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Impact of Nucleoside Reverse Transcriptase Inhibitors on Mitochondria in Human Immunodeficiency Virus Type 1-Infected Children Receiving Highly Active Antiretroviral Therapy▽

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Mitochondrial toxicity induced by nucleoside reverse transcriptase inhibitors (NRTIs) has been reported to be responsible for various adverse effects. The relative impact of NRTIs on the mitochondria of human immunodeficiency virus (HIV) type 1 (HIV-1)-infected children receiving highly active antiretroviral therapy (HAART) is unknown. Mitochondrial DNA (mtDNA) levels were quantified longitudinally from peripheral blood mononuclear cells (PBMCs) in 31 HIV-1-infected children from Pediatric AIDS Clinical Trial Group Study 382 who were receiving HAART, including nelfinavir, efavirenz, and different NRTIs, and who had had undetectable plasma HIV-1 RNA levels for >2 years. The median mtDNA levels in PBMCs increased from 137 copies/cell at the baseline to 179 copies/cell at week 48 ($P = 0.01$) and 198 copies/cell at week 104 ($P < 0.001$). Before the initiation of HAART, children who received regimens containing didanosine had mtDNA levels persistently lower than those in children not receiving didanosine (106 versus 140 copies/cell; $P = 0.008$). During HAART, the median increase in the mtDNA level from the baseline to week 104 was the lowest in children who received regimens containing didanosine (+26 copies/cell) compared to those in children who received other regimens (+79 copies/cell) ($P = 0.02$). A multivariate analysis also demonstrated that didanosine, as part of HAART, was the only NRTI associated with the change in mtDNA levels ($P = 0.007$). Children receiving didanosine-containing antiretroviral regimens have the lowest mtDNA levels in PBMCs and may be at greater risk for long-term adverse effects due to mitochondrial toxicity. This may be of particular importance in resource-limited countries where didanosine is widely used for the treatment of HIV-infected children.

The morbidity and mortality associated with human immunodeficiency virus (HIV) type 1 (HIV-1) infection of children have improved dramatically with the availability of highly active antiretroviral therapy (HAART) (22, 47). Along with the clear benefits of HAART, important adverse effects of antiretrovirals are increasingly being recognized (5). In some patients, these side effects require the discontinuation or a change of the antiretroviral therapy.

In combination with protease inhibitors (PIs) or nonnucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs) remain the backbone for many HAART regimens. NRTIs have been shown to deplete mitochondrial DNA (mtDNA) by selectively inhibiting DNA polymerase γ (28, 30), which is crucial for the replication of mtDNA. The depletion of mtDNA, which causes mitochondrial dysfunction, is at least partly responsible for various NRTI-associated adverse effects (21).

The "gold standard" for the diagnosis of mitochondrial toxicity is examination of biopsy materials from muscle, liver, or nerve; however, the collection of these biopsy specimens is not practical, especially for vulnerable children. Peripheral blood mononuclear cells (PBMCs) are easily obtained from patients, and several reports have suggested clinical correlations between toxicity and the mtDNA levels in the PBMCs of HIV-infected adults receiving antiretroviral therapy (11, 17, 18, 31, 34). In contrast, no clinical correlations between the mtDNA levels in PBMCs and lipodystrophy, lactate levels, or the toxicities of antiretroviral regimens have been identified (9, 24, 32, 38, 41, 48). The clinical use of the assay for mtDNA levels in PBMCs is still controversial (3, 45); therefore, more research is required to elucidate the importance of the mtDNA levels in PBMCs in the clinical setting, especially for children for whom the levels of sample materials are limited.

Children may be more vulnerable than adults to the adverse effects of antiretrovirals because of the potential negative impact on growth and development with their long-term exposure (33); however, information regarding the frequency and severity of long-term adverse effects in children is limited. In addition, only one cross-sectional study that has examined mtDNA levels in the PBMCs of children receiving antiretroviral therapy is available (14). That study showed no difference in mitochondrial function and mitochondrial content in PBMCs between children with lipodystrophy and those without lipodystrophy.

