidentiality from the
National Institutes of Health (NIH).

Data collection
The study population comprised individuals from 6 CNICS
sites: University of Alabama, Birmingham; University of Califor-
nia, San Diego; University of California, San Francisco; University
of Washington; Case Western Reserve University; and Harvard
University [18]. All patients in the CNICS database have provided
informed written consent, as reviewed and approved by the
institutional review boards of all participating sites, to have their
clinical information used for the purposes of research. HIV-1 pol
sequences in the region encoding protease and/or part of reverse
transcriptase (i.e., the first 246–400 codons of RT) were obtained at
each site by conventional Sanger sequencing directly from RT-
PCR amplification products from sample extractions of viral
RNA. The exact bulk sequencing protocol varied among sites, as
some sites used commercial laboratory services (e.g., Monogram,
Quest, Virco, LabCorp) whereas others used in-house assays based
on commercial kits. At the time of this study, 4914 codon or amino
acid sequences were available in the CNICS database but many
were obtained when patients were on-therapy. Amino acid
sequences were reconstituted from reports of residue polymor-
phisms relative to the HXB2 reference sequence. Sequences were
linked to: unique anonymized patient identifiers; CNICS site;
sampling date; age; ARV regimens; demographic factors (self-
reported gender at baseline, race/ethnicity); HIV risk factors (men
who have sex with men, MSM; injection drug use, IDU); baseline
plasma HIV RNA titres (pVL, virus/mL plasma); and baseline
CD4 cell counts (cells/mL). A patient record was included in this
study if: (1) a sequence was sampled before therapy, and (2) CD4
and/or pVL were sampled within 120 days of the sequence sample
date. To normalize the distributions of pVL and CD4, we used
log_{10} and cube-root transformations [21], respectively. Race and
ethnicity records were aggregated into the following groups: non-
Hispanic white, non-Hispanic black, Hispanic, and other. Non-
random associations between demographic and risk factors were
identified using log-linear models.

Sequence analysis
Sequences were aligned pairwise against the NL4-3 pol reference
sequence using an implementation of the Gotoh algorithm in
HyPhy [22,23] under default settings. Surveillance drug resistance
mutations (SDRMs) were tallied for each amino acid sequence
according to the 2009 update of the World Health Organization
list of surveillance drug resistance mutations (SDRMs) [24]. Amino
acid polymorphisms (e.g., bulk sequence mixtures) were resolved to
the resistant residue when applicable.

Statistical analysis
Resistance scores by drug class (protease inhibitors, PI; nuclease and non-nuclease reverse transcriptase inhibitors,
NRTIs/NNRTIs) were calculated according to the Stanford HIV
Drug Resistance database algorithm [25]. The Stanford scoring
system is a linear predictor of drug resistance phenotypes that
works by assigning integer weights to a sequence for encoding
resistance-associated amino acid residues at specific positions.
Weights (or ‘scores’) for a given sequences were calculated for
every drug in a given drug class. We used the highest score of any
drug in a drug class as the class-specific score. These quantitative
scores, denoted by S_{PI}, S_{NRTI} and S_{NNRTI} respectively, were used
to analyze the aggregate effects of TDR on baseline CD4 and
pVL. Aggregate or position-specific effects of TDR on baseline
CD4 or pVL were evaluated alongside demographic and risk
factors in linear models. In each case we used a bi-directional
stepwise algorithm in R (stepAIC [26]) to select a ‘best-fitting’ linear
model based on the Akaike information criterion (AIC), which
penalizes model likelihood by the number of parameters. We
seeded the algorithm with the full additive model (without
interaction terms) and limited model complexity to second-order
interactions. To avoid over-fitting the data from evaluating a large
number of alternative models, we averaged parameter estimates
and P values over an ensemble of models (best +5 next-best) using
Akaike weights [27].
To estimate causal effects of individual SDRMs, we used inverse probability-of-treatment weight (IPTW) estimators [19]. The conditional probability \( Pr(A|A) \) for a given ‘treatment’ variable \( A \) was computed by fitting a logistic model of \( A \) against the set of all potential confounding variables \( A \). To account for compensatory interactions among mutations, we combined the corresponding presence/absence variables into a single multinomial treatment as suggested to us by Miguel A. Hernán (pers. comm.). For example:

