

Use of a High Resolution Melting Assay to Analyze HIV Diversity in HIV-infected Ugandan Children

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Background: We used a novel high resolution melting (HRM) diversity assay to analyze HIV diversity in Ugandan children (age 0.6–12.4 years) who were enrolled in an observational study of antiretroviral treatment (ART). Children were maintained on ART if they were clinically and immunologically stable.

Methods: HIV diversity was measured before ART (baseline) in 76 children and after 48 or 96 weeks of ART in 14 children who were not virally suppressed. HIV diversity (expressed as HRM scores) was measured in 6 regions of the HIV genome (2 in *gag*, 1 in *pol*, 3 in *env*).

Results: Higher baseline HRM scores were significantly associated with older age (≥ 2 years, $P \leq 0.001$ for all 6 regions). HRM scores from different regions were weakly correlated. Higher baseline HRM scores in 3 regions (1 in *gag*, 2 in *env*) were associated with ART failure. HIV diversity was lower in 4 regions (2 in *gag*, 1 in *pol*, 1 in *env*) after 48–96 weeks of nonsuppressive ART compared with baseline.

Conclusions: Higher levels of HIV diversity were observed in older children before ART, and higher levels of diversity in some regions of the HIV genome were associated with ART failure. Prolonged exposure to nonsuppressive ART was associated with a significant decrease in viral diversity in selected regions of the HIV genome.

Key Words: HIV, diversity, children, antiretroviral therapy

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HIV-infected infants usually have homogenous viral populations, suggesting that 1 or a few HIV variants initiate the infection.¹ Several factors promote rapid evolution of HIV viruses following infection, including the short half-life of HIV virions, the large number of virions in an infected individual (large viral population size), lack of proofreading by HIV reverse transcriptase and frequent viral recombination.² Children with established HIV infection generally harbor diverse viral populations.³ The high level of viral diversity and the capacity of HIV to evolve rapidly in response to selective pressures can complicate HIV treatment.^{4,5}

To date, studies of HIV diversity in HIV-infected infants and children have been limited because of the time, effort and cost associated with analyzing sequences from individual HIV variants. We recently developed a rapid assay for analysis of HIV diversity that is based on high resolution melting (HRM) of DNA duplexes.⁶ The HRM diversity assay uses the LightScanner instrument (Idaho Technology, Inc., Salt Lake City, UT) to measure the range of melting temperatures for DNA duplexes amplified from HIV. The assay provides a simple, single numeric measure of the level of genetic diversity in each region analyzed, which we refer to as the HRM score.⁶ DNA duplexes with different sequences have different melting properties, and DNA heteroduplexes also have unique melting characteristics. In the HRM diversity assay, more diverse DNA populations (eg, amplicons from more diverse HIV populations in clinical samples) melt over a broader range of temperatures, which is reflected in higher HRM scores.

Our recent work demonstrates that the HRM diversity assay can detect single base mutations that are present at levels as low as 5% in plasmid mixtures, and can also detect insertions and deletions at low levels.⁷ The HRM diversity assay is highly reproducible,⁸ and HRM scores are significantly associated with sequence-based measures of HIV diversity obtained from analysis of cloned HIV DNA⁶ and next generation sequencing.⁹ This assay facilitates studies of factors associated with HIV diversity and diversification in different regions of the viral genome.

In a previous study, we used the HRM diversity assay to compare the level of diversity in the HIV *gag* region in HIV-infected Ugandan infants and their mothers.⁶ That study confirmed that the viral populations in 6- to 8-week-old HIV-infected infants were very homogeneous.⁶ In a subsequent study, we used the HRM diversity assay to analyze diversity in 3 regions of the HIV genome (2 regions in HIV *gag* and 1 region in HIV *pol*) in 31 HIV-infected Ugandan infants 6 weeks to 18 months of age. Higher HRM scores in all 3 regions were associated with older age, demonstrating diversification of the virus in these regions over time following infection. Furthermore, higher HRM scores at 6–8 weeks of age (scores above the 75th percentile) were associated with a significantly increased risk of death by 5 years of age.⁸

In this report, we examined HIV diversity in the *gag*, *pol* and *env* regions in 76 Ugandan children from 0.6 to 12.4 years of age who were enrolled in an observational study of antiretroviral therapy (ART). We analyzed the association of pretreatment HRM

scores with age (as a surrogate for the duration of HIV infection), the relationship between HIV diversity and treatment outcome and the impact of exposure to nonsuppressive ART on HIV diversity.

