Perinatal Exposure to Antiretroviral Therapy Is Associated with Increased Blood Mitochondrial DNA Levels and Decreased Mitochondrial Gene Expression in Infants

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Background. The effects of perinatal antiretroviral therapy (ART) on infant mitochondrial function are not well known. We compared blood mitochondrial DNA (mtDNA) levels and mtDNA gene expression (mtRNA) in human immunodeficiency virus (HIV)–uninfected, ART-exposed infants born to HIV-positive mothers with mtDNA levels and mtDNA gene expression in control infants born to uninfected women.

Methods. In this prospective cohort study, longitudinal mtDNA:nuclear DNA and mtRNA:β-actin mRNA ratios were compared in blood samples obtained at various time points from birth to 8 months, using generalized estimating equation linear regression models.

Results. Log_{10} mtDNA levels at birth were higher in ART-exposed infants, compared with levels in control infants, although the difference did not reach statistical significance ($P = .07$ for comparison of samples obtained 0–3 days after birth). ART-exposed infants' mtDNA levels increased further during the zidovudine prophylaxis period—from age 4 days to age 6 weeks—($P = .001$) and remained significantly higher than the levels observed in control infants until the end of the study. In contrast, log_{10} mtRNA levels at birth were lower in ART-exposed infants than in control infants ($P = .03$), but were not statistically different later.

Conclusions. When control infants and ART-exposed infants were compared, the mtDNA level was increased but mitochondrial gene expression was decreased in ART-exposed infants. These differences persisted after zidovudine was discontinued, suggesting that changes in mitochondrial proliferation and/or expression take place during and after ART exposure. These changes are likely the effects of the antiretroviral drugs on mitochondria. The clinical relevance and long-term impact of these alterations must be studied.

The use of highly active antiretroviral therapy (HAART) during pregnancy and antiretroviral therapy (ART) during delivery and shortly after birth, have successfully reduced the risk of transplacental and perinatal mother-to-child transmission (MTCT) of HIV to less than 2% [1]. This approach may result in up to 40 weeks of in utero exposure to antiretrovirals, including nucleoside reverse transcriptase inhibitors (NRTIs) such as zidovudine (ZDV), which can cross the placenta and accumulate in amniotic fluid [2, 3]. Given that NRTIs are known

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to cause mitochondrial toxicity, there is growing concern about the short- and long-term effect of perinatal exposure to NRTIs for infants who are not HIV infected [4, 5].

Although the benefit of ART for the prevention of MTCT remains undeniable, concerns have been raised about potential long-term mitochondrial dysfunction [4–9]. In animals, transplacental and perinatal exposure to NRTIs can induce genotoxicity [10], mitochondrial DNA (mtDNA) damage and depletion [11–14], and increased DNA oxidative damage [15] in various tissues. For human infants exposed to NRTI perinatally, the literature is scarce and inconsistent. Clinical signs suggestive of mitochondrial toxicity have included hyperlactatemia [16, 17], long-lasting alterations of hematological parameters [18–20], and abnormal brain imaging [21]. Small studies have found no evidence of serious adverse effects [22] or altered neurodevelopment [23] in HIV-uninfected children who were exposed to ART perinatally. Furthermore, a large US observational cohort of children exposed to ART ($N = 5468$) did not attribute any death to mitochondrial dysfunction [24], and a follow-up study on children from the AIDS Clinical Trials Group 076 trial ($N = 324$) detected no statistically significant difference between ZDV-exposed and placebo groups with respect to growth, neurologic parameters, or neurodevelopmental parameters [22]. However, other cohort studies have recently presented evidence of an unusually high incidence of mitochondrial dysfunction phenotype in children exposed to ART perinatally [25–28].

At the cellular and molecular level, NRTI exposure during pregnancy has been associated with abnormal cord mitochondrial morphology and mtDNA depletion in cord cells [29], cord blood, and the placenta [30], whereas for peripheral blood cell mtDNA, results have been inconsistent [27, 31]. The goal of this study was to carry out a prospective cohort study of longitudinally sampled levels of blood mtDNA and mtDNA gene expression (mtRNA) in HIV- and ART-exposed infants, and compare them to those of control infants.