\[
A_{M184V} = \begin{cases} 
0 & \text{if } M184X = 0 \text{ and } (K103X = 0 \text{ and } K219X = 0) \\
1 & \text{if } M184X = 1 \text{ and } (K103X = 0 \text{ and } K219X = 0) \\
2 & \text{if } M184X = 1 \text{ and } (K103X = 1 \text{ or } K219X = 1)
\end{cases}
\]

where \( X \) signifies any SDRM residue at that position. Conditional probabilities for multinomial treatments were obtained by fitting a multinomial logistic model with the \textit{multinom} function in the R package \textit{nnet}. Because weighting observations by the inverse of \( Pr(A|A) \) can introduce excessive variability, we used stabilized weights that normalize \( Pr(A|A) \) by the marginal probability \( Pr(A) \), which was estimated by fitting a logistic model containing only the intercept term [19]. The stabilized weights were incorporated into a linear model of baseline CD4 or pVL on treatment \( A \) using the \textit{svyglm} function in the R package \textit{survey} [28], which uses an estimating equation-based approach to fitting generalized linear models with robust standard errors. Cases with missing data were omitted from these analyses.

A Bayesian network analysis to detect co-transmission of TDR mutations was carried out using a Markov chain Monte Carlo (MCMC) procedure in HyPhy [29]. A Bayesian network is a compact representation of the joint probability distribution of multiple variables (i.e., the presence/absence of TDR mutations), where ‘joint’ implies that the probability distribution of a variable may be conditional on other variables in the network [30]. In other words, a Bayesian network provides an efficient framework for exploring the statistical interactions among variables. Each variable is represented by a ‘node’ in the network, and connections between nodes (‘edges’) are drawn to indicate that one variable is conditioned on the second. The MCMC procedure obtains a random sample of Bayesian networks from the posterior probability distribution defined by the data. The Markov chain was propagated for 10⁵ steps, discarding the first half as burn-in and thinning the remaining sample to 100 steps at equal intervals. Edges in the network signifying co-transmission were accepted if they were present in 90% or more of the networks in the sample.

**Results**

**Population**

The study population was comprised of 14111 individuals with a median baseline CD4 of 300 cells/mL (\( n = 14011 \)) and a median baseline pVL of 24 400 copies/mL (\( n = 13953 \)). Median year of birth was 1964. The majority of the population self-reported as ‘white’ (\( n = 8694 \)), 3725 individuals self-reported as ‘black’, 207 as ‘Asian/Pacific Islander’ and 833 as ‘other’; there were 650 non-respondents. At the time of this study, the CNICS database contained 1585 sequence records that we classified as therapy-naive. Out of these, 1575 naive sequences were linked to baseline CD4 and pVL records. 1526 naive sequences were associated with baseline CD4 records within 120 days of the sequence sampling date and were otherwise censored from subsequent analyses. Likewise, 1516 naive sequences were associated with baseline pVL within 120 days of sampling and were otherwise censored. The median baseline CD4 and pVL of patients with naive sequences in this data set were 285 cells/mL and 10⁷ copies/mL, respectively. Composition of the naive sample with respect to race was significantly associated with gender (\( \chi^2 = 68.2, df = 3, P = 1.03 \times 10^{-14}; \) e.g., the sample included a disproportionately greater number of naive sequences from white males (odds ratio, \( OR = 3.1 \)) and black females (\( OR = 3.0 \)). Sample composition by race was also significantly associated with MSM (\( \chi^2 = 138.8, df = 3, P = 7.05 \times 10^{-30} \); largely because of a greater number of white MSMS than expected by marginal frequencies (\( OR = 2.85 \)).