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TABLE 1. NRTI regimens before and during the study

NRTI regimen before the study	No. (%) of children on the following NRTI regimen during the study:						
	Non-ddI-containing regimens			ddI-containing regimens			Total
	d4T	ZDV + 3TC	d4T + 3TC	ddI + d4T	ZDV + ddI	ddI + 3TC	
Non-ddI-containing regimen							
ZDV + 3TC	7 (23)	2 (7)[a]		4 (13)			13 (42)
d4T + 3TC	2 (7)		5 (16)[a]				7 (23)
ddI-containing regimens							
ZDV + ddI	1 (3)	2 (7)			2 (7)[a]		5 (16)
ddI + d4T		2 (7)					2 (7)
ddI	1 (3)		1 (3)				2 (7)
ddI + 3TC						1 (3)[a]	1 (3)
Treatment naïve		1 (3)					1 (3)
Total	11 (35)	7 (23)	6 (19)	4 (13)	2 (7)	1 (3)	31 (100)

[a] Children who did not change NRTI regimens before and during HAART (n = 10; 32%).

In order to optimize current and future antiretroviral therapies, there is an urgent need to determine not only which regimens provide sustained virologic and immunologic benefits but also those interventions that are the least likely to produce long-term toxicity. We investigated the effect of specific NRTIs given in combination with an NNRTI (efavirenz) and a PI (nelfinavir) on mtDNA levels in the PBMCs of a cohort of children who achieved sustained virologic suppression.

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MATERIALS AND METHODS

Subjects. Thirty-one children (median age, 5.6 years; age range, 3.2 to 16.8 years) who were a subset of Pediatric AIDS Clinical Trial Group (PACTG) Study 382, which consisted of treatment with efavirenz, nelfinavir, and one or two NRTIs, participated in this study. They were selected because they had achieved persistent and undetectable plasma HIV-1 RNA levels while receiving HAART for >2 years after the initiation of HAART (44). All 31 patients reached undetectable HIV-1 RNA levels (<50 copies/ml) by week 48 and continued to have undetectable HIV-1 RNA levels up to week 104. Before they were enrolled in the study, children were PI and NNRTI treatment naïve, but they could be NRTI experienced and were NRTI experienced as follows: 6% (n = 2) had received one NRTI and 91% (n = 28) had received two NRTIs. One child (3%) was antiretroviral naïve. The NRTI regimens that the children received during the study were either continued (32%; n = 10) or changed (68%; n = 21), based on the clinical judgment of the primary care physicians (Table 1). Informed consent was obtained from the study participants and their legal guardians. This study followed the human experimentation guidelines of the U.S. Department of Health and Human Services and the University of California San Diego review board.

Quantitation of mtDNA and nDNA by real-time PCR. mtDNA and nuclear DNA (nDNA) were quantified by real-time PCR with a LightCycler instrument (Roche Applied Science, Indianapolis, IN), as described previously (17). The results are expressed as the ratio of the mean mtDNA level (in numbers of copies per cell) to the mean nDNA level for a given extract, in duplicate. The ratios were quantified at the baseline and at weeks 2, 4, 8, 48, and 104 after the initiation of HAART.

Measurement of HIV-1 intracellular DNA, plasma HIV-1 RNA, and CD4$^+$ T-lymphocyte counts and percentages. HIV-1 intracellular DNA (HIV-1 DNA) and HIV-1 RNA levels were quantified by using the Amplicor Monitor HIV-1 DNA assay and the standard and ultrasensitive Amplicor HIV-1 Monitor assay (version 1.0) (12, 44, 46), respectively (Roche Molecular Systems, Alameda, CA). The absolute numbers and percentages of CD4$^+$ T lymphocytes were determined in PACTG-certified laboratories by flow cytometry.

