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

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Abstract

BACKGROUND—We used a novel high resolution melting (HRM) diversity assay to analyze HIV diversity in Ugandan children (ages 0.6 to 12.4 years) who were enrolled in an observational

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Competing Interests
None of the authors have a commercial or other association that might pose a conflict of interest.

Authors' Contributions
All authors participated in preparation of the manuscript, provided input, and approved the final version. Additional contributions are described below.
Maria M. James: Performed the HRM assays, analyzed the data, prepared the figures
Lei Wang: Performed the statistical analysis for this study
Deborah Donnell: Senior statistician for this study
Matthew M. Cousins: Developed the HRM assay, assisted with HRM analysis
Linda Barlow-Mosha: Study coordinator and pediatrician for the observational study
Jessica M. Fogel: Assisted with analysis of HRM data
William I. Towler: Developed the HRM assay, assisted with the HRM analysis
Allison L. Agwu: Contributed to analysis of immunologic outcomes and to the discussion of the relevance of the findings to use of non-suppressive antiretroviral drug regimens.
Danstan Bagenda: Data analyst for the observational study
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Philippa Musoke: PI of the observational study; contributed to analysis and interpretation of study results
Susan H. Eshleman: Designed the study, coordinated all aspects of the project, lead author

study of antiretroviral treatment (ART). Children were maintained on ART if they were clinically and immunologically stable.

METHODS—HIV diversity was measured prior to 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 six regions of the HIV genome (two in *gag*, one in *pol*, three in *env*).

RESULTS—Higher baseline HRM scores were significantly associated with older age (≥ 2 years, $P \leq 0.001$ for all six regions). HRM scores from different regions were weakly correlated. Higher baseline HRM scores in three regions (one in *gag*, two in *env*) were associated with ART failure. HIV diversity was lower in four regions (two in *gag*, one in *pol*, one in *env*) after 48 to 96 weeks of non-suppressive ART compared to baseline.

CONCLUSIONS—Higher levels of HIV diversity were observed in older children prior to ART and higher levels of diversity in some regions of the HIV genome were associated with ART failure. Prolonged exposure to non-suppressive ART was associated with a significant decrease in viral diversity in selected regions of the HIV genome.

Keywords

HIV; diversity; children; antiretroviral therapy

HIV-infected infants usually have homogenous viral populations, suggesting that one 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 proof-reading 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 (e.g., 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 DNA [6] 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 6–8 week old HIV-infected infants were very

homogeneous [6]. In a subsequent study, we used the HRM diversity assay to analyze diversity in three regions of the HIV genome (two regions in HIV *gag* and one region in HIV *pol*) in 31 HIV-infected Ugandan infants 6 weeks to 18 months of age. Higher HRM scores in all three 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 pre-treatment 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 non-suppressive 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 (WHO) criteria for ART and received a regimen of stavudine (d4T), lamivudine (3TC), 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. Pre-treatment (baseline) HIV viral loads were measured the day of ART initiation; pre-treatment 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 six regions of the HIV genome: two regions in *gag* (GAG1, GAG2), one region of *pol* (POL), and three 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[fluorescence]/d[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 prior to 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 >1,000 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 [IQR] for continuous variables; frequency distributions for categorical variables) were provided for clinical characteristics

and diversity measurements; comparisons used Fisher's exact test and the Wilcoxon rank sum test. Pearson correlation coefficients were computed for HRM scores at six regions. Univariate and multivariate logistic regression analyses were used to explore associations between baseline HRM scores and age, using Firth's penalized likelihood approach to avoid bias in parameter estimates caused by small sample size and a potential separation problem. Principal components of the six regions were computed on non-standardized HRM scores. Two composite baseline scores were computed: (1) a "mean HRM score" defined as the average of the six baseline HRM scores, and (2) "any high HRM score", defined as 1 if any of the six 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 >1,000 copies/mL HIV RNA 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 was used to perform all statistical analyses.