**Prevalence of transmitted SDRMs**

Overall, 225 out of 1585 naive sequences (14.2%) contained one or more SDRMs. Of these 225 sequences, 66 (4.2%) contained at least one PI-associated SDRM, 130 (8.2%) contained at least one NRTI-associated SDRM, and 131 (8.3%) contained at least one NNRTI-associated SDRM. In addition, 17 (1.1%) sequences contained SDRMs in all three drug classes, which is substantially greater than the number expected by chance (<0.5 sequences) if the joint transmission of mutations was independent of drug class. A decline from pre-2003 levels in the fraction of naive sequences with at least 1 SDRM was mirrored by a similar decline in the mean number of SDRMs per naive sequence (Figure 1). However, we observed an increase in the most recent sample year (2006) in the upper 95% quartile of the distribution in the number of SDRMs. Neither measure of prevalence was significantly associated with demographic or risk factors in generalized linear models (\( P > 0.05 \)). Counts of SDRMs by residue position are provided in Table 1. A Bayesian network analysis detected significant co-transmission of SDRMs in four groups (Figure 2). The largest group generally corresponded to NRTI resistance-associated
mutations (with the exception of K103, I54 and V82) and contained well-characterized mutational pathways, e.g., D67/T69/K70/K219 and T215/M41/L210. Two smaller groups (M46/I84/I85 and D30/N88) comprised mutations associated with PI resistance, the latter group being associated with resistance to nelfinavir in particular. The remaining group (K101/G190) comprised mutations associated with resistance to NNRTIs. In sum, co-transmitted SDRMs tended to confer resistance to the same drug or drug class.

### Aggregate SDRM predictors of baseline CD4 and HIV RNA

The summated Stanford scores for NRTI and NNRTI ($S_{\text{NRTI}}$, $S_{\text{NNRTI}}$) and race/ethnicity were significantly ($\alpha = 0.05$) associated with baseline CD4 (Table 2). For every 10 units of $S_{\text{NRTI}}$ and $S_{\text{NNRTI}}$, the model predicted a decline in $\sqrt{\text{CD4}}$ by about 0.07 and 0.04 units, respectively. For example, if a hypothetical patient whose CD4 count matched the study median (285 cells/mL) became infected by an HIV-1 variant carrying SDRMs such that $S_{\text{NRTI}} \sim 10$ and $S_{\text{NNRTI}} \sim 10$, we would predict a baseline CD4 count of $\sqrt{\text{285}} \sim 16$ cells/mL. The joint effect of transmitted RTI resistance on CD4 is illustrated in Figure 3. Only 2% of the variance in log_{10} pVL was explained by this model. $S_{\text{PI}}$ was not significantly associated with either CD4 or pVL at baseline.

### Position-specific SDRM predictors of baseline CD4 and HIV RNA

Although aggregate SDRM-based statistics such as Stanford scores are convenient and easy to interpret as proximate measures of drug class-specific resistance, they may mask the effects of individual mutations on clinical outcome. Consequently, we assessed the effects of position-specific SDRMs in linear models of baseline CD4 and pVL. To minimize the number of predictor variables and avoid over-fitting of the data, we limited our analysis to positions with a minimum SDRM frequency of 1% in naïve sequences (see Table 1). These positions were: M46, I54, and L90 in protease, and; M41, D67, M184, L210, T215, K219, K103, Y181, and G190 in reverse transcriptase. The presence or absence of SDRMs at each position was represented by a binary variable (herein denoted by appending an ‘X’ to the amino acid positional notation, e.g., M46X = {0,1}). D67X and K219X were each associated with significantly lower baseline CD4 ($P < 0.04$; Table 3); both sets of SDRMs are thymidine analog mutations (TAMs) conferring resistance to NRTIs. For example, the model would predict a hypothetical decline from 400 cells/mL to 146 and 235 cells/mL with the introduction of SDRMs at positions D67 and K219, respectively. In addition, there was a significant interaction effect between D67X and K219X associated with a compensatory increase by

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**Table 1.** Number and percentage prevalence of putatively transmitted surveillance drug resistance mutations (SDRMs) by residue position in antiretroviral (ARV)-naïve sequences.