Physical parameters, lipid profiles, and adverse effects. The following data were extracted from the database: (i) body weight and height at the baseline and at weeks 48 and 104, with body mass index (BMI) calculated as weight (kg)/(height [m])^2, and z scores were used to express the deviation in the standard deviation units, calculated to adjust for age, weight, height, and race/ethnicity; (ii) lipid profiles, including the fasting total cholesterol, triglyceride, low-density lipoprotein, and high-density lipoprotein levels measured at weeks 112 to 160 of HAART; and (iii) significant adverse effects (grade 3 or higher) related to NRTI use.

Statistical analysis. The Wilcoxon matched paired signed-rank test was used for comparisons within the subject over time. The Wilcoxon sum rank test was used for comparison of numerical variables in two independent groups, and the Kruskal-Wallis test was used for comparison of numerical variables in more than three independent groups. Correlations between mtDNA levels and the various laboratory findings were calculated by using Spearman's correlation test. Fisher's exact test was used to compare two groups with respect to categorical variables. The multivariate regression analyses on the change in mtDNA levels from the baseline to week 104 were performed to evaluate the contributions of covariates, including age, gender (male versus female), race/ethnicity (African American versus others), mtDNA levels at the baseline, and the use of didanosine (ddI) during HAART (ddI versus non-ddI). Of note, the values of the changes in mtDNA levels were log transformed before calculation of the differences because the distribution of the untransformed values was skewed to lower values. The same multivariate analyses on the change in mtDNA levels from the baseline to week 104 were also performed for the use of each NRTI, including stavudine (d4T; d4T versus non-d4T), zidovudine (ZDV; ZDV versus non-ZDV), or lamivudine (3TC; 3TC versus non-3TC), and other covariates.

RESULTS

Impacts of NRTI regimens on mtDNA levels before initiation of HAART. The children were divided into two groups on the basis of their use of ddI before enrolling the study: regimens containing no ddI (n = 20) and regimens containing ddI (n = 10) (Table 2). The median mtDNA level was significantly decreased in children who received regimens containing ddI (106 copies/cell; range, 76 to 176 copies/cell) compared with the level in those who did not receive ddI (140 copies/cell; range, 110 to 431 copies/cell) (P = 0.008). Notably, patients who received the ddI and d4T combination (n = 2) had the lowest mtDNA levels (76 and 92 copies/cell). Of note, the background characteristics among the two groups did not differ significantly by age (P = 0.98), sex (P = 0.76), race/ethnicity (P = 0.85), the duration of therapy (P = 0.40), plasma HIV-1 RNA levels (P = 0.17), or CD4$^+$ T-lymphocyte counts (P =

TABLE 2. mtDNA levels in subjects on the basis of the NRTI regimen before initiation of HAART

NRTI regimen	No. (%) of subjects	Median (range) mtDNA level (no. of copies/cell) at baseline	*P* value[a]	Median (range) duration (wk) of NRTI therapy	*P* value[b]
Non-ddI-containing regimens	20 (65)	140 (110–431)	0.008	57 (8–285)	0.40
ZDV + 3TC	13 (42)	170 (116–238)		79 (23–285)	
d4T + 3TC	7 (23)	127 (110–431)		53 (8–101)	
ddI-containing regimens	10 (32)	106 (76–176)		85 (7–274)	
ZDV + ddI	5 (16)	104 (76–124)		103 (7–274)	
ddI + d4T	2 (7)	85 (78–92)		63 (52–73)	
ddI alone	2 (7)	157 (137–176)		79 (24–138)	
ddI + 3TC	1 (3)	149		49	
Treatment naïve	1 (3)	137		0	

[a] Comparison of the mtDNA levels between children with non-ddI-containing regimens and those with ddI-containing regimens before the initiation of HAART.
[b] Comparison of the duration of therapy between children with non-ddI-containing regimens and those with ddI-containing regimens before the initiation of HAART.