Informed Consent

Guidelines of the U.S. Dept. 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 prior to 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, IQR: 1.2–2.4 years, vs. median: 7.7 years, IQR: 5.9–9.5 years, $P < 0.0001$, Wilcoxon rank sum test), reflecting the fact that many of the older children were born prior to widespread availability of sdNVP prophylaxis for prevention of mother to child transmission.

HIV diversity in samples collected prior to antiretroviral therapy

We used the HRM diversity assay to analyze HIV diversity in six regions of the HIV genome (two regions in *gag*, one region in *pol*, three regions in *env*, see Methods). The median HRM scores obtained from samples collected prior to ART initiation (baseline samples) ranged from 4.6–5.9 for the six 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 six regions after removing one outlier that had unusually high HRM scores for ENV1 and ENV2, with lower scores for other regions (15 pairwise correlations of six regions, Figure 1). The HRM scores obtained for different genomic regions were correlated. However, the correlation coefficients were relatively low (range: 0.2 to 0.6).

Association of HIV diversity and age

Since 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 (e.g., ≤ 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 six regions were significantly associated with older age (< 2 years vs. ≥ 2 years of age, Table 2).

Multivariate analysis of all six regions with age as an outcome was attempted, but showed great instability in the parameter estimates, indicating underlying multi-collinearity between the HRM scores (data not shown). Principal component analysis of the six scores confirmed low dimension in the multivariate HRM predictors 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 use of two composite variables: (1) mean HRM score for the six 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 >1,000 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 three of the six 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 non-suppressive 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 above). 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 multi-class drug resistance [11]. We analyzed whether prolonged exposure to non-suppressive ART was also associated with a reduction in the diversity of the viral population. For all 14 children, an HIV viral load of >1,000 copies/mL was documented for at least 6 months prior to analysis (median: 16.5 months, range 6–24 months). In this subset of 14 children, we compared baseline HRM scores to 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 pre-treatment scores for four of the six genomic regions analyzed (GAG1, GAG2, POL and ENV3). We considered the possibility that the low HRM scores obtained for children on non-suppressive treatment may have been artifactual (i.e., 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 log10 HIV viral load in the on-treatment sample was associated with the HRM score for only one region (GAG1, r=0.49, P=0.03); correlations for the other five 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 (one 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 100–5,000 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 six 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 of age, we demonstrated that higher HRM scores in three of the six 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 non-suppressive ART regimen.

We found that higher HRM scores in all six 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 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 under 2 years of age. In contrast, our studies suggest that HIV usually diversifies in adults within 1-2 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 (e.g., 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 younger than 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 prior to 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 prior to 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. Interestingly, 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]. While 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 non-suppressive 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 non-suppressive antiretroviral drug regimens while addressing treatment adherence and other issues, rather than interrupt treatment [14]. In this study, maintenance of children on a non-suppressive ART regimen for 48 or 96 weeks was associated with a significant reduction in viral diversity in four of the six genomic regions analyzed (two in *gag*, one in *pol*, and one 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 non-suppressive treatment regimens developed multi-class 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 al. observed a similar reduction in genetic diversity by analyzing cloned HIV *pol* variants from seven adults failing ART [16]. Interestingly, 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 two regions in *gag* and one region of *env*. Furthermore, the bottlenecking that we observed was not a generalized phenomenon, since diversity remained high in two other regions of *env*. Further studies are needed to evaluate the effect of non-suppressive 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 one genomic region only. While 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 more than 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 non-suppressive ART on viral populations. A novel finding of this study is that the genetic bottlenecking that resulted from prolonged exposure to non-suppressive 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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REFERENCES