SUBJECTS, MATERIALS, AND METHODS

Study design and population. Infants were enrolled from 2 Canadian sites (Vancouver and Toronto). ART-exposed infants were eligible for inclusion in the study if they satisfied both of the following criteria: (1) they were born to an HIV-infected woman who received HAART during pregnancy and intravenous ZDV during labor, and (2) they received oral ZDV prophylaxis during the first 6 weeks of life starting within ~12 hours of birth, as per Canadian guidelines [32]. Control infants were born to HIV-uninfected mothers and enrolled from 3 sources: (1) infants (1–6 months old) having blood work done prior to elective minor pediatric surgery, (2) neonates (0–3 days old) born at Children's and Women's Health Centre of British Columbia, and (3) infants (1 day–8 months old) having blood work done for various minor medical reasons. Controls were excluded if they were known to have a mitochondrial disorder or had a serious and/or febrile illness. Voluntary written informed consent was obtained from mothers or guardians for all study and control infants. All procedures were approved by the Research Ethics Boards of the University of British Columbia and the Hospital for Sick Children in Toronto, as well as by the Hospital Research Review Committee of the Children's and Women's Health Centre of British Columbia.

Sample collection. All blood samples were collected in EDTA and frozen rapidly, without processing. For neonates, heel-stick blood samples (~0.5 mL) were collected at the time of phenylketonuria testing. Beyond the neonatal period, blood samples were collected at approximately 4, 8, 12, and 26 weeks of age. Serial samples were collected from ART-exposed infants concomitantly with the performance of routine clinical lab tests. For ethical reasons, control infants provided a single blood sample each.

Routine laboratory testing. Routine laboratory testing of ART-exposed infants included complete blood counts as well as evaluation of serum lactate and alanine aminotransferase levels.

mtDNA assay. DNA was extracted from 0.1 mL of whole blood using the QiaAmp DNA kit (Qiagen). A mitochondrial gene (cytochrome C oxidase subunit I [CCOI]) and a nuclear gene (accessory subunit of the polymerase γ [ASPG]) were quantified by real-time polymerase chain reaction (PCR) on a LightCycler 480 (Roche), by use of the Faststart probe master mix (Roche Diagnostics). A standard curve was built by serial dilution of a plasmid containing the 2 genes of interest. The probes and primers are described elsewhere [33], and the PCR conditions were as follows: 95°C for 10 min followed by 45 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 5 s. mtDNA levels were expressed as the ratio of the number of copies of CCOI to the number of copies of ASPG, and the assay showed a coefficient of variation (CV) of <15%.

mtRNA assay. Only samples frozen and stored at −80°C were used for the mtRNA analyses. Whole blood was thawed rapidly, and 0.25 mL was immediately mixed with 0.75 mL of Trizol (Invitrogen). Total RNA was precipitated according to the manufacturer's protocol; the pellet was resuspended in 0.2 mL of lysis buffer and applied to an RNeasy column (Qiagen). The RNA was treated with DNAse (Qiagen) directly on the column and eluted in 20 μL of water. Randomly-primed (N_6) cDNA was then prepared from 5 μL of RNA using Expand reverse transcriptase (Roche). The absence of DNA in the RNA preparation was confirmed and the cDNA was subjected to real-time PCR on a LightCycler 480 to quantify mitochondrial mRNA (CCOI) and a housekeeping mRNA (β-actin). The primers and probes used for CCOI are described elsewhere [33]. For β-actin, BACTF (5' TCCTATGTGGGCGACGAGG 3') and BACTR (5' GGTGTTGAAGGTCTCAAACATG 3') were used with the following probes (5' CCCRTGCTGCTGACCRAGGCC-F 3') and (5' LC_{640}- CCTGAACCCCAAGGCCAACCGY-P 3') (Sigma-Proligo). A standard curve was built as de-

scribed above, and the β-actin reaction conditions were as before, both genes showing identical PCR efficiencies. mtRNA levels were expressed as the ratio of CCOI mRNA to β-actin mRNA and the assay CV was <15%.

