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Accessibility
Baseline resistance to nucleoside reverse transcriptase inhibitors fails to predict virologic response to combination therapy in children (PACTG 338)

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Abstract

Background: The association between baseline drug resistance mutations and subsequent increase in viral failure has not been established for HIV-infected children. We evaluated drug resistance mutations at 39 codon sites (21 protease inhibitor (PI) resistant codons and 18 nucleoside reverse transcriptase inhibitor (NRTI) resistant codons) for 92 clinically stable NRTI-experienced, PI-naive HIV-infected children 2 to 17 years of age who were initiating new therapy with ritonavir plus zidovudine (ZDV) and lamivudine or plus stavudine. The association between baseline drug resistance mutations and subsequent viral failure after 12 and 24 weeks of highly active antiretroviral therapy (HAART) was studied.

Results: There were few primary PI associated mutations in this PI-naive population, but 84% had NRTI mutations – codons 215 (66%), 41 (42%), 67 (37%), 210 (33%) and 70 (32%). None of the specific baseline drug resistance mutations were associated with a higher rate of virologic failure after 12 or 24 weeks of HAART. Median week 12 viral load decreased as the total number of NRTI mutations at baseline increased (P = 0.006). Specifically, a higher level of baseline ZDV resistance mutation was associated with a decrease in viral failure after 12 weeks on a ZDV-containing HAART regimen (P = 0.017).

Conclusion: No increase was seen in the rate of viral failure after HAART associated with the presence of resistance mutations at baseline. This paradoxical result may be due to adherence, replicative capacity, or ZDV hypersusceptibility to the new regimen.

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Background
Nucleoside reverse transcriptase inhibitors (NRTI) were
the first antiretroviral drugs available and continue to be
a component of anti-retroviral therapy (ART), despite the
emergence of drug resistance over time. Few studies have
investigated the role of pre-existing drug resistance and
response to therapy in children [1-4] compared to similar
studies in adults [5,6]. The largest published drug resist-
ance study of HIV-infected children found a high rate of
primary mutations associated with resistance to zidovu-
dine (ZDV), didanosine (ddI) and zalcitabine (ddC), but
concluded that none of the baseline drug mutations were
associated with a higher rate of virologic failure [2]. It is
possible that HIV drug resistance may evolve differently in
children because of differences in pharmacokinetics in
children, fewer drug options, and higher viral burden,
specially in younger children [7,8] and unique chal-
lenges to therapy compliance. Pediatric AIDS Clinical Tri-
als Group (PACTG) 338 was one of the first clinical trials
to evaluate highly active anti-retroviral therapy (HAART)
which included a protease inhibitor, ritonavir (RTV), in
children [9]. We investigated the role of baseline HIV drug
resistance mutations and response to therapy.

Results
There were very few primary resistance mutations to PIs in
this PI-naive population, although 88% of the children
had polymorphisms that included secondary minor resist-
ance mutations. The most frequent secondary PI muta-
tions were at codons 63 (78%), 77 (37%), 36 (17%) and
10 (12%) (data not shown). Only two children had a pri-
mary PI resistance mutation (V82A). Other PI mutations
(71, 33 and 20) were present in less than 10% of the study
subjects. The most common NRTI mutations occurred at
codons 215 (66%), 41 (42%), 67 (37%), 210 (33%), 70
(32%), 69 (22%), 118 (21%) and 219 (21%). The
median numbers of baseline NRTI, thymidine analog
mutations (TAM), PI and total mutations were 3, 3, 2 and
4.5, respectively (both primary and secondary mutations
were included in the analysis for the PI mutations).

After 12 weeks on study, 51 (55%) subjects had viral loads
suppressed below 400 copies/ml. The number of subjects
with viral suppression dropped to 31 (34%) and 29
(32%) at weeks 24 and 48, respectively.

The association between the presence of a specific base-
line mutation and virologic failure after 12 weeks of
HAART was studied (Table 1). There was the suggestion
of a potential association with virologic failure for only one
baseline mutation, the NRTI codon 215 (unadjusted P =
0.019) for the three-drug combination regimen. However,
in this case the presence of resistance mutations was asso-
ciated with a decreased (rather than an increased) rate of
viral failure at week 12.