1. Verhofstede C, Demecheleer E, De Cabooter N, et al. Diversity of the human immunodeficiency virus type 1 (HIV-1) env sequence after vertical transmission in mother-child pairs infected with HIV-1 subtype A. J Virol. 2003; 77:3050–3057. [PubMed: 12584330]
2. Tebit DM, Nankya I, Arts EJ, et al. HIV diversity, recombination and disease progression: how does fitness "fit" into the puzzle? AIDS Rev. 2007; 9:75–87. [PubMed: 17694675]
3. Leal E, Janini M, Diaz RS. Selective pressures of human immunodeficiency virus type 1 (HIV-1) during pediatric infection. Infect Genet Evol. 2007; 7:694–707. [PubMed: 17719854]
4. Richman DD, Little SJ, Smith DM, et al. HIV evolution and escape. Trans Am Clin Climatol Assoc. 2004; 115:289–303. [PubMed: 17060974]
5. McBurney SP, Ross TM. Viral sequence diversity: challenges for AIDS vaccine designs. Expert Rev Vaccines. 2008; 7:1405–1417. [PubMed: 18980542]
6. Towler WI, James MM, Ray SC, et al. Analysis of HIV diversity using a high resolution melting assay. AIDS Research and Human Retroviruses. 2010; 26:913–918. [PubMed: 20666583]
7. Cousins MM, Donnell D, Eshleman SH. Impact of mutation type and amplicon characteristics on genetic diversity measures generated using a high resolution melting diversity assay. Journal of Molecular Diagnostics. 2012 Submitted.
8. James MM, Wang L, Musoke P, et al. Association of HIV diversity and survival in HIV-infected Ugandan infants. PLoS One. 2011; 6:e18642. [PubMed: 21533179]
9. Cousins, M.; Redd, A.; Porcella, S., et al. Comparison of a high resolution melting (HRM) assay to next generation sequencing for analysis of HIV diversity. 19th Conference on Retroviruses and Opportunitic Infections; Seattle, WA. March 5 - 8 2011; Abstract# V-123
10. Barlow-Mosha, L.; Ajuna, P.; Mubiru, M., et al. Early effectiveness of a NVP-based HAART regimen among HIV-infected children with and without prior single-dose NVP exposure. 15th Conference on Retroviruses and Opportunistic Infections; Boston, MA. February 3 - 2008; Abstract #358
11. Towler WI, Barlow-Mosha L, Church JD, et al. Analysis of drug resistance in children receiving antiretroviral therapy for treatment of HIV-1 infection in Uganda. AIDS Res Hum Retroviruses. 2010; 26:563–568. [PubMed: 20455758]
12. Cousins MM, Laeyendecker O, Beauchamp G, et al. Use of a high resolution melting (HRM) assay to compare gag, pol, and env diversity in adults with different stages of HIV infection. PLoS One. 2011; 6:e27211. [PubMed: 22073290]
13. CDC Global AIDS Program. Early Infant Diagnosis of HIV Implementation Guide Book: Guide for Program Managers. http://womenchildrenhiv.org/pdf/p08-ch/ch-09-01/Book1_Early_Implementation.pdf.
14. Agwu A, Lindsey JC, Ferguson K, et al. Analyses of HIV-1 drug-resistance profiles among infected adolescents experiencing delayed antiretroviral treatment switch after initial nonsuppressive highly active antiretroviral therapy. AIDS Patient Care STDS. 2008; 22:545–552. [PubMed: 18479228]
15. Maron G, Gaur AH, Flynn PM. Antiretroviral therapy in HIV-infected infants and children. Pediatr Infect Dis J. 2010; 29:360–363. [PubMed: 20351530]
16. Hong S, Cao J, Tu YT. Evolution of HIV-1 in a patient population failing multiple-drug therapy. Microbiol Immunol. 2009; 53:535–539. [PubMed: 19703248]