For both mtDNA and mtRNA determination, all samples from a given study infant were extracted and assayed together, randomized with control samples.

Statistical analysis. For statistical analyses, all mtDNA and mtRNA values were $\log_{10}$ transformed. Analyses of variance were used to compare mtDNA and mtRNA among ethnic groups within exposure categories. Generalized estimating equation (GEE) linear regression models were used to examine the effect of ART exposure in utero on mtDNA and mtRNA levels while controlling for correlation among repeated measurements for the ART-exposed infants and potential confounding of other covariates [34]. All available mtDNA and mtRNA values were included in the GEE models.

Comparisons of demographic characteristics and laboratory values for the ART-exposed and unexposed groups were done using the Kruskal-Wallis and Mann-Whitney tests (2-tailed) for continuous variables and the χ^2 test for categorical variables. Spearman's correlation was used to investigate the relationships between the $\log_{10}$ mtDNA and $\log_{10}$ mtRNA ratios and in utero ART exposure, infant platelet count, and infant lactate level.

RESULTS

Study populations. A total of 73 ART-exposed infants and 81 control infants born between July 2003 and June 2006 were eligible for the study (41 ART-exposed infants and 16 control infants in Toronto and 32 ART-exposed infants and 65 control infants in Vancouver) . A total of 87 control infants were initially enrolled, but 6 were excluded because they had serious or febrile illnesses with grade 3 and/or 4 abnormal laboratory values (as per the grading criteria of the National Institute of Allergy and Infectious Diseases, Division of AIDS [35]). All ART-exposed infants had at least 1 sample collected during the first 8 months of life. Birth samples (obtained 0–3 days after birth) were collected primarily at the Vancouver site (table 1).

Demographic characteristics are presented in table 1. The groups were similar with respect to age, sex, gestational age at delivery, birth weight, and Apgar scores. For the subgroup of infants with a birth sample (0–3 days), gestational age was less, birth weight was lower, and Apgar score tended to be lower in the ART-exposed group, compared with control infants (table 1). Maternal cigarette smoking, marijuana use, and use of drugs of addiction during pregnancy also tended to be higher in the ART-exposed group. In addition, mothers of ART-exposed infants were more likely to be of Aboriginal or black–African-Canadian ethnicity.

Exposure to antiretroviral therapy during pregnancy and the postnatal period. All 73 HIV-infected pregnant women received combination ART during pregnancy; 16 received therapy from the time of conception, 11 started therapy in the first trimester of pregnancy (0–13 weeks), 35 started in the second trimester (>13–26 weeks), and 11 in the third trimester (>26 weeks). The median duration of in utero exposure to ART was 22.3 weeks (interquartile range, 16.3–28.6 weeks). If a regimen was changed early in pregnancy, the later regimen was used for analysis. The majority of HIV-infected pregnant women received ZDV+3TC, with a protease inhibitor ($n = 41$), a non-NRTI ($n = 20$), or both ($n = 4$). There were 4 women who received nucleoside analogue–only regimens or nucleotide analogue–only regimens, and 11 received a regimen that contained stavudine (d4T) or didanosine (ddI) (none took d4T and ddI combined). All infants born to HIV-infected mothers received ZDV prophylaxis; 18 infants (25%) stopped prophylaxis early (on average, after 4.2 weeks) as a result of abnormal laboratory values indicative of conditions such as anemia, neutropenia, or hyperlactatemia.

Clinical outcome. There were no cases of HIV transmission among the study infants. None of the infants demonstrated long-term clinical symptoms suggestive of mitochondrial dysfunction, and no deaths occurred during the study period.