MATERIALS AND METHODS

Source of Samples Used for Analysis

Plasma samples were obtained from 76 HIV-infected children enrolled in an observational study in Uganda (enrollment: 2004–2006, Table 1).¹⁰ These children met the 2003 World Health Organization criteria for ART and received a regimen of stavudine, lamivudine and nevirapine (NVP)¹⁰; none of the children were switched to a second-line regimen during the study. HIV viral loads, CD4 cell count and CD4 cell % were measured in the observational study. Pretreatment (baseline) HIV viral loads were measured the day of ART initiation; pretreatment CD4 cell counts and CD4 cell % were measured within 30 days of ART initiation. HIV viral load, CD4 cell count and CD4 cell % were also assessed every 12 weeks between 24 and 96 weeks after ART initiation. Detection of HIV RNA did not lead to change of therapy if the children were clinically and immunologically stable. Most of the children had a significant decline in HIV RNA after ART initiation, and the response to ART was similar among children with and without prior single-dose NVP (sdNVP) exposure.¹⁰

Laboratory Methods

HIV subtyping was performed in a previous study.¹¹ The HRM diversity assay was performed as described to analyze HIV diversity in 6 regions of the HIV genome: 2 regions in *gag* (GAG1, GAG2), 1 region of *pol* (POL) and 3 regions in *env* (ENV1, ENV2, ENV3).¹² The HXB2 coordinates for these regions are: GAG1: 1998–2097, GAG2: 2068–2278, POL: 2373–2597, ENV1: 7798–8036, ENV2: 7950–8119, ENV3: 8016–8299. In this assay, regions of the HIV genome were amplified in the presence of a fluorescent dye, which was incorporated into the amplified products. The resulting amplicons were analyzed on a LightScanner instrument (Model HR 96) to produce a melting curve for each sample ($-d[\text{fluorescence}]/d[\text{temperature}]$). The difference in temperature between the initiation and completion of melting is defined as the HRM score. The HRM diversity assay was performed using samples from all 76 children before ART initiation (baseline), and using samples from a subset of those children who were not virologically suppressed (defined as children who had plasma HIV viral load >1000 copies/mL after 48 and 96 weeks of ART); 14 of 26 children who were not virologically suppressed had a 48-week or 96-week sample available for analysis.

Statistical Methods

Summary statistics (median and interquartile range for continuous variables; frequency distributions for categorical variables) were provided for clinical characteristics and diversity measurements; comparisons used Fisher exact test and the Wilcoxon rank sum test. Pearson correlation coefficients were computed for HRM scores at 6 regions. Univariate and multivariate logistic regression analyses were used to explore associations between baseline HRM scores and age, using the Firth penalized likelihood approach to avoid bias in parameter estimates caused by small sample size and a potential separation problem. Principal components of the 6 regions were computed on nonstandardized HRM scores. Two composite baseline scores were computed: (1) a “mean HRM score” defined as the average of the 6 baseline HRM scores, and (2) “any high HRM score”, defined as 1 if any of the 6 baseline HRM scores exceeded 6, and 0 if all HRM scores were ≤ 6 . HRM scores were also obtained for samples collected at the 48-week or 96-week study visit for 14 children who had >1000 copies/mL HIV RNA

TABLE 1. Characteristics of Children Before Initiation of Antiretroviral Treatment (2004–2006)

Variable	N	Result
Median age at ART initiation in years (IQR)	76	4.8 (1.7–7.9)
Median baseline CD4 cell count (IQR)		
All children	68	475 (236–733)
Children ≥ 5 years of age	34	252 (52–396)
Median baseline CD4 cell % (IQR)		
All children	69	11.0 (7.3–14.9)
Children <5 years of age	35	9.0 (3.8–13.6)
Median baseline \log_{10} HIV viral load (IQR)	72	5.6 (5.3–5.8)
HIV subtype	76	
A		36 (47.4%)
C		1 (1.3%)
D		22 (28.9%)
Intersubtype recombinant		12 (15.8%)
Unknown		5 (6.6%)
Prior single-dose nevirapine exposure	76	34 (44.7%)
Median baseline HRM score (IQR)		
GAG1	76	5.8 (5.0–7.1)
GAG2	76	5.8 (4.7–7.4)
POL	75 ^a	5.2 (4.5–5.6)
ENV1	76	4.6 (4.3–5.1)
ENV2	76	5.0 (4.4–5.6)
ENV3	76	5.9 (5.3–6.6)