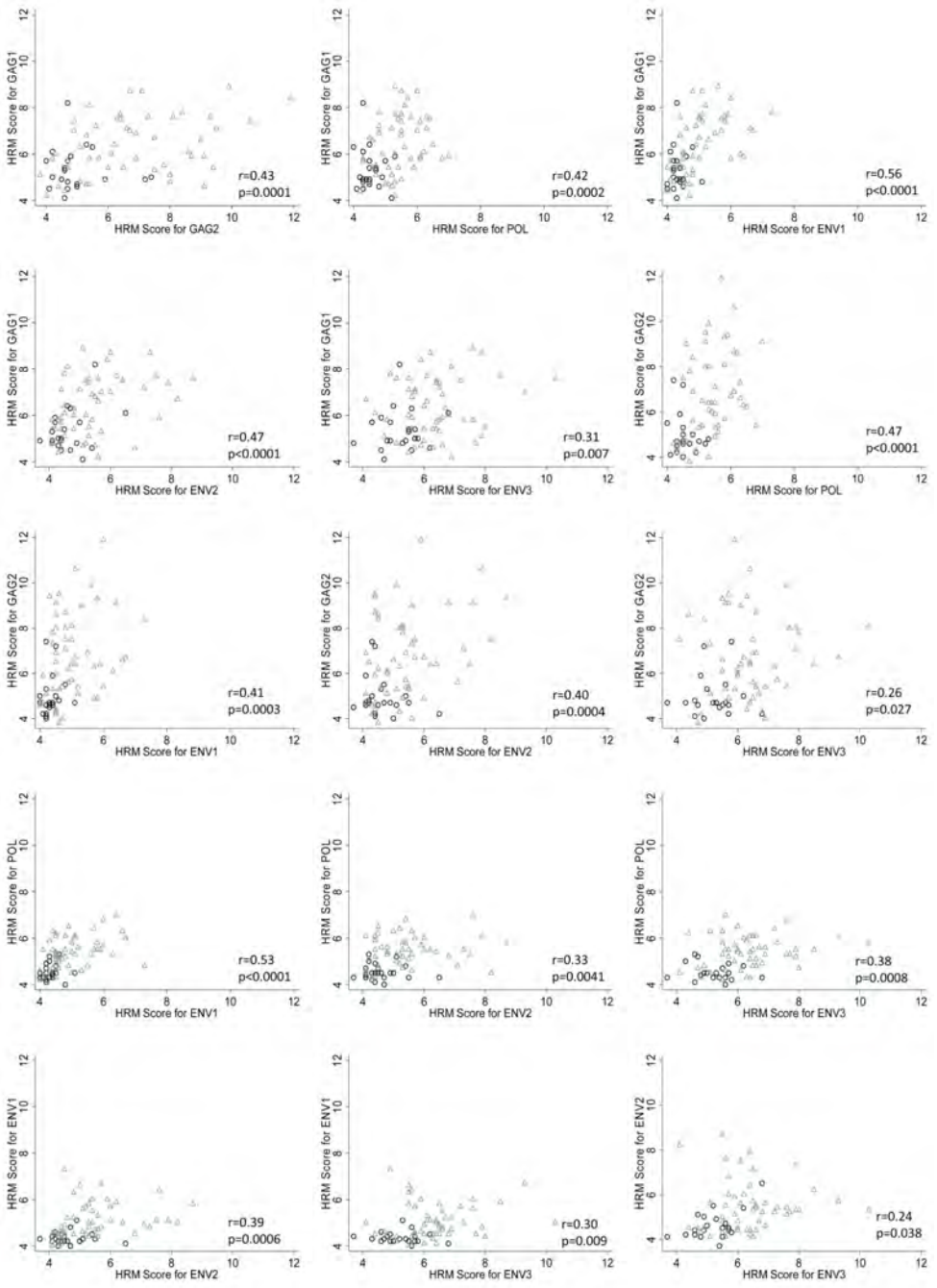

Figure 1. Correlation of HRM scores in six regions of the HIV genome

The figure shows the results of correlation analyses for HRM scores in the six 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.