Abnormal laboratory values. The abnormal values observed during routine laboratory testing are presented in table 1. Ninety-seven percent of ART-exposed infants had at least 1 laboratory test result outside the normal range, although the majority of these results were only mildly abnormal. These included neutropenia (20 infants [27%] total; grade of value not reported), anemia (41 infants [56%] total; 6 infants [8%] had grade 2 values and 7 [9%] had grade 3 values), and hyperlactatemia (65 infants [89%] total; 14 [19%] had grade 2 values and 3 [4%] had grade 3 values) [35]. There were no grade 4 abnormal laboratory values.

mtDNA and mtRNA level. A total of 319 blood samples from 73 ART-exposed infants and 81 control infants were analyzed for mtDNA content, and 197 were analyzed for mtRNA level. Regarding the latter, 15 were below the β-actin standard curve, so that a total of 182 samples from 32 study and 64 control infants were ultimately analyzed for mtRNA content. For the GEE modeling, samples were grouped into 4 time periods according to infant age at the time of blood sampling: early neonatal period (0–3 days), ZDV prophylaxis period (4 days-6 weeks), 6 to 16 weeks, and >16 weeks to~8 months. Figure 1*A* and 1*B* show the mean $\log_{10}$ mtDNA and mtRNA values for each age group. In accordance with the study design, these values were measured repeatedly for ART-exposed infants and a single time for control infants.

GEE linear regression models (table 2) showed that during the 6–16 weeks period, blood mtDNA levels in control infants were significantly higher than the levels at birth. When ART-exposed and control infants were compared at birth (0–3 days) blood mtDNA levels were not quite statistically different ($P = .07$),

Table 1. Demographic characteristics, clinical characteristics, and laboratory values for HIV-infected mothers and their antiretroviral therapy (ART)–exposed infants, as well as control infants born to HIV-uninfected mothers.

Characteristic or value	All infants			Infants for whom a birth blood sample was available[a]		
	ART-exposed (n = 73)	Control (n = 81)	P	ART-exposed (n = 26)	Control (n = 27)	P
Male sex	35 (48)	48 (59)	.16	13 (50)	14 (52)	.89
Gestation time, weeks	38 (38–40)	39 (37–40)	.64	38 (38–40)	40 (39–41)	.02
Birth weight, kg	3.06 (2.72–3.41)	3.21 (2.69–3.58)	.5	3.01 (2.72–3.47)	3.59 (3.29–3.92)	.001
Apgar score at 1 min	9 (8–9)	9 (8–9)	.64	8 (8–9)	9 (8–9)	.07
Apgar score at 5 min	9 (9–9)	9 (9–9)	.54	9 (9–9)	9 (9–9)	.71
Mother coinfected with HBV or HCV	10 (14)	. . .		5 (19)	. . .	
Maternal ethnicity[b]			<.001			NT
Aboriginal, First Nations, Metis, or Inuit	8 (11)	0 (0)		5 (19)	0 (0)	
White	18 (25)	44 (54)		9 (35)	13 (48)	
Black–African Canadian	34 (47)	0 (0)		9 (35)	0 (0)	
Hispanic	4 (5.5)	5 (6.2)		0 (0)	1 (3.7)	
Asian	1 (1.4)	13 (16)		1 (3.8)	4 (15)	
South, East, and Western Asian	6 (8.2)	6 (7.4)		2 (7.7)	2 (7.4)	
Other or missing	2 (2.7)	13 (16)		0 (0)	7 (26)	
Maternal use or consumption during pregnancy[b]						
Prescription drugs	0 (0–3)	0 (0–2)	.90	3 (0–5)	4 (1–6)	.24
Alcohol	10 (14)	7 (9)	.32	6 (32)	1 (3.7)	.05
Cigarettes	17 (23)	6 (7)	.01	11 (42)	2 (7)	.003
Marijuana	8 (11)	0 (0)	.003	4 (15)	0 (0)	.05
Drugs of addiction						
Any	7 (10)	1 (1.3)	.03	4 (15)	0 (0)	.05
Cocaine	4 (5.3)	1 (1.3)	.20	2 (7.7)	0 (0)	.24
Crack	2 (2.7)	0 (0)	.23	1 (3.8)	0 (0)	.49
Heroin	2 (2.7)	0 (0)	.23	1 (3.8)	0 (0)	.49
LSD	0 (0)	0 (0)	. . .	0 (0)	0 (0)	. . .
Methadone	4 (5.3)	0 (0)	. . .	2 (7.7)	0 (0)	.24
Antiretroviral exposure						
Duration of maternal ART during pregnancy, weeks	22.3 (16.3–28.6)	NA		19.5 (12.7–31.4)	NA	
Duration of infant ZDV prophylaxis, weeks	6.0 (5.7–6.0)	NA		5.5 (4.1–6.0)	NA	
Infant stopped ZDV prophylaxis <6 weeks after birth	18 (25)	NA		13 (50)	NA	
Abnormal infant laboratory values						
Neutropenia, WBC count <LLN	20 (27)	. . .		6 (23)	. . .	
Anemia Hgb <LLN	41 (56)	. . .		8 (31)	. . .	
Hyperlactatemia at any age, lactate level >ULN	65 (89)	8 (50)[c]		24 (92)	. . .	
Lactate level at 1 month ± 2 weeks of age	2.8 (2.0–4.0)	. . .		3.0 (2.5–4.5)	. . .	