0.20). In addition, the distribution of the NRTI switch (ddI-containing regimens versus non-ddI-containing regimens) before and after the study was not significantly different ($P = 0.68$) (Table 1). No difference in mtDNA levels were observed at the baseline between children who received ZDV and those who did not receive ZDV ($P = 0.51$) or between children who received d4T and those who did not receive d4T ($P = 0.16$) before HAART; however, children who received 3TC ($n = 21$) had higher mtDNA levels than those who did not receive 3TC ($n = 10$) ($P = 0.003$). Of note, among the 21 patients who received 3TC, only 1 patient (4.8%) received ddI, reflecting the difference in the mtDNA levels in children who did or who did not receive ddI and not a specific beneficial effect of 3TC.

mtDNA levels increase during HAART in children with sustained virologic suppression. Overall, the median mtDNA levels increased from 137 copies/cell (interquartile range [IQR], 115 to 171 copies/cell) at the baseline to 179 copies/cell (IQR, 145 to 209 copies/cell) at week 48 ($P = 0.01$) and 198 copies/cell (IQR, 166 to 290 copies/cell) at week 104 ($P < 0.001$).

Changes in mtDNA levels in children receiving ddI in combination with HAART regimens are least increased compared to those in children receiving other NRTIs. We next examined the data on the basis of the use of ddI during HAART: (i) children who received ddI ($n = 7$) and (ii) children who did not receive ddI ($n = 24$). The background characteristics between the two groups were similar by age ($P = 0.60$), gender ($P = 0.56$), race/ethnicity ($P = 0.20$), HIV-1 RNA levels ($P = 0.27$), $CD4^+$ T-cell counts ($P = 0.23$), and mtDNA levels at the baseline ($P = 0.70$). The median changes in mtDNA levels from the baseline to week 48 in children receiving ddI (+16 copies/cell) was lower than those in children not receiving ddI (+57 copies/cell) ($P = 0.20$). Furthermore, the median changes in mtDNA levels from the baseline to week 104 in children receiving ddI were significantly lower (+26 copies/cell) than those in children not receiving ddI (+79 copies/cell) ($P = 0.02$) (Fig. 1).

When children were divided on the basis of the use of ZDV, 3TC, and d4T during HAART, we found no significant difference in the changes in mtDNA levels from the baseline to week 48 ($P = 0.14$ to 0.65). However, at week 104, children receiving ZDV (+101 copies/cell) had the highest increases in mtDNA levels compared to the levels in those not receiving ZDV (+52 copies/cell) ($P = 0.03$) (Fig. 1). No difference in mtDNA levels was observed in children receiving 3TC and those not receiving 3TC (+85 copies/cell versus +51 copies/cell; $P = 0.30$) (Fig. 1). In contrast, children receiving d4T (+51 copies/cell) experienced marginally lower mtDNA increases compared to those in children not receiving d4T (+98 copies/cell) ($P = 0.06$) (Fig. 1).

We also analyzed the data on the basis of the combination of the NRTIs that were part of a child's HAART regimen. The subjects were divided into four groups: (i) those receiving d4T, (ii) those receiving ZDV plus 3TC, (iii) those receiving d4T plus 3TC, and (iv) those receiving ddI-containing regimens. Although no significant differences in the change in mtDNA levels were observed from the baseline to week 48 ($P = 0.40$), at week 104, the changes in mtDNA levels differed significantly between children who received ZDV plus 3TC (+127 copies/cell; $n = 7$) and those who received ddI-containing regimens