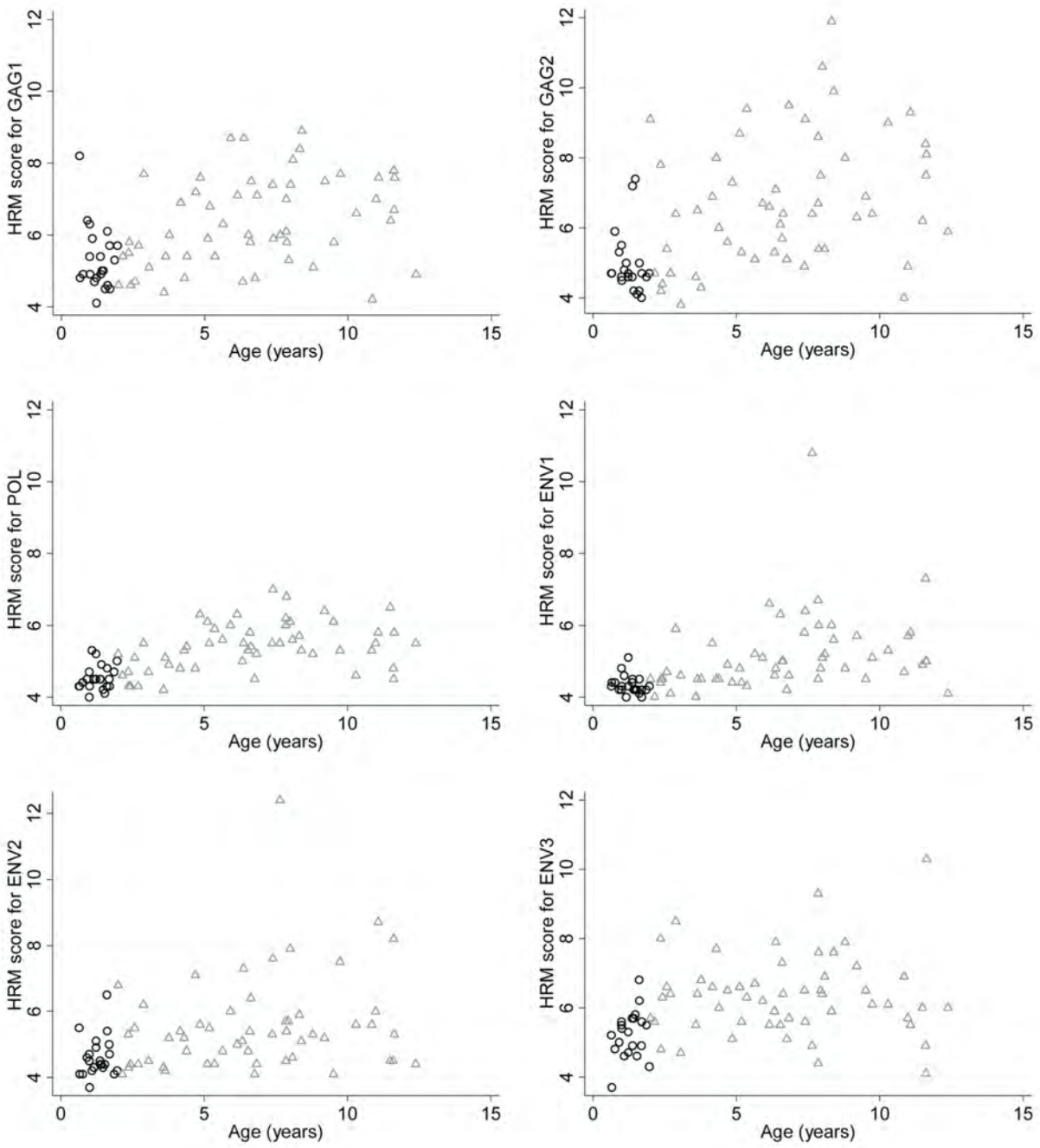

Figure 2. Association of HRM scores with age

Plots of HRM score and age in years are shown for each of the six 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.