<table>
<thead>
<tr>
<th>Class</th>
<th>position</th>
<th>count</th>
<th>% prevalence</th>
<th>Class</th>
<th>position</th>
<th>count</th>
<th>% prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>L23</td>
<td>1</td>
<td>0.06</td>
<td>NRTI(cont’d)</td>
<td>K70</td>
<td>9</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>L24</td>
<td>1</td>
<td>0.06</td>
<td></td>
<td>L74</td>
<td>11</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>D30</td>
<td>15</td>
<td>0.95</td>
<td></td>
<td>V75</td>
<td>2</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>V32</td>
<td>2</td>
<td>0.13</td>
<td></td>
<td>F77</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>M46</td>
<td>20</td>
<td>1.26</td>
<td></td>
<td>Y115</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>I47</td>
<td>2</td>
<td>0.13</td>
<td></td>
<td>F116</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>G48</td>
<td>0</td>
<td>0</td>
<td></td>
<td>Q151</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>I50</td>
<td>2</td>
<td>0.13</td>
<td></td>
<td>M184</td>
<td>40</td>
<td>2.52</td>
</tr>
<tr>
<td></td>
<td>F53</td>
<td>1</td>
<td>0.06</td>
<td></td>
<td>L210</td>
<td>29</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>I54</td>
<td>16</td>
<td>1.01</td>
<td></td>
<td>T215</td>
<td>77</td>
<td>4.86</td>
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<tr>
<td></td>
<td>G73</td>
<td>3</td>
<td>0.19</td>
<td></td>
<td>K219</td>
<td>30</td>
<td>1.89</td>
</tr>
<tr>
<td></td>
<td>L76</td>
<td>1</td>
<td>0.06</td>
<td></td>
<td>L100</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>V82</td>
<td>12</td>
<td>0.76</td>
<td></td>
<td>K101</td>
<td>7</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>N83</td>
<td>0</td>
<td>0</td>
<td></td>
<td>K103</td>
<td>82</td>
<td>5.17</td>
</tr>
<tr>
<td></td>
<td>I84</td>
<td>10</td>
<td>0.63</td>
<td></td>
<td>V106</td>
<td>2</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>I85</td>
<td>2</td>
<td>0.13</td>
<td></td>
<td>V179</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>N88</td>
<td>15</td>
<td>0.95</td>
<td></td>
<td>Y181</td>
<td>24</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td>L90</td>
<td>30</td>
<td>1.89</td>
<td></td>
<td>Y188</td>
<td>10</td>
<td>0.63</td>
</tr>
<tr>
<td>NRTI</td>
<td>M41</td>
<td>49</td>
<td>3.09</td>
<td></td>
<td>G190</td>
<td>24</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td>K65</td>
<td>3</td>
<td>0.19</td>
<td></td>
<td>P225</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>D67</td>
<td>28</td>
<td>1.77</td>
<td></td>
<td>M230</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T69</td>
<td>9</td>
<td>0.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$PI$ = protease inhibitor; $NRTI$ = nucleoside reverse transcriptase inhibitor; $NNRTI$ = non-nucleoside reverse transcriptase inhibitor.

doi:10.1371/journal.pone.0021189.t001
Figure 2. Bayesian network depicting conditional dependencies among surveillance drug resistance mutations (SDRMs) in ARV-naïve sequences. Each labeled node corresponds to a position in protease (rectangular) or RT (rounded). Nodes without dependencies (L23, L24, V32, I47, I50, F53, G73, L76, K65, V75, F77, Y115, F116, L100, V106, V179, Y181, and Y188) were omitted from the graph for clarity. Connections between nodes (edges) are labeled with the log_{10} odds ratio of the 2 x 2 contingency table for the presence/absence of SDRMs at the respective sites, e.g., an SDRM at position PR-D30 is estimated to be 10^{1.7} \approx 5000 times more likely to be present in an ARV-naïve sequence that contains an SDRM at position PR-N88. Line widths for edges are also drawn in proportion to the log_{10} odds ratios. Inf= infinity, i.e., unable to estimate because of zero count(s) in the contingency table.

doi:10.1371/journal.pone.0021189.g002

about +2.5 units \sqrt{\text{CD4}} (P=0.006; Figure 5). This model accounted for 6.8% of the variance in \sqrt{\text{CD4}}.