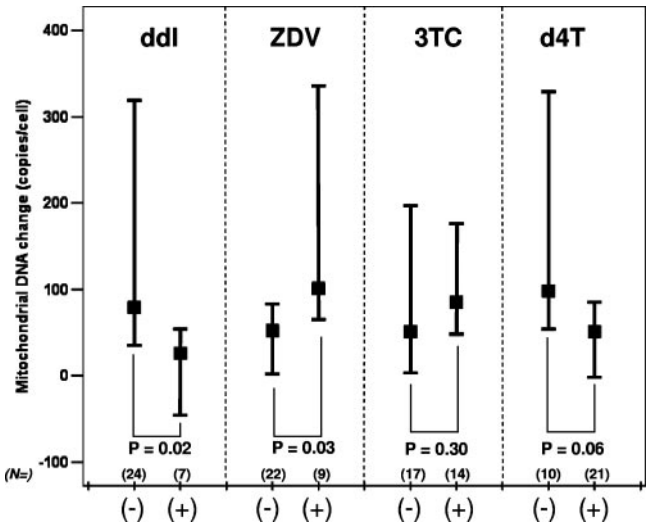

FIG. 1. Changes in mtDNA levels from the baseline to week 104 between children who received specified NRTI and those who did not receive specified NRTI during HAART. Squares, median values of the changes in mtDNA levels from the baseline to week 104; bars, IQRs of changes in mtDNA levels from the baseline to week 104. The Wilcoxon sum rank test was used to compare the mtDNA levels between the two groups.

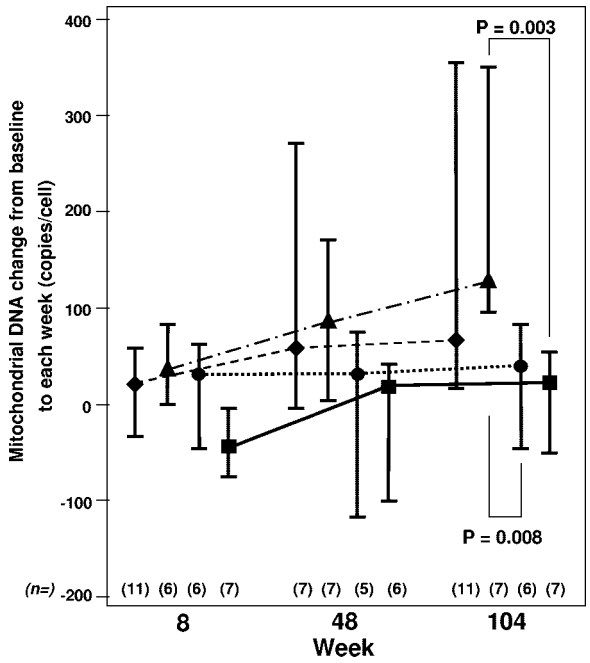

FIG. 2. Changes in mtDNA levels from the baseline to weeks 8, 48, and 104 on the basis of the NRTI regimens received during HAART. The different symbols indicate the median values of the changes in mtDNA levels from the baseline to each week in children who received (i) d4T (◆), (ii) ZDV plus 3TC (▲), (iii) d4T plus 3TC (●), and (iv) ddI-containing regimens (■). Bars indicate the IQRs of changes in mtDNA levels from the baseline to each week. The Wilcoxon sum rank test was used to compare the mtDNA levels in two groups.

(+26 copies/cell; $P = 0.003$; $n = 7$) or d4T plus 3TC (+43 copies/cell; $P = 0.008$; $n = 6$) (Fig. 2).

In multivariate analyses, ddI remains an important predictor of change in mtDNA levels from the baseline to week 104. Multivariate regression analyses were performed to assess the impact of ddI on the change in mtDNA levels from the baseline to week 104. The use of ddI during HAART ($P = 0.007$) and baseline mtDNA levels ($P < 0.001$) were independently associated with a change in mtDNA levels from the baseline to week 104 when other factors were controlled for, including age, sex, and race/ethnicity (Table 3). The use of ZDV, 3TC, or d4T was not associated with a change in mtDNA levels from the baseline to week 104 ($P = 0.32$, $P = 0.40$, and $P = 0.27$, respectively). Thus, the use of ddI during HAART was an important determinant of changes in mtDNA levels after other factors were controlled for.