Criteria for inclusion in the observational study were as follows: HIV-infected child aged 6 months to 12 years, symptoms of HIV infection (Centers for Disease Control and Prevention stage C/World Health Organization stage III-2003 version), CD4 cell % <15%, or CD4 cell % <20% with stage II World Health Organization/Centers for Disease Control and Prevention stage B, caretaker willing to give informed consent and follow study procedures, adherent to the last 3 clinic visits, antiretroviral drug-naïve except for antiretroviral drugs used for prevention of mother-to-child transmission of HIV (with <1 week of drug exposure).

Criteria for exclusion from the observational study were as follows: hemoglobin <7.0 g/dL, platelet count <49,000/mm³, absolute neutrophil count <250/mm³, serum creatinine >1.7 mg/dL, aspartate aminotransferase or alanine aminotransferase >5 times upper limits of normal, known hypersensitivity to diazepam or the last month preceding screening for entry into the study.

^aOne child who was >2 years of age did not have an HRM score obtained for the *pol* region.

N indicates number of children with data available for analysis; IQR, interquartile range.

at both of those visits. The Wilcoxon signed rank test was used to compare the difference in HRM scores between specimens obtained before and after ART initiation in this subset. If the 48-week sample was not available, the sample collected at 96 weeks was used for analysis. In analyses using plasma HIV viral load, left-censored results (viral load <400 copies/mL) were assigned a value of 200, and right-censored results (viral load >750,000 copies/mL) were assigned a value of 750,000. SAS version 9.2 (SAS Institute Inc., Cary, NC) was used to perform all statistical analyses.

Informed Consent

Guidelines of the U.S. Department of Health and Human Services and the authors' institutions were followed in the conduct of this research. Approval for the observational study was obtained from the Makerere University Faculty of Medicine Research and Ethics Committee in Uganda, and written informed consent was obtained from the mothers for participation in the study.

RESULTS

Characteristics of Children Before Antiretroviral Therapy

Characteristics of the children, including age at ART initiation, baseline CD4 cell count, baseline CD4 cell %, baseline HIV viral load, HIV subtype and prior sdNVP exposure are shown

in Table 1. In this cohort, the median age of the sdNVP-exposed children was lower than that of the sdNVP-unexposed children (median: 1.6 years, interquartile range: 1.2–2.4 years versus median: 7.7 years, interquartile range: 5.9–9.5 years; $P < 0.0001$, Wilcoxon rank sum test), reflecting the fact that many of the older children were born before widespread availability of sdNVP prophylaxis for prevention of mother-to-child transmission.

HIV Diversity in Samples Collected Before Antiretroviral Therapy

We used the HRM diversity assay to analyze HIV diversity in 6 regions of the HIV genome (2 regions in *gag*, 1 region in *pol*, 3 regions in *env*; see Methods). The median HRM scores obtained from samples collected before ART initiation (baseline samples) ranged from 4.6 to 5.9 for the 6 regions analyzed (Table 1). These values are higher than those typically obtained for cloned plasmid DNA.¹²

We assessed linear correlations among baseline HRM scores for the 6 regions after removing 1 outlier that had unusually high HRM scores for ENV1 and ENV2, with lower scores for other regions (15 pairwise correlations of 6 regions; Fig. 1). The HRM scores obtained for different genomic regions were correlated. However, the correlation coefficients were relatively low (range: 0.2–0.6).

Association of HIV Diversity and Age

Because children in the observational cohort were known or assumed to have perinatally acquired HIV infection, age can be considered a surrogate for duration of HIV infection in this cohort. Figure 2 shows the baseline HRM scores plotted as a function of age. HRM scores were generally low (eg, ≤ 6) in children < 2 years of age; a greater range of HRM scores was seen in children ≥ 2 years of age. Higher median baseline HRM scores for each of the 6 regions were significantly associated with older age (< 2 years versus ≥ 2 years of age, Table 2).