When the association between the number of baseline
resistance mutations and virologic failure after 12 weeks
of HAART was investigated, the only significant relation-
ship observed was in the ZDV/lamivudine (3TC)/RTV
combination regimen. A higher median number of NRTI
mutations at baseline was associated with virologic sup-
pression (1.5 versus 4.0, P = 0.016). There was no appar-
tent association between the number of baseline PI
resistance mutations and the level of viral load after 12
weeks of HAART, although as the number of baseline
NRTI mutations increased the level of viral load at week
12 decreased (Figure 1). A linear regression with adjust-
ment for censoring of viral loads at the detection limit
showed that after 12 weeks on HAART there was a
decrease of 0.245 in log_{10} RNA (copies/mL) associated
with each additional NRTI resistance mutation (P =
0.006).

Association between the level of baseline ZDV resistance
mutations and virologic failure after 12 weeks on HAART
was investigated (Table 2). The level of baseline ZDV resist-
ance was categorized as i) high level (NRTI codon 215), ii)
intermediate level (at least one of the NRTI codons 41 or
210, but no high level mutation), iii) low level (at least
one of the NRTI codons 67, 70 or 219, but no high or
intermediate level mutation), or iv) no ZDV resistance
according to an algorithm specified prior to any data anal-
ysis. There was no association between baseline ZDV
resistance levels and viral failure after 12 weeks on HAART
for the two-drug HAART arm, although for the ZDV-con-
taining three-drug arm a higher level of baseline ZDV
resistance was associated with a lower chance of viral fail-
ure (P = 0.017). From Table 2 it should be noted that
whenever a codon 41 or 210 mutation was observed a
codon 215 mutation was also observed and the case was
classified as having a high level of resistance. Thus the
association between a high level of resistance and a lower
chance of viral failure should not be ascribed to only the
occurrence of a codon 215 mutation, but rather to the
occurrence of codon 215, 41 and/or 210 mutations.

The association between the presence of a specific base-
line mutation and virologic suppression during the first
24 weeks on HAART was explored. Viral response was
categorized into three ordered levels: full suppression (viral
load no more than 400 copies/mL at week 12 or 24), par-
tial suppression (viral load dropped 0.75 log_{10} copies/mL
or more at week 12 or 24 from baseline, but no full sup-
pression), and no suppression. According to this defini-
tion, 56 (60%) subjects achieved full viral suppression
and an additional 13 (14%) achieved partial viral sup-
pression during the first 24 weeks on HAART. There
was no statistically significant (at the unadjusted 5% level)
association between any baseline mutation and virologic
suppression during the first 24 weeks on HAART.
For the 69 children with full or partial viral suppression as defined above, the association between the presence of a specific baseline mutation and viral rebound between the suppression and week 48 was evaluated. A rebound was declared if there was an increase in RNA of more than one log₁₀ at any time between the time of suppression and week 48 from the nadir viral load value at or before the time of suppression, or if the child came off the assigned protocol treatment at or before week 48. Of the 69 children who achieved full or partial virologic suppression during the first 24 weeks on HAART, 37 (54%) had a viral rebound. In the three-drug arm children with mutation at the NRTI codon 67 seemed to be less likely to have a viral rebound after virologic suppression (unadjusted P = 0.002). There was also the suggestion of a potential association with virologic failure for the presence of any PI mutation (unadjusted P = 0.020) in the three-drug arm and for mutation at the NRTI codon 69 (unadjusted P = 0.028) in the two-drug arm, but these findings need to be treated cautiously due to the problem of multiple comparisons (see statistical analysis section).

Discussion
We evaluated the association between resistance mutations and their potential effect on the ability of antiviral medications to reduce the viral burden in children with HIV disease. As expected, there was little PI resistance and considerable NRTI resistance in this PI-naïve, NRTI-experienced population. However, contrary to our expectations, we did not find an increase in the rate of viral failure after HAART associated with the presence of resistance mutations at baseline. The two specific associations identified at week 12 in our study (Table 1) were of borderline significance and were in the direction of a decrease, rather than an increase, in the rate of viral failure. As the number of NRTI mutations at baseline increased, the median viral load at week 12 decreased (P = 0.006) and as the baseline level of ZDV resistance increased viral failure decreased after 12 weeks on a ZDV-containing HAART regimen (P = 0.017). Our results were consistent with the drug resistance study of 135 HIV-1 infected children by Eschleman et al. [2]. Neither study produced significant evidence linking a specific baseline drug resistance mutation to a subsequent increase in viral failure. These results suggest that baseline genotyping may not be necessary prior to switching to a PI based regimen. This finding has particular pertinence in resource limited settings.

Possible explanations for these observations include adherence, replicative capacity, and hypersusceptibility to NRTIs. It is likely that children who were adherent to a non-suppressive NRTI regimen and thus developed resistance, were more likely to be adherent to a HAART regimen that contained a brand new class of drugs. Friedland and Williams [10] have suggested that the relationship between the development of resistance and adherence to the drugs is bell-shaped, such that both high and low degrees of adherence decrease the likelihood of the development of resistance. This concept has been supported by several recent studies [11-13]. The RNA results from

| Baseline resistance mutation codons | RNA > 400 at week 12 (N = 19) | RNA ≤ 400 at week 12 (N = 26) | RNA > 400 at week 12 (N = 22) | RNA ≤ 400 at week 12 (N = 25) | Total number (%) with a resistance mutation at baseline (N = 92 children) |
|-----------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|------------------------------------------------------------------------|
| d4T plus RTV group                |                               |                               |                               |                               |                                                                         |
| Number with a mutation            |                               |                               |                               |                               |                                                                         |
| NRTI resistance mutations         |                               |                               |                               |                               |                                                                         |
| 215                               | 14                            | 19                            | 9b                            | 19                            | 61 (66)                                                                |
| 41                                | 8                             | 11                            | 6                             | 14                            | 39 (42)                                                                |
| 67                                | 4                             | 13                            | 6                             | 11                            | 34 (37)                                                                |
| 210                               | 4                             | 12                            | 4                             | 10                            | 30 (33)                                                                |
| 70                                | 4                             | 10                            | 6                             | 9                             | 29 (32)                                                                |
| 69                                | 4                             | 4                             | 5                             | 7                             | 20 (22)                                                                |
| 118                               | 2                             | 7                             | 5                             | 5                             | 19 (21)                                                                |
| 219                               | 3                             | 8                             | 3                             | 5                             | 19 (21)                                                                |
| 74                                | 2                             | 6                             | 1                             | 4                             | 13 (14)                                                                |
| 184                               | 2                             | 1                             | 1                             | 1                             | 5 (5)                                                                  |
| 44                                | 0                             | 2                             | 0                             | 2                             | 4 (4)                                                                  |
| 151                               | 0                             | 0                             | 0                             | 2                             | 2 (2)                                                                  |
| 65                                | 0                             | 1                             | 0                             | 0                             | 1 (1)                                                                  |
| 75                                | 0                             | 1                             | 0                             | 0                             | 1 (1)                                                                  |
| 116                               | 0                             | 0                             | 0                             | 1                             | 1 (1)                                                                  |
| Any NRTI                          | 16                            | 23                            | 15                            | 23                            | 77 (84)                                                                |
| Any PI or NRTI                    | 19                            | 25                            | 21                            | 25                            | 90 (98)                                                                |

a. No cases with mutations were observed for the following NRTI mutations: 62, 77 and 115.
b. P = 0.019, Fisher’s exact test
PACTG 338, where most children had detectable, even high viral loads at baseline, suggest that the drug(s) they were taking were less effective in suppressing viral replication, resulting in ongoing viral production in the presence of ART. Thus, children who were more adherent may have been more likely to develop resistance to their non-suppressive initial therapy regimens. The children's viral loads rapidly became undetectable once they were placed on drugs that were more suppressive and to which they had no baseline resistance (RTV and 3TC). Only 5 of the children had the M184V and only 2 had the V82A mutations at baseline. The effect would be less striking in children in the two drug regimen since the mutations associated with stavudine (d4T) resistance are primarily those seen with ZDV, ddI and dDC to which the children had been previously exposed (codons 41, 67, 70, 210, 215, and 219).