Virologic and immunologic responses between children who received ddI as a component of HAART and those who did not receive ddI. The proportions of children who reached HIV-1 RNA levels <50 copies/ml within 8 weeks of HAART were similar for children who received ddI (57%) and those who did not receive ddI (50%) ($P = 0.78$). The median $CD4^+$ T-cell counts in children receiving ddI tended to be lower than those in children not receiving ddI during HAART at week 48 (613 and 1,054/μl, respectively; $P = 0.04$) and week 104 (751 and 1,075/μl, respectively; $P = 0.12$).

mtDNA levels and intracellular HIV-1 DNA levels during early stage of HAART. Overall, there was a modest negative correlation between the mtDNA levels and the HIV-1 DNA levels ($r = -0.29$; $P < 0.001$; $n = 173$). When the correlation was evaluated at each week, no correlation was seen at the baseline ($r = -0.10$; $P = 0.60$); however, there were significant negative correlations between the mtDNA levels and the HIV-1 DNA levels at week 2 ($r = -0.44$; $P = 0.02$) and week 4 ($r = -0.43$; $P = 0.02$). No correlations were seen during the rest of the study visits, at week 8 ($r = 0.19$; $P = 0.32$), week 48 ($r = -0.19$; $P = 0.35$), and week 104 ($r = -0.25$; $P = 0.19$).

mtDNA levels and plasma HIV-1 RNA levels and $CD4^+$ T-cell counts. Although HIV-1 infection itself appears to lower the mtDNA contents of PBMCs in antiretroviral agent-naïve patients (6, 11, 36), in our study, no correlations were observed between the mtDNA and the HIV-1 RNA levels at the baseline and weeks 2 and 4 ($r = -0.29$ to -0.34; $P = 0.06$ to 0.91) when the majority of subjects had detectable plasma HIV-1 RNA. In addition, no correlations were observed between the mtDNA levels and the $CD4^+$ T-cell counts at any study visit ($P = 0.06$ to 0.64).

mtDNA levels and physical parameters, lipid profiles, adverse effects, and age. No significant correlations were seen between the mtDNA levels and (i) the changes in BMIs ($P = 0.84$) or z scores ($P = 0.24$) at week 48 and the changes in BMIs ($P = 0.24$) or z scores ($P = 0.68$) at week 104 and (ii) fasting lipid profiles, including the cholesterol ($P = 0.15$), triglyceride ($P = 0.68$), high-density lipoprotein ($P = 0.87$), and low-density lipoprotein ($P = 0.06$) levels at week 104. Six adverse events in five children were reported as grade 3 or greater, including neutropenia ($n = 3$), hepatitis ($n = 2$), and thrombocytopenia ($n = 1$), during HAART. The mtDNA levels in children who experienced adverse effects were not different from the ones in children who did not experience the adverse effects each week ($P = 0.33$ to 0.74). Furthermore, no correlations were observed between age and the mtDNA levels at the baseline ($P = 0.15$), the changes in mtDNA levels from the baseline to week 48 ($P = 0.67$), and the changes in mtDNA levels from the baseline to week 104 ($P = 0.87$).

DISCUSSION

We have shown that ddI-containing HAART regimens are associated with the greatest degree of mtDNA suppression in PBMCs compared to those achieved by regimens not containing ddI in HIV-1-infected children who sustained undetectable HIV-1 RNA levels during HAART.