Table 1

Characteristics of children prior to 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)

*Abbreviations: N: number of children with data available for analysis; ART: antiretroviral therapy; IQR: interquartile range; HRM: high resolution melting. Note: 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 [CDC] stage C/World Health Organization [WHO] stage III-2003 version), CD4 cell percent < 15%, or CD4 cell percent <20% with stage II WHO/CDC stage B, caretaker willing to give informed consent and follow study procedures, adherent to the last three 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: haemoglobin < 7.0 g/dl, platelet count <49,000 / mm^3, absolute neutrophil count < 250/mm^3, 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 preceeding screening for entry into the study).

[a]One child who was > 2 years of age did not have an HRM score obtained for the *pol* region.

Table 2

Comparison of median HRM scores for children < 2 vs. ≥ 2 years of age*

	Median HRM diversity score (IQR)		
Region analyzed	**Age < 2 years N=22**	**Age ≥ 2 years N=54**	**p*a***
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

*Abbreviations: HRM: high resolution melting; IQR: interquartile range; N: number of children in each group.

*a*P values were obtained using the Wilcoxon rank sum test.

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 value	Adj. OR (95% CI)	P value
1	Mean HRM score[a] CD4 cell count (per 100 cells)	32.7 (6.2, 172.6)	< 0.001	15.6 (2.6, 94.1) 0.6 (0.5, 0.9)	0.003 0.02
2	Any high HRM score[b] CD4 cell count (per 100 cells)	23.1 (5.9, 89.9)	< 0.001	24.6 (4.0, 150.7) 0.6 (0.4, 0.8)	0.001 0.002

*Abbreviations: OR: odds ratio; CI: confidence intervals; Adj. OR: adjusted odds ratio; HRM: high resolution melting. Eight observations were excluded due to missing CD4 cell count.

[a]Mean HRM score indicates the mean of the HRM scores obtained for the six regions analyzed.

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

Table 4

Association between baseline HRM scores and lack of virologic suppression.

	Median HRM diversity score (IQR)		
Region analyzed	**Suppressed N=50**	**Non-suppressed N=26**	**P**
GAG1	5.4 (4.9, 6.4)	6.5 (5.7, 7.5)	**0.02**[a]
GAG2	5.8 (4.6, 8.0)	5.8 (4.7, 6.7)	0.90[a]
POL	5.2 (4.5, 5.6)	5.2 (4.5, 5.5)	0.92[a]
ENV1	4.5 (4.2, 4.9)	5.0 (4.4, 5.7)	**0.03**[a]
ENV2	4.5 (4.4, 5.4)	5.4 (4.8, 6.0)	**0.01**[a]
ENV3	5.9 (5.1, 6.5)	5.9 (5.5, 6.8)	0.35[a]
Mean HRM score[b]	5.4 (4.7, 6.0)	5.9 (5.2, 6.4)	0.10[a]
Any high score[c]	34 (68%)[d]	21 (81%)[d]	0.29[d]

[*] Abbreviations: HRM: high resolution melting; IQR: interquartile range; N: number of children in each age group.

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

[b] Mean HRM score indicates the mean of the HRM scores obtained for the six regions analyzed.

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

[d] Frequency and column percentage; P value from Fisher's exact test.

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)[a]	P value[b]
GAG1	1.2 (0.1, 2.5)	0.002
GAG2	0.6 (0.0, 1.6)	0.01
POL	0.5 (0.1, 1.1)	0.01
ENV1	0.3 (−.01, 1.1)	0.06
ENV2	0.5 (−0.3, 1.2)	0.2
ENV3	1.3 (0.6, 2.2)	0.003

*Abbreviations: HRM: high resolution melting; IQR: interquartile range.

[a]Values are shown for the difference between HRM scores obtained prior to ART initiation (baseline) and after 48 or 96 weeks of non-suppressive antiretroviral therapy; four of the 14 children analyzed did not have a 48-week result; the 96-week result was used for analysis for those four children.

[b]Wilcoxon signed rank test.