M184X was significantly associated with an approximately ten-fold lower pVL at baseline (P=1.3 \times 10^{-7}; Table 3). M46X and L210X were also significantly associated with lower baseline pVL (P<0.01) and D67X was associated with greater pVL (P=0.009).

We also observed several significant mutational interaction terms in the model. For instance, interactions between M184X and either K103X or K219X were associated with a compensatory increase in pVL, (P=0.018 and P=0.023, respectively; Figure 5). Similarly, a decline in pVL associated with M46X was compensated by T215X (P=0.033). An interaction term between D67 and K219, which was encountered in the preceding model on CD4, was also statistically significant in this model of pVL.

Table 2. Coefficient estimates from Akaike Information Criterion (AIC)-selected linear models of baseline \sqrt{\text{CD4}} and plasma HIV RNA on Stanford scores by drug class and demographic and risk factors.

<table>
<thead>
<tr>
<th></th>
<th>\sqrt{\text{CD4}}</th>
<th>\log_{10} \text{plasma HIV RNA}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>S_{\text{NRTI}}</td>
<td>-6.9 (-12.2, -1.1) x 10^{-3}</td>
<td>0.02</td>
</tr>
<tr>
<td>S_{\text{NNRTI}}</td>
<td>-4.4 (-8.4, -0.5) x 10^{-3}</td>
<td>0.035</td>
</tr>
<tr>
<td>Male</td>
<td>NS</td>
<td>0.19 (0.06, 0.32)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>-0.35 (-0.61, -0.09)</td>
<td>0.008</td>
</tr>
<tr>
<td>Hispanic</td>
<td>-0.57 (-0.86, -0.28)</td>
<td>1.1 \times 10^{-4} NS</td>
</tr>
</tbody>
</table>

All estimates were averaged over an ensemble of linear models using Akaike weights. Only statistically significant (α=0.05) terms after weighting are reported. Linear effects of factors were estimated relative to the model intercept, i.e., a hypothetical female white individual. NS = not significant.

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NRTI and NNRTIs (plasma viral load (pVL) as a function of Stanford scores for associated with an unweighted univariate linear model, M184 was significantly M184, and M184 compensated by K103X and/or K219X). In for the respective levels 0, 1 and 2 (i.e. AM multinomial outcome regression with smoothing parameter \( \alpha = 0.8 \)). Stabilized weights adjusting for the conditional probability of stabilized weights) to disentangle the causal effects of SDRMs.

Causal inference

We used an inverse probability weighting scheme (using stabilized weights) to disentangle the causal effects of SDRMs from confounding variables that influence both the probability of the ‘treatment’ (an SDRM at the position in question) and CD4 or pVL. Based on the results of the preceding section, we focused on the compensated and uncompensated effects of M184X on baseline pVL, and of D67X on both pVL and \( \sqrt{CD4} \). K103X and K219X were assumed to be interchangeable with respect to their compensatory interactions with M184X. Consequently, the multinomial outcome \( A_{M184} \) comprised \( n=1445 \), 19 and 15 cases for the respective levels 0, 1 and 2 (i.e., wildtype, uncompensated M184X, and M184X compensated by K103X and/or K219X). In an unweighted univariate linear model, M184X was significantly associated with a \(-1.1\) decline in \( \log_{10} pVL \) in the absence of K103X and/or K219X (Table 4). Stabilized weights adjusting for the conditional probability of carrying M184X ranged from 0.02 to 18.72 with a mean of 1.016. Adding stabilized weights to the model exposed a greater deleterious effect of M184X on \( \log_{10} pVL \) (\(-1.69\) units) when uncompensated by K103X and/or K219X. Similarly, re-weighting linear models resulted in larger estimated effects of uncompensated D67X on both baseline CD4 (\(-2.1\) units \( \sqrt{CD4} \)) and pVL (2.5-fold increase; Table 4). Stabilized weights adjusting for the conditional probability of carrying D67X ranged from 0.006 to 12.3 with a mean of 1.003. For both pVL and CD4, the effects of D67X compensated by K219X became statistically significant and reversed sign relative to the unadjusted models (Table 4).