To our knowledge, the current study is the first longitudinal evaluation of mtDNA levels in the PBMCs of HIV-1-infected

TABLE 3. Multivariate analyses of mtDNA changes from the baseline to week 104

Covariate	Regression coefficient ± SE[a]	P value
Constant	2.3000 ± 0.5491	0.0003
Age (yr)	0.0071 ± 0.0117	0.5509
Sex (male)	0.0403 ± 0.0811	0.6235
Race (African American)	0.1406 ± 0.0793	0.0884
Baseline mtDNA (log copies/cell)	−1.0121 ± 0.2534	0.0005
ddI use	−0.2782 ± 0.0946	0.0070

[a] SE, standard error.

children receiving HAART. We demonstrated a gradual increase in mtDNA levels in the PBMCs of children who sustained undetectable HIV-1 RNA levels during HAART, which is also supported by the data achieved in studies with adults (8). Our current study was able to control for several important factors compared to those used in other longitudinal studies (17, 24, 34, 38, 41, 48) and cross-sectional studies (9, 11, 15, 18, 32) with adults that could potentially affect the mtDNA levels in PBMCs. First, we selected children with sustained undetectable HIV-1 RNA levels during HAART, decreasing the likelihood that patients with detectable HIV-1 RNA can significantly reduce the mtDNA content of PBMCs (6, 11, 36). Second, although the majority of previous studies have shown the lack of an association between the mtDNA levels in PBMCs and lipodystrophy (9, 11, 14, 24, 32, 38, 41, 48), the children in our study did not have obvious symptoms or signs of lipodystrophy. Third, among the previously published studies and this study, our study had the longest period of observation of patients receiving HAART (up to 104 weeks). Finally, although the effects of PIs and NNRTIs combined with NRTIs on the mtDNA levels in PBMCs are unknown, our cohort of children received the same combination of a PI (nelfinavir) and an NNRTI (efavirenz), with only the NRTI being different. Thus, having controlled for the PI and NNRTI treatment background, we were able to differentiate the effects of specific NRTIs on mtDNA levels.

Clinically, d4T and ddI are known to be associated with higher incidences of adverse effects in HIV-1-infected patients receiving antiretroviral therapy. Cross-sectional studies with adults have suggested that subjects receiving d4T in combination with ddI experience low mtDNA levels in PBMCs compared to the levels in those receiving other regimens (11, 18). This clinical observation is supported by in vitro studies illustrating that the inhibition of DNA polymerase γ in association with mitochondrial toxicity is differentially affected by NRTIs: d4T = ddI $\gg$ 3TC > tenofovir > ZDV (25, 28). In our current study with children, ddI was the most important determinant of mitochondrial toxicity, as defined by the lowest mtDNA levels before and during HAART. These findings were also confirmed by multivariate analyses that controlled for other confounding factors, including d4T use.

In contrast, the mtDNA levels in children receiving a ZDV-containing regimen (ZDV plus 3TC) were significantly increased compared to those in children receiving other NRTI regimens. Similarly, although the duration of therapy is only 6 weeks for infants receiving ZDV to prevent the mother-to-child transmission of HIV-1, mtDNA levels have been observed to increase in the PBMCs of infants who are exposed to ZDV perinatally (1, 16). The mechanisms of increase in mtDNA levels are still unknown, but our in vitro data indicate that the mtDNA levels in human skeletal muscle myoblasts treated with ZDV show the highest increase compared to those in untreated cells or cells treated with different NRTIs when the cells were incubated for 2 days (43). These findings are also consistent with the increase in mtDNA levels observed with ZDV in other in vitro models, including human hepatoblastoma (HepG2) cells (20) and human skeletal muscle cells (2). In total, these results strongly suggest that ZDV upregulates genes encoding mtDNA. Because ZDV has been reported to cause significant mitochondrial toxicity in different in vitro models (23), this may reflect a compensatory response to mitochondrial dysfunction caused by a different pathway. Importantly, an increase in mtDNA does not necessarily mean an increase in mitochondrial function; several studies have shown inhibition of the respiratory function by NRTIs independently through mtDNA polymerase γ (29), oxidative stress (26), and increases in mtDNA levels and mitochondrial mass by oxidative stress (27, 51). In addition, treatment with NRTIs, including 3TC, abacavir, emtricitabine, and tenofovir, which are known to be less toxic to mitochondria, have been shown to result in increases in mtDNA levels in HepG2 cells (20), human skeletal muscle cells (2), and lymphoblasts (40). Further studies are needed to delineate the mechanisms associated with changes in mtDNA levels by NRTIs and mitochondrial function and the clinical significance of an increases in PBMC mtDNA levels.