Multivariate analysis of all 6 regions with age as an outcome was attempted, but showed great instability in the parameter estimates, indicating underlying multicollinearity between the HRM scores (data not shown). Principal component analysis of the 6 scores confirmed low dimension in the multivariate HRM predictor scores obtained for the different genomic regions (data not shown). These findings suggest that use of a composite score for HIV diversity (combining HRM scores from multiple regions of the HIV genome) is more informative than analysis of single regions. We explored the use of 2 composite variables: (1) mean HRM score for the 6 regions, and (2) any high HRM score (see Methods). In multivariate models that adjusted for baseline CD4 cell count, for both composite variables, higher composite HRM scores (reflecting higher levels of HIV diversity) were independently associated with older age (≥ 2 years, Table 3).

Association of Baseline HIV Diversity and Treatment Outcome

We next analyzed the association of baseline HRM scores and treatment outcome. In this cohort, children were maintained on their initial ART regimen if they were immunologically and clinically stable. Twenty-six of the 76 children had an HIV viral load > 1000 copies/mL after 48 and 96 weeks on ART and were classified as not virologically suppressed. Lack of virologic suppression was associated with higher baseline HRM scores for 3 of the 6 regions analyzed; children who failed to achieve virologic suppression on ART had higher baseline scores for the GAG1, ENV1 and ENV2 regions (Table 4). We did not find a significant association between baseline HRM score and treatment outcome using either

of the composite baseline variables described above (Table 4). We explored the association of virologic suppression with other baseline variables (CD4 cell count, CD4 cell %, age, prior exposure to single-dose NVP); none of those associations was statistically significant (data not shown).

Effect of Nonsuppressive Antiretroviral Treatment on HIV Diversity

Samples collected at 48 or 96 weeks were available for 14 of the 26 children who were not virologically suppressed (see preceding section). In these children, continued exposure to ART in the absence of virologic suppression was previously shown to be associated with selection of HIV strains with multiclass drug resistance.¹¹ We analyzed whether prolonged exposure to nonsuppressive ART was also associated with a reduction in the diversity of the viral population. For all 14 children, an HIV viral load of > 1000 copies/mL was documented for at least 6 months before analysis (median: 16.5 months, range: 6–24 months). In this subset of 14 children, we compared baseline HRM scores with HRM scores obtained after 48 or 96 weeks of ART (Table 5). On-treatment HRM scores (after 48 or 96 weeks of ART) were significantly lower than the pretreatment scores for 4 of the 6 genomic regions analyzed (GAG1, GAG2, POL and ENV3). We considered the possibility that the low HRM scores obtained for children on nonsuppressive treatment may have been artifactual (ie, that the lower HRM scores obtained for these samples may have been obtained because these samples had lower HIV viral loads). This did not appear to be the case. Among the 14 children studied, the \log_{10} HIV viral load in the on-treatment sample was associated with the HRM score for only 1 region (GAG1, $r = 0.49$, $P = 0.03$); correlations for the other 5 regions were not statistically significant. Furthermore, all HRM diversity assays performed in this study used at least 100 copies of HIV RNA as templates for reverse transcription and amplification with only one exception (1 sample collected at the 96-week visit had < 100 copies of HIV RNA). We previously demonstrated that results obtained with the HRM diversity assay are not significantly different when the HIV RNA template number ranges from 100 to 5000 copies.⁸

DISCUSSION

The HRM diversity assay used in this study is a low-cost, rapid method for analysis of HIV diversity without sequencing. This method facilitates analysis of HIV diversity in larger sample sets and comparison of diversity and diversification in different regions of the HIV genome. In this study, analysis was performed for 6 genomic regions in baseline samples from 76 children and in on-treatment samples from 14 of those children (> 500 diversity measures). In a previous study of infants aged 6 weeks to 18 months, we demonstrated that higher HRM scores in 3 of the 6 genomic regions analyzed in this report (GAG1, GAG2, POL) were significantly associated with older age.⁸ That study also demonstrated that higher HRM scores in those regions at 6–8 weeks of age were significantly associated with death by age 5.⁸ This report extends that study by including analysis of the *env* region, by analyzing HIV diversity in untreated children up to 12.4 years of age, by examining the association of HRM scores and response to ART, and by examining changes in HIV diversity associated with maintenance on a nonsuppressive ART regimen.