Discussion

The overall prevalence of transmitted drug resistance (TDR) mutations in therapy-naïve patients has been reported in a large number of study populations around the world and ranges from 0 to 25% [1]. On the other hand, the transmission of a specific mutation is a relatively infrequent event whose prevalence requires very large samples (over 1000 individuals) to estimate. This requirement has generally been achieved by integrating study populations from similar geographic regions, such as the Combined Analysis of Resistance Transmission over Time of Chronically and Acute Infected HIV Patients (CATCH) study encompassing 27 countries in Europe [31], the Swiss HIV Cohort Study [32], the UK Collaborative HIV cohort study [14], and the Centers for AIDS Research (CFAR) Network of Integrated Clinical Systems (CNICS) cohort reported in this study. Here we briefly compare and contrast the prevalence of drug class-specific and site-specific TDR across these integrated cohorts. First, NRTI-associated SDRMs generally tend to be more prevalent than SDRMs associated with PIs or NNRTIs. Our sample deviates slightly from this trend in that the prevalence of naïve sequences with \( \geq 1 \) NNRTI-associated SDRM is similar to sequences with \( \geq 1 \) NRTI-associated SDRM, largely due to an atypically high frequency of transmitted mutations at RT-K103 (5.2%) in our sample. Second, SDRMs that are slow to revert in the absence of selection (e.g., M41L, K103N, and T215X in RT and L90M in protease), implying a negligible cost to fitness [1], tend to have relatively high prevalence in therapy-naïve sequences.

The foremost and best-documented clinical implication of TDR is that it impedes the virological response to subsequent drug therapy [6–8]. In contrast, the clinical implications of TDR in the absence of drug therapy are subtle and less well understood. These effects have been difficult to elucidate because the transmission of SDRMs remains an infrequent event at the scale of individual mutations. Investigators have attempted to overcome the limitations of insufficient sample sizes by evaluating the effects of aggregate statistics of TDR, such as an ‘all-or-none’ statistic that groups sequences carrying one or more SDRMs [14,16,17]. While this approach is convenient and easy to interpret, it is subject to confounding because different drug classes or mutations can have opposite effects on prognostic clinical markers. Our use of a large data set from a multi-center cohort enabled us to overcome some limitations of sample size, and to break down the effects of TDR on baseline CD4 and pVL by drug class or amino acid position. For example, we found that \( S_{NRTI} \) and \( S_{NNRTI} \) had opposing associations with pVL; because these quantities are correlated, their effects on pVL would have been confounded in an ‘all-or-none’ analysis. Similarly, we have observed several compensatory interactions among mutations that would otherwise mask the actual effects of individual mutations on CD4 or pVL (e.g., D67N/G and K219Q/E).

Because this study population was comprised of seroprevalent rather than seroincident individuals, each baseline measurement of CD4 and pVL was sampled at a point in time following an unknown date of transmission. Consequently, there is no guarantee that these baseline measurements were taken at a consistent stage of infection. In other words, the CD4 count or pVL at baseline is the result of dynamics over an unknown period of time, starting from an unknown value upon infection. These missing data would be required to calculate rates of change, which would be easier to generalize to specific cases in clinical practice where these quantities (time since infection and prognostic markers at infection) may be known. This is a common problem of observational cohort studies and may explain why a substantial
Figure 5. Box-and-whisker plots illustrating (A) the effects of SDRMs at positions D67 and K219 on CD4 cell count and (B) the effects of SDRMs at positions M184 and K103 on plasma viral load (pVL). ‘Wildtype’ denotes sequences lacking SDRMs at both positions, irrespective of whether any other SDRMs were present at other positions in protease or RT. Solid lines indicate the group median and open circles denote outliers that fall outside the region defined by 1.5 times the interquartile range. Plot (A) was rescaled with respect to untransformed CD4 counts to emphasize differences among groups, trimming 4 outliers from the ‘wildtype’ group (1602, 1626, 1838, and 2073 cells/mL) from the plot region. Sample sizes per group are annotated on each plot.

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Table 3. Coefficient estimates from AIC-selected linear models of baseline $\sqrt{CD4}$ and $\log_{10}$ plasma HIV RNA on position-specific SDRMs and demographic and risk factors.