NOTE. Data are no. (%) of subjects or median (interquartile range). Hgb, hemoglobin; LLN, lower limit of normal; LSD, lysergic acid diethylamide; NA, not applicable; NT, not tested (number of events too small for a statistical test); ULN, upper limit of normal; WBC, white blood cells; ZDV, zidovudine.

[a] Birth blood samples were collected 0–3 days after birth.

[b] Fisher's exact test was used when there were fewer than 5 subjects in a given category.

[c] Only 16 control infants had lactate levels evaluated.

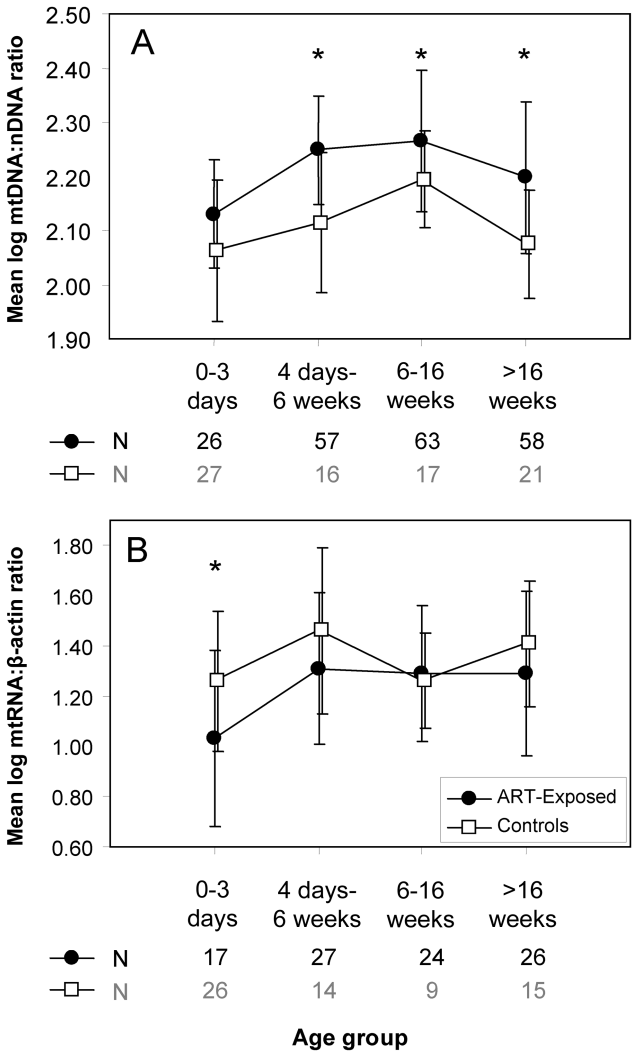

Figure 1. Mean longitudinal $\log_{10}$-transformed ratio of mtDNA to nDNA *(A)* and mtRNA to β-actin *(B)* divided over 4 time periods: 0–3 days (birth), 4 days–6 weeks (approximate zidovudine prophylaxis period), 6–16 weeks, and >16 weeks. The bars represent the standard deviation. The number of infants included in each period is indicated under the graph. *P<.05, for comparison of study and control groups.

but the levels in ART-exposed infants became significantly higher during the 4 days-6 weeks period ($P = .001$) and remained so during the 6–16 weeks period ($P = .01$) and the 16 weeks to ~8 months period ($P < .001$). When mothers who used heroin, cocaine, crack, or methadone during their pregnancy (7 study subjects and 1 control subject) were excluded from the GEE model, the difference between exposed and control infants with respect to the mtDNA levels at birth also became statistically significant ($P = .05$), although the differences were unchanged for the other time periods.

Overall mtDNA values were lower for ART-exposed infants from the Vancouver site, compared with the values observed at lethe Toronto site, possibly because 14 of 17 infants who stopped ZDV prophylaxis early were from the Vancouver site. Although the 2 groups were unbalanced with respect to ethnicity, there was no statistical difference in mtDNA or mtRNA values among racial subgroups within the study group or the control group, so ethnicity did not confound the relationship between ART exposure and mtDNA level. After adjusting for research site, length of in utero exposure to ART (all regimens included), maternal infection with hepatitis C virus (HCV) or hepatitis B virus (HBV), birth weight, and gestational age at delivery were not associated with higher mtDNA level. Smoking during pregnancy was associated with higher mtDNA level ($\beta = 0.05$; $P = .01$) but this association was not seen after adjusting for ART exposure (study vs. control group; $\beta = 0.01$; $P = .57$).

GEE modeling showed that mtRNA levels increased in control infants during the 4 days-6 weeks period ($P = .04$), compared with values at birth (0–3 days). In contrast to mtDNA levels, birth mtRNA levels were significantly lower in ART-exposed infants than in control infants ($P = .03$). A trend toward lower mtRNA in ART-exposed infants was maintained through the 4 days-6 weeks period ($P = .14$) and the >16 weeks period ($P = .08$), but not during the 6–16 weeks period ($P = .63$), probably due to the low number of control infants in the latter period. These results were unchanged if mothers who used drugs of addiction were excluded from the analyses.

Because d4T and ddI have been more prominently implicated in mitochondrial toxicity, the 0–3 days ART-exposed group was divided into those who were exposed in utero to these drugs and those who were not (figure 2). In agreement with the GEE modeling, birth mtDNA levels, irrespective of the length of in utero exposure, did not reach statistical significance when the 3 groups were compared ($P = .07$). There was a marginal difference between neonates exposed to a non-d4T or non-ddI regimen ($P = .03$) (figure 2*A*). In contrast, mtRNA levels were statistically different between the 3 groups at birth ($P = .001$) (figure 2*B*). Pairwise comparisons also showed that neonates exposed to a non-d4T or non-ddI regimen had significantly lower mtRNA levels than both control infants ($P = .001$) and neonates exposed to d4T or ddI ($P = .003$). The latter group was not significantly different from control infants ($P = .22$).

Length of in utero exposure. Sixteen women conceived while receiving HAART; the others initiated therapy during their pregnancy, for the purpose of preventing MTCT. Figure 3 presents the relationships between the length of in utero exposure and mtDNA level (figure 3*A*) or mtRNA level (figure 3*B*) at 0–3 days for ART-exposed infants whose mothers started ART after conception and received a ZDV+3TC–based regimen. Among this subgroup, a statistically significant positive correlation was observed between the length of in utero ART exposure and mtDNA levels ($n = 18$) ($R = 0.7$; $P = .002$) (figure 3*A*) but not mtRNA levels ($n = 12$) ($R = -0.165$; $P = .61$) (figure 3*B*). When the same analysis was done on birth samples from all infants, including those whose mothers were receiving HAART prior to becoming pregnant and who continued various regi-

Table 2. Generalized estimating equation linear regression models comparing the effect of in utero exposure to antiretroviral therapy (ART) on mitochondrial DNA (mtDNA) and mitochondrial gene expression levels (mtRNA).

	Log_{10} mtDNA:nDNA ratio				Log_{10} mtRNA:β-actin mRNA ratio[a]			
	Samples from ART-exposed infants, no.	Samples from control infants, no.	β	P	Samples from ART-exposed infants, no.	Samples from control infants, no.	β	P
Research site, Toronto vs. Vancouver	. . .	. . .	0.06	.001	. . .	. . .	NA	NA
Comparison of time period								
4 days-6 weeks vs. 0–3 days	. . .	. . .	0.04	.26	. . .	. . .	0.21	.04
6–16 weeks vs. 0–3 days	. . .	. . .	0.10	.001	. . .	. . .	0.01	.94
>16 weeks vs. 0–3 days	. . .	. . .	−0.01	.88	. . .	. . .	0.15	.06
Comparison of ART-exposed group and control group, by time period								
0–3 days	26	27	0.06	.07	17	26	−0.22	.03
4 days-6 weeks	67	26	0.12	.001	35	14	−0.15	.14
6–16 weeks	77	17	0.06	.01	33	9	0.04	.63
>16 weeks	68	21	0.11	<.001	33	15	−0.15	.08

NOTE. NA, not applicable.

[a] Vancouver site only.

mens throughout their pregnancy, no relationship was observed between mtDNA level and length of in utero exposure ($n = 25$) ($R = 0.096$; $P = .65$), data not shown.

Additional analyses. Whole blood was used, as it was impossible to isolate specific blood cells from the small volumes of blood available (often less than 0.2 mL for neonates). Because platelets contain some mtDNA [36], their relationship with mtDNA levels was investigated. No correlation was found between platelet count and mtDNA level ($R = 0.11$; $P = .10$) or mtRNA level ($R = 0.01$; $P = .88$). Lactate measurement at 1 month of age (±2 weeks) showed no correlation with mtDNA level ($N = 55$) ($R = 0.056$; $P = .68$) (data not shown). There was no overall correlation between mtDNA and mtRNA levels ($N = 182$) ($R = 0.02$; $P = .82$) or between the

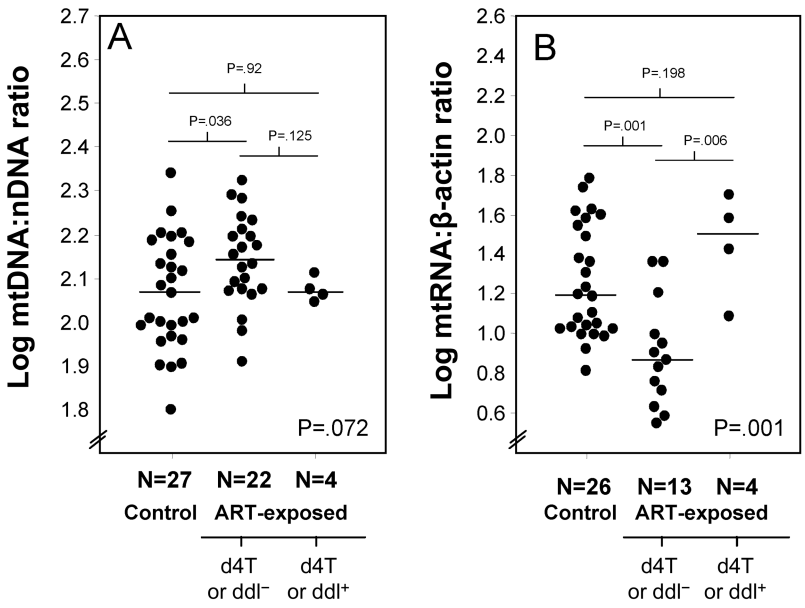

Figure 2. Ratio of mitochondrial DNA (mtDNA) to nuclear DNA at birth (0–3 days) *(A)* and ratio of mtDNA gene expression (mtRNA) to β-actin mRNA at birth (0–3 days)*(B)*. Infants who were exposed to antiretroviral therapy (ART) were subdivided into 2 groups: those who were exposed to stavudine (d4T) or didanosine (ddI) in utero (d4T or ddI$^+$) and those who were not (d4T or ddI$^-$). *Horizontal bars*, medians. *P* values in lower right corner of each panel are for 3-group comparison.