We found that higher HRM scores in all 6 genomic regions analyzed (indicating higher HIV diversity) were significantly associated with older age, independent of immunologic status (CD4 cell count, CD4 cell %). This indicates that the viral population tends to increase in diversity in *gag*, *pol* and *env* over time in HIV-infected children. HRM scores from different genomic

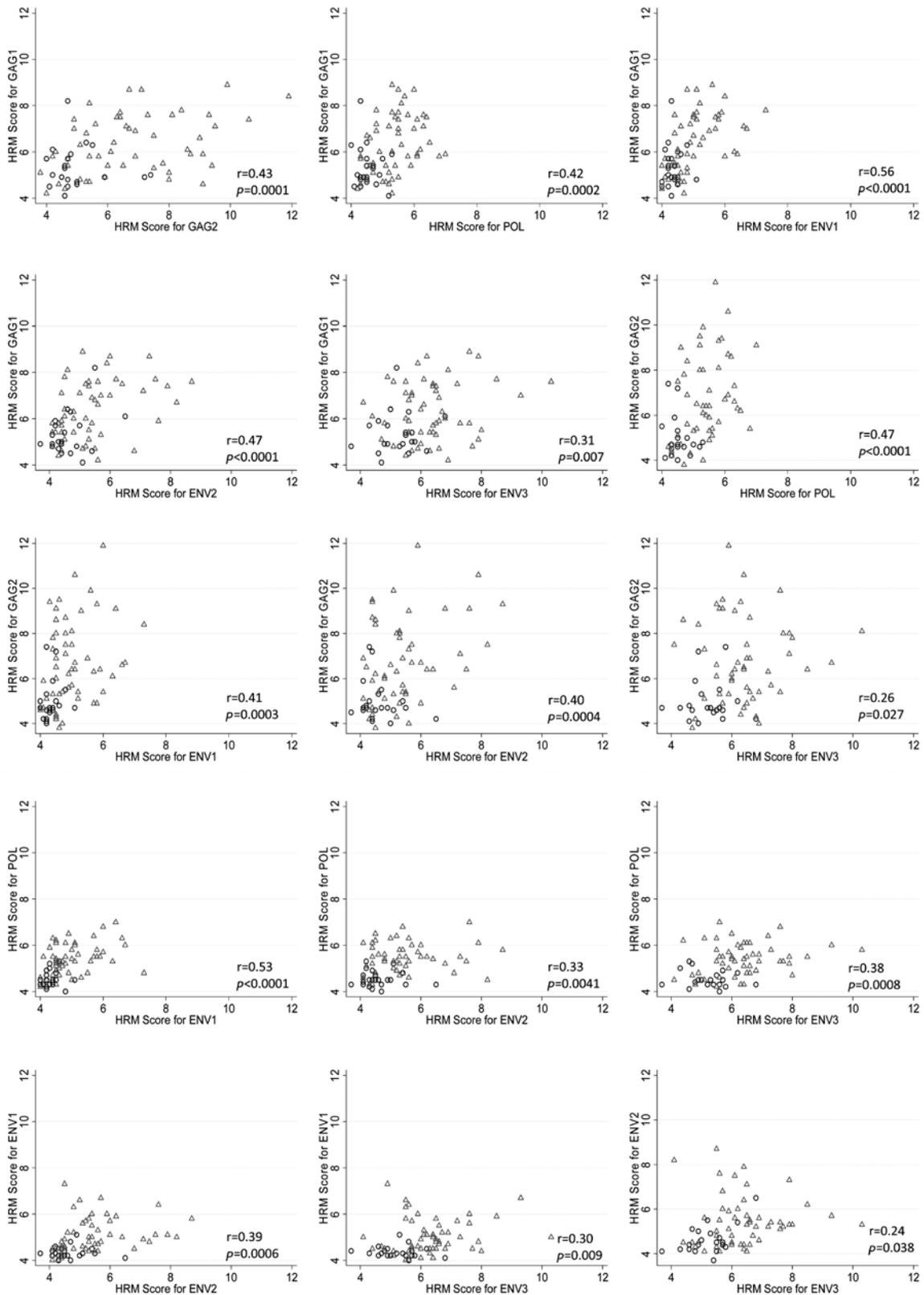


FIGURE 1. Correlation of HRM scores in 6 regions of the HIV genome. The figure shows the results of correlation analyses for HRM scores in the 6 genomic regions analyzed (see text); 15 pairwise comparisons are shown. The correlation coefficients (*r*) and the *P* values for the pairwise comparisons are shown in the figure panels. Data for children <2 years of age are shown with circles; data from children ≥2 years of age are shown with triangles.