<table>
<thead>
<tr>
<th></th>
<th>$\sqrt{CD4}$</th>
<th>$\log_{10}$ plasma HIV RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate (95% CI)</td>
<td>$P$ value</td>
</tr>
<tr>
<td>M41X</td>
<td>1.0 (0.05, 1.95)</td>
<td>0.04</td>
</tr>
<tr>
<td>M46X</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>D67X</td>
<td>−2.1 (−3.9, −0.4)</td>
<td>0.026</td>
</tr>
<tr>
<td>M184X</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>L210X</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>K219X</td>
<td>−1.2 (−2.4, −0.08)</td>
<td>0.037</td>
</tr>
<tr>
<td>D67X:K219X</td>
<td>2.5 (0.14, 4.8)</td>
<td>0.006</td>
</tr>
<tr>
<td>M184X:K103X</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>M184X:K219X</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>K219X:G190X</td>
<td>−3.0 (−5.6, −0.4)</td>
<td>0.014</td>
</tr>
<tr>
<td>L90X:M41X</td>
<td>−1.7 (−4.4, 0.1)</td>
<td>0.007</td>
</tr>
<tr>
<td>I54X:L210X</td>
<td>2.0 (−0.59, 4.7)</td>
<td>0.04</td>
</tr>
<tr>
<td>M46X:T215X</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>M184X:T215X</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>M219X:T215X</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>K219X:Y181X</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Male</td>
<td>−0.58 (−0.9, −0.24)</td>
<td>7.5 $\times 10^{-4}$</td>
</tr>
<tr>
<td>Sample date</td>
<td>23.3 (7.4, 38.9) $\times 10^{-5}$</td>
<td>0.004</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td>Black</td>
<td>−0.35 (−0.61, −0.10)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>−0.57 (−0.85, −0.29)</td>
<td>8.3 $\times 10^{-5}$</td>
</tr>
<tr>
<td>other</td>
<td>−0.53 (−0.98, −0.08)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

SDRMs are annotated by HXB2 reference residue and position (gene-specific numbering), followed by an ‘X’ to indicate a non-reference residue. Positions 46, 54, and 90 are in protease and all others are in reverse transcriptase. All estimates were averaged over an ensemble of linear models using Akaike weights. Only terms that were statistically significant ($z < 0.05$) are reported here. NS = not significant.

doi:10.1371/journal.pone.0021189.t003
fraction (~90%) of variation in baseline CD4 or pVL remained unaccounted for in the linear models. Furthermore, because the baseline samples were taken either upon entry into care or prior to initiating ARV therapy (ART), they may constitute a biased sample of the patient population with respect to CD4 and pVL. Recent guidelines, for example, indicate that patients should be initiated on ART when their CD4 cell count falls below a threshold of 500 cells per μL regardless of HIV RNA [33]. However, treatment guidelines have evolved over time [34] and using a large sample size covering a decade of clinical practice can ameliorate some of these biases. As guidelines have pushed initiation of therapy to earlier time points, it could be argued that less fit virus, with lower HIV RNA and higher CD4, would not merit clinical significance since virtually every patient should start therapy soon after identification; in other words, an association of TDR with lower viral load would not result in a longer delay for treatment failure [1]. Taking such an approach, we have found that uncompen-
sated mutations are present; these estimates are unchanged in the adjusted model and are not repeated for clarity of presentation. Significant (z = 0.05) effect estimates are bolded.

doi:10.1371/journal.pone.0021189.t004

Table 4. Unadjusted and stabilized weight-adjusted linear models of multinomial exposure variables $A_{M184}$ (M184X and (K103X or K219X)) and $A_{D67}$ (D67X and K219X) on $\log_{10}$ plasma HIV RNA and $\sqrt[3]{\text{CD4}}$, respectively.