In a recent study with adults, d4T and ddI use was associated with fat mtDNA depletion, whereas ddI exposure was the only observed association with mtDNA depletion in PBMCs (10). In an in vitro model, the thymidine analogues, including d4T and ZDV, but not ddI, decreased mitochondrial activity and cell survival in adipose tissue (4). In contrast, ddI significantly decreased the mtDNA content in neuronal cells (19), human hepatoma cell lines (37, 50), and human skeletal muscle cells (2), whereas no significant change in mtDNA levels was observed with d4T, ZDV, or 3TC. In our preliminary studies with human skeletal muscle myoblasts, ddI significantly decreased mtDNA levels as well as mtRNA levels (43). In summary, the currently available data show that the effects of NRTIs on mitochondria seem to differ in different tissues. Therefore, the evaluation of mtDNA in future studies should include the possible anatomical sites where adverse effects are potentially the greatest.

Our findings also showed a negative correlation between mtDNA and HIV-1 DNA levels in PBMCs during the early stages of HAART. Although our data clearly showed that ddI-containing regimens can alter mtDNA levels, the negative effect of ddI may be masked by the beneficial effect of decreasing intracellular virus levels during the early stage of HAART. Our finding that the mtDNA levels no longer correlated with the HIV-1 DNA levels during prolonged HAART suggests that at steady state the use of a specific NRTI has a greater impact on the mtDNA levels than relatively small differences in HIV-1 DNA levels. Interestingly, the correlations between the mtDNA and the HIV-1 DNA levels were not observed at the baseline, which likely reflects the failure of mono-NRTI or dual NRTI therapy to suppress viral replication in the study participants.

Although improved growth is usually associated with effective HAART in HIV-1-infected children (35, 49), the changes in the mtDNA levels in PBMCs did not correlate with changes in BMIs or *z* scores, suggesting that the degree of mitochondrial toxicity did not affect the growth of the children over the 104 weeks of effective HAART. However, no measurement of peripheral or visceral fat was performed to determine the presence or absence of lipodystrophy in this study; therefore, the ability of the mtDNA levels in PBMCs to predict lipodystrophy was not possible.

We appreciate that there are several potential limitations of the current study. First, the number of children evaluated was

relatively small; a larger cohort may help to define the impact of low mtDNA levels on HIV-infected children. Second, we quantified the mtDNA levels in PBMCs containing different cells at different stages of activation, during which it has been reported that the levels of mtDNA change (7). Third, it has been suggested that platelet contamination of PBMCs could be a potential factor confounding mtDNA levels (13), and this possibility was not investigated in the current study. Finally, although we showed that ddI significantly affects the mtDNA levels in PBMCs in children, it is still unknown how well the mtDNA levels in PBMCs reflect the mtDNA levels at other anatomical sites in vivo. In addition, information regarding the mtDNA levels in PBMCs in healthy children is limited (16, 42). Thus, future research should focus on the impact of mtDNA levels in PBMCs on physical growth, peripheral or visceral fat levels, muscle mass, toxicity, and neurodevelopmental progress in children.

In conclusion, we have shown that ddI has a significant impact on the mtDNA levels found in the PBMCs of children with sustained virologic suppression during HAART. Our findings that ddI is associated with the greatest suppression of mtDNA are of particular importance in resource-limited countries where ddI is a common component of antiretroviral therapy (39, 52). A prospective study is urgently needed to elucidate the long-term clinical significance of mtDNA levels in children and the NRTI regimens causing the least mitochondrial toxicity.

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