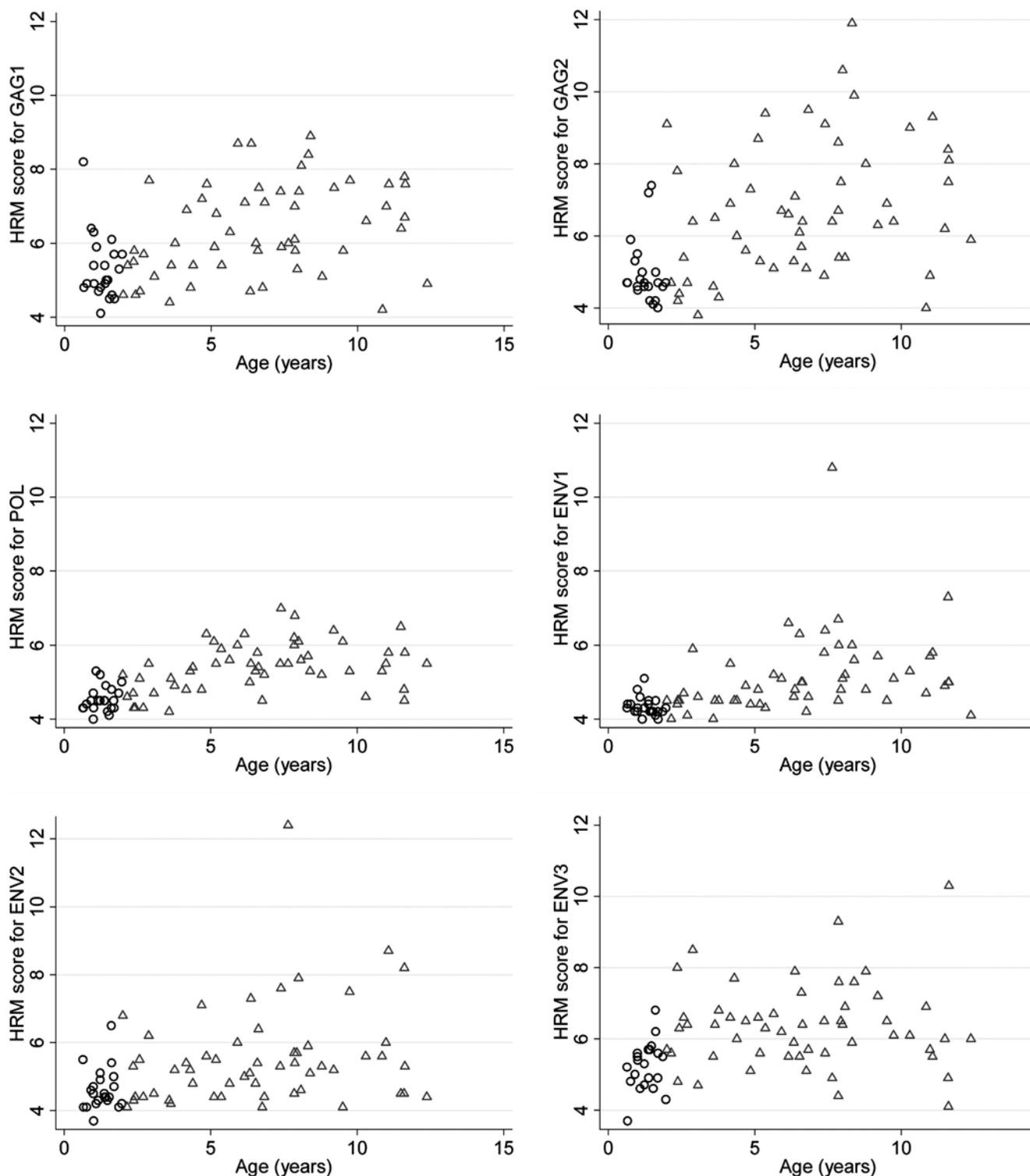


FIGURE 2. Association of HRM scores with age. Plots of HRM score and age in years are shown for each of the 6 genomic regions analyzed. Data for children <2 years of age are shown with circles; data from children ≥2 years of age are shown with triangles.