<table>
<thead>
<tr>
<th>Level</th>
<th>Unadjusted model</th>
<th>Adjusted model</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\log_{10}$ plasma HIV RNA $\sim A_{M184}$</td>
<td>$4.63$ (4.58, 4.67)</td>
<td>$4.85 \times 10^{-4}$</td>
</tr>
<tr>
<td>$A_{M184} = 0$</td>
<td>$4.63$ (4.58, 4.67)</td>
<td>$4.85 \times 10^{-4}$</td>
</tr>
<tr>
<td>$A_{M184} = 1$</td>
<td>$-1.10$ (-1.57, -0.63)</td>
<td>$-2.69$ (-2.63, -0.74)</td>
</tr>
<tr>
<td>$A_{M184} = 2$</td>
<td>$-0.236$ (-0.568, 0.096)</td>
<td>$0.16$</td>
</tr>
<tr>
<td>$\sqrt[3]{\text{CD4}} \sim A_{D67}$</td>
<td>$6.22$ (6.12, 6.33)</td>
<td>$7.5 \times 10^{-16}$</td>
</tr>
<tr>
<td>$A_{D67} = 0$</td>
<td>$-1.77$ (-2.8, -0.74)</td>
<td>$-2.14$ (-2.66, -1.63)</td>
</tr>
<tr>
<td>$A_{D67} = 1$</td>
<td>$-0.768$ (-1.93, 0.40)</td>
<td>$0.196$</td>
</tr>
<tr>
<td>$A_{D67} = 2$</td>
<td>$0.51$ (0.07, 0.89)</td>
<td>$0.016$</td>
</tr>
<tr>
<td>$\log_{10}$ plasma HIV RNA $\sim A_{D67}$</td>
<td>$4.61$ (4.56, 4.65)</td>
<td>$&lt;2 \times 10^{-16}$</td>
</tr>
<tr>
<td>$A_{D67} = 0$</td>
<td>$0.41$ (0.21, 0.62)</td>
<td>$0.34$</td>
</tr>
<tr>
<td>$A_{D67} = 1$</td>
<td>$0.40$ (0.32, 0.48)</td>
<td>$0.23$ (0.05, 0.41)</td>
</tr>
<tr>
<td>$A_{D67} = 2$</td>
<td>$-0.21$ (-0.64, 0.22)</td>
<td>$0.011$</td>
</tr>
</tbody>
</table>

The model intercepts are taken to be $\beta = 0$ by default, i.e., none of the designated mutations are present; these estimates are unchanged in the adjusted model and are not repeated for clarity of presentation. Significant ($z = 0.05$) effect estimates are bolded.
Previous studies have reported no greater than a 3-fold (10^{-0.5}) reduction in pVL due to M184V/I [39,40,41], while M184I itself has been estimated to reduce fitness by 23% relative to M184V [42]. This discrepancy may be due to failing to account for interactions with other mutations such as the compensatory interactions with K103N and K219, or confounding by other genetic and non-genetic factors affecting pVL. On the other hand, experimental studies of M184V/I-containing recombinant HIV-1 are potentially not subject to these confounding factors, and M184V has been reported to reduce fitness by as much as 16-fold in vitro [11]. However, it is difficult to estimate the fitness effect of a mutation in vitro from in vitro measures of its effect on replication efficiency as there are other components of RT functionality that can be affected [43].

In contrast, the clinical consequences of D67N/G and K219Q/E are not as well known. The effects of D67N/G and K219Q/E on CD4 in our study were mirrored by significant effects on pVL; i.e., substitutions at D67 were causally associated with a 2.5-fold increase in pVL that was only partially compensated by substitutions at K219. These residues potentially form a salt bridge and likely affect the formation of the RT 3’ pocket during polymerization [44]. Substitutions at these sites have previously been suggested to incur a moderate fitness cost in vitro [11,43], although these previous studies had not employed statistical methods to separate out the effects of these substitutions from confounding by other mutations and demographic or risk factors. Our results give the first evidence that the transmission of D67N/G may have deleterious virological and immunological consequences in therapy-naive patients that may be compensated by K219Q/E.

Author Contributions
Conceived and designed the experiments: RHH SDWF AFYP JLA. Performed the experiments: AFYP. Analyzed the data: AFYP. Contributed reagents/materials/analysis tools: WCM MK JSK MSS BR SLB. Wrote the paper: AFYP.

References