Another explanation for these results might be reduced replication capacity of the virus found in the children at baseline. The NRTI mutations typically associated with reduced fitness are M184V [14] and K65R [15]; however, only 1 child had the K65R mutation and only 5 had the M184V mutation at baseline. In addition, viral loads at baseline were relatively high: the median baseline viral load was 20,500 copies/mL and 20% of the children had baseline viral loads of 100,000–1,000,000 copies/mL.

Lastly, it has been shown that the L74V and M184V mutations confer hypersusceptibility to ZDV [16,17]. However, in the 338 data set only 5 of the 47 children randomized to ZDV/3TC/RTV for whom we have data, had the 74V mutation. This could, however, be an explanation for why children on the ZDV/3TC/RTV arm who had more baseline NRTI mutations were more likely to have a viral load <400 copies/mL at 12 weeks than those with fewer mutations.

Limitations of our study include the fact that we have baseline resistance data on only a subset of children from the entire study and lack of information on adherence to
the drug regimens, replicative capacity and hypersusceptibility of the viruses. We did not determine the occurrence of new resistance mutations after the initiation of HAART or investigate the virologic impact of these subsequent resistance mutations.

**Conclusion**

Despite considerable baseline NRTI resistance, the children in PACTG 338 who were treated with a brand new class of drugs (e.g. ritonavir, the protease inhibitor) in combination therapy responded favorably and rapidly. We did not observe an increase in the rate of viral failure after HAART linked to the presence of resistance mutations at baseline. In fact, viral loads at the 12 week time point were inversely correlated with the number of baseline NRTI mutations. It is important to remember that resistance is not an all-or-none phenomenon, and treatment failure is not defined by a resistance test. In order to better understand both the virology of mutated viruses in vivo and their response to anti-retroviral drugs as well as the use of resistance testing, prospective randomized clinical trials linked with pathogenesis-related in vitro analysis should be performed in children.

**Methods**

**Patient population**

PACTG 338 was a multicenter, randomized clinical trial that enrolled 297 children aged 2–17 years. All subjects were HIV-infected, had been receiving continuous, unchanged antiretroviral therapy for the 16 weeks before study entry, and were naïve to protease inhibitors and lamivudine (3TC) or had received no more than 6 weeks of ZDV plus 3TC in the year prior to study entry and none in the 4 months prior to study entry. Children were randomly assigned to receive either ZDV/3TC (n = 100), stavudine (d4T)/RITV (n = 97) or ZDV/3TC/RITV (n = 100). Children initially assigned to the ZDV/3TC regimen were not included in this evaluation of drug resistance and subsequent viral failure as this regimen was demonstrated to result in a suboptimal virologic suppression compared to protease inhibitor-based regimens.

A total of 92 children assigned to a RTV-containing regimen had both baseline resistance information and viral load measurement at week 12, and they were included in any analysis involving the primary endpoint of viral failure at week 12. Of these 92 subjects, the median age was 7.3 years, median CD4 cell count was 602 cells/mm³, proportion with CD4 cell count <500 cells/ul was 29%, proportion with CD4 percent <25% was 38%, median plasma HIV-1 RNA was 20,500 copies/mL, and the proportion with HIV-1 RNA copy number > 4 log₁₀ was 75%. The majority of the children were African-American (66%) and 51% were male. Children had received prior treatment with ZDV monotherapy (46%) and 51% were male. Children had received prior treatment with ZDV monotherapy (46%), the combination of ZDV and ddi (37%) or other combination therapy (15%). Baseline characteristics of the children in this resistance analysis were very similar to the baseline characteristics for the overall PACTG 338 study of 297 children, except for minor differences in the CD4 cell count (648 cells/mm³), the proportion of African-American children (51%) and the proportion with HIV-1 RNA copy number > 4 log₁₀ (64%). Children with viral loads <1,000 copies/mL were excluded from this resistance study. For the secondary analyses of viral suppression at weeks 12 or 24 and viral rebound after 48 weeks on HAART, two additional subjects were included, who did not have viral load measured at week 12 but did have information at week 24. The institutional review board at each institution approved the study and informed consent was obtained from all patients or their guardians.

**HIV-1 genotyping**

Sequencing was determined in batch at the conclusion of the study in two laboratories that participated in the National Institute of Allergy and Infectious Diseases (NIAID) Virology Quality Assurance Program. For HIV-1 sequencing, plasma RNA was extracted using the QIAam-

### Table 2: Association of the level of baseline ZDV resistance mutations and viral failure after 12 weeks on HAART

| Level of baseline ZDV resistance mutation | d4T plus RTV group | ZDV plus 3TC plus RTV group |
|-----------------------------------------|-------------------|-----------------------------|
| **RNA > 400 at week 12** (N = 19)       | High              | 14                           |
| **RNA ≤ 400 at week 12** (N = 26)       | Intermediate      | 0                            |
| **RNA > 400 at week 12** (N = 22)       | Low               | 1                            |
| **RNA ≤ 400 at week 12** (N = 25)       | None              | 4                            |
| **P-value**                             | 0.904             | 0.017                        |

a. At least codon 215.
b. At least one of codons 41 and 210, but no high level mutation (none at codon 215).
c. At least one of codons 67, 70 and 219, but no high or intermediate level mutations (none at codons 215, 41 or 210).
d. Mantel-Haenszel test for trend.
pViral RNA Mini Kit (Qiagen Inc., Valencia, CA). Reverse transcriptase polymerase chain reaction (RT-PCR) and direct DNA sequencing of protease and reverse transcriptase genes were performed using the TruGene HIV-1 Genotyping Kit (Visible Genetics-Bayer Diagnostics, Toronto, Canada) according to the manufacturer’s instructions. The International AIDS Society-USA recommended guidelines for resistance to protease inhibitor (PI) and nucleoside reverse transcriptase inhibitors (NRTI) were used [18,19].

Viral load
HIV-1 RNA copy number was assessed using the NucliSens Assay (Organon Teknika, Durham, NC) [20], which has a lower limit of quantification of 400 copies/mL. All RNA assays were performed at a single laboratory at the University of North Carolina, Chapel Hill, NC that was certified as proficient by the NIAID Virology Quality Assurance Program [21]. Assay results from the NucliSens Assay were adjusted using Virology Quality Assurance external standards [22].

Statistical analysis
Fisher's exact test was used for associations between specific baseline resistance mutations and viral failure, the Kruskal-Wallis test was used to assess the association between the number of resistance mutations and viral failure, and the Mantel-Haenszel test for trend was used to evaluate the association between level of baseline ZDV resistance and viral failure defined as viral load > 400 cp/ml [23,24]. Further, the Kruskal-Wallis test was used to correlate specific baseline resistance mutations and viral suppression, Fisher’s exact test was used to investigate the association of baseline mutations and viral rebound, and the association between the total number of resistance mutations and viral load was assessed using the locally weighted scatter plot smooth [25] and linear regression with adjustment for left censoring. All P values were two-sided and were not adjusted for multiple comparisons. Because 39 codon sites were evaluated in this analysis, caution should be exercised in the interpretation of the P values. A conservative solution to the multiple comparisons problem is the Bonferroni method that multiplies the nominal P value times the overall number of statistical tests [26]. If the result is still < 0.05, then the result is clearly statistically significant. Using the Bonferroni approach for any analysis involving individual resistance codons, a P value between 0.0013 (0.05/39) and 0.05 should be interpreted as suggestive but not necessarily definitive. P < 0.0013 should be considered clear evidence of statistical significance.

Competing interests
The author(s) declare that they have no competing interests.

Authors’ contributions
AAW, LMF, RY, KM, SIP, KES and SAN conceived, designed and implemented the primary study (PACTG 338), upon which this secondary resistance study was based. SF organized this secondary study and contributed to its design and implementation along with SAF, AK, LAP and SN. CH performed the statistical analysis. SF drafted the manuscript jointly with KES. All authors provided review comments and textual modifications during manuscript development. All authors read and approved the final manuscript.

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