regions were weakly correlated in pairwise analyses. Multivariate and principal component analysis of the association of HRM scores with age also indicated that the levels of HIV diversity in the different regions analyzed were not independent. These findings indicate that HIV diversifies in children in a generalized fashion, rather than selectively (in specific regions or genes), but that the pattern of diversification varies from child to child. For this reason, consideration of composite HRM scores from multiple genomic regions may be more informative for some analyses than consideration of the HRM score from any single region. HRM scores were generally low in children <2 years of age. In contrast, our studies suggest that HIV usually diversifies in adults within 1 to 2 years after infection (data not shown). A variety of factors are likely to influence viral diversification in children. These include host factors (humoral immunity, cellular immunity and other factors) as well as viral factors (eg, replication rate, mutation rate). The delayed serologic response to HIV infection in infants and young children is one reason why serologic assays are not recommended for diagnosis of HIV in children <18 months of age,¹³ and may be one reason why viral diversification occurs later in children than adults (relative to the time of HIV infection). One limitation of this study is the potential confounding of sdNVP. In this cohort, the median age of the sdNVP-exposed children was lower than that of the sdNVP-unexposed children, reflecting the fact that many of the older children were born before widespread availability of sdNVP. In a previous study, however, we did not find an association between sdNVP and HRM score in the GAG2 region.⁶

In this cohort, children who had higher levels of *env* and *gag* diversity before ART initiation were less likely to achieve virologic suppression. Higher levels of diversity in these regions could reflect higher viral mutation rates and/or higher rates of viral replication,

either of which could facilitate escape from antiretroviral drug suppression. It is interesting to note that we did not see an association of higher baseline *pol* diversity with treatment failure. The HRM diversity assay can detect single-point mutations, even when they are present at levels as low as 5% in plasmid mixtures.⁷ Although we did not see an association between higher baseline *pol* diversity and treatment outcome, it is possible that HIV variants with resistance mutations in *pol* were present at higher frequencies at baseline in the children who subsequently failed ART, but that these variants were present at levels that are too low to detect with the HRM diversity assay.

In settings with limited access to second-line ART regimens, children may be maintained on nonsuppressive regimens to reduce the risk of clinical and immunologic decline.^{14,15} In higher resource settings where second-line regimens are available, it may also be preferable in some cases to maintain children and adolescents on nonsuppressive antiretroviral drug regimens while addressing treatment adherence and other issues, rather than interrupt treatment.¹⁴ In this study, maintenance of children on a nonsuppressive ART regimen for 48 or 96 weeks was associated with a significant reduction in viral diversity in 4 of the 6 genomic regions analyzed (2 in *gag*, 1 in *pol* and 1 in *env*). This most likely represents bottlenecking of the viral population as a result of persistent selective pressure. In a previous study of the same cohort, we demonstrated that the children who were maintained on nonsuppressive treatment regimens developed

TABLE 2. Comparison of Median HRM Scores for Children ≤2 vs. ≥2 years of age

Region Analyzed	Median HRM Diversity Score (IQR)		P*
	Age <2 years N = 22	Age ≥ 2 years N = 54	
GAG1	5.0 (4.8–5.7)	6.1 (5.4–7.4)	0.001
GAG2	4.7 (4.6–5.0)	6.4 (5.3–8.0)	<0.0001
POL	4.5 (4.3–4.7)	5.4 (4.9–5.8)	<0.0001
ENV1	4.3 (4.2–4.4)	4.9 (4.5–5.6)	<0.0001
ENV2	4.5 (4.2–4.9)	5.3 (4.5–5.9)	0.001
ENV3	5.3 (4.7–5.6)	6.3 (5.6–6.8)	<0.0001

IQR indicates interquartile range; N, number of children in each group.
*P values were obtained using the Wilcoxon rank sum test.

TABLE 4. Association Between Baseline HRM Scores and Lack of Virologic Suppression

Region Analyzed	Median HRM Diversity Score (IQR)		P
	Suppressed N = 50	Nonsuppressed N = 26	
GAG1	5.4 (4.9–6.4)	6.5 (5.7–7.5)	0.02*
GAG2	5.8 (4.6–8.0)	5.8 (4.7–6.7)	0.90*
POL	5.2 (4.5–5.6)	5.2 (4.5–5.5)	0.92*
ENV1	4.5 (4.2–4.9)	5.0 (4.4–5.7)	0.03*
ENV2	4.5 (4.4–5.4)	5.4 (4.8–6.0)	0.01*
ENV3	5.9 (5.1–6.5)	5.9 (5.5–6.8)	0.35*
Mean HRM score [†]	5.4 (4.7–6.0)	5.9 (5.2–6.4)	0.10*
Any high score [‡]	34 (68%) [§]	21 (81%) [§]	0.29 [§]

*P value from Wilcoxon rank sum test. Statistically significant values (P < 0.05) are shown in bold text.

[†]Mean HRM score indicates the mean of the HRM scores obtained for the 6 regions analyzed.

[‡]Any high HRM score indicates that 1 or more of the baseline HRM scores was > 6.

[§]Frequency and column percentage; P value from Fisher exact test.

IQR indicates interquartile range; N, number of children in each age group.

TABLE 3. Association Between Composite Baseline HRM Scores and Age (≤2 vs. ≥2 years), Adjusting for CD4 Cell Count

Model	Baseline Covariate	Univariate Analysis		Multivariate Analysis	
		OR (95% CI)	P	Adjusted OR (95% CI)	P
1	Mean HRM score [†]	32.7 (6.2–172.6)	<0.001	15.6 (2.6–94.1)	0.003
	CD4 cell count (per 100 cells)			0.6 (0.5–0.9)	0.02
2	Any high HRM score [‡]	23.1 (5.9–89.9)	<0.001	24.6 (4.0–150.7)	0.001
	CD4 cell count (per 100 cells)			0.6 (0.4–0.8)	0.002

[†]Mean HRM score indicates the mean of the HRM scores obtained for the 6 regions analyzed.

[‡]Any high HRM score indicates that 1 or more of the baseline HRM scores was > 6.

Eight observations were excluded due to missing CD4 cell count.

OR indicates odds ratio; CI, confidence intervals.

TABLE 5. Decrease in HRM Scores After 48 or 96 weeks of ART in 14 Children Who Were not Virally Suppressed on Treatment

Region Analyzed	Median Decline in HRM Score (IQR)*	P†
GAG1	1.2 (0.1 to 2.5)	0.002
GAG2	0.6 (0.0 to 1.6)	0.01
POL	0.5 (0.1 to 1.1)	0.01
ENV1	0.3 (−0.01 to 1.1)	0.06
ENV2	0.5 (−0.3 to 1.2)	0.2
ENV3	1.3 (0.6 to 2.2)	0.003

*Values are shown for the difference between HRM scores obtained before ART initiation (baseline) and after 48 or 96 weeks of nonsuppressive antiretroviral therapy; 4 of the 14 children analyzed did not have a 48-week result; the 96-week result was used for analysis for those 4 children.

†Wilcoxon signed rank test.

IQR indicates interquartile range.

multiclass antiretroviral drug resistance.¹¹ Selection of drug-resistant HIV variants in this cohort was not associated with an increase in *pol* diversity. Instead, prolonged selection by antiretroviral drugs led to a reduction in *pol* diversity (bottlenecking). Hong et al¹⁶ observed a similar reduction in genetic diversity by analyzing cloned HIV *pol* variants from 7 adults failing ART. It is interesting to note that we observed a reduction in diversity not only in the *pol* region (the region targeted by the antiretroviral drugs in the treatment regimen), but also in 2 regions in *gag* and 1 region of *env*. Furthermore, the bottlenecking that we observed was not a generalized phenomenon, because diversity remained high in 2 other regions of *env*. Further studies are needed to evaluate the effect of nonsuppressive therapy on viral evolution, and to determine whether reductions in viral diversity in this setting have any impact on subsequent antiretroviral treatment responses.

Existing data on HIV diversity in children is very limited. Most studies have included very small numbers of children and have examined diversity in 1 genomic region only. Although it is generally assumed that HIV diversity increases over time, our findings present a more complex picture of viral diversification. We found that increases in diversity occur in different regions of the HIV genome in different children. There was no single pattern of diversification, and the virus remained highly homogeneous in some regions, even in children who were >10 years old. We also found that higher baseline HIV diversity was associated with higher likelihood of virologic failure on ART. There are also very limited data on the effects of prolonged nonsuppressive ART on viral populations. A novel finding of this study is that the genetic bottlenecking that resulted from prolonged exposure to nonsuppressive ART was not a generalized phenomenon; instead, reductions in diversity were observed in specific regions of the HIV genome, including some regions that were not directly targeted by antiretroviral drugs. Results from this study provide new insights into factors associated with HIV diversity. At this time, we do not envision use of the HRM diversity assay as a tool for clinical decision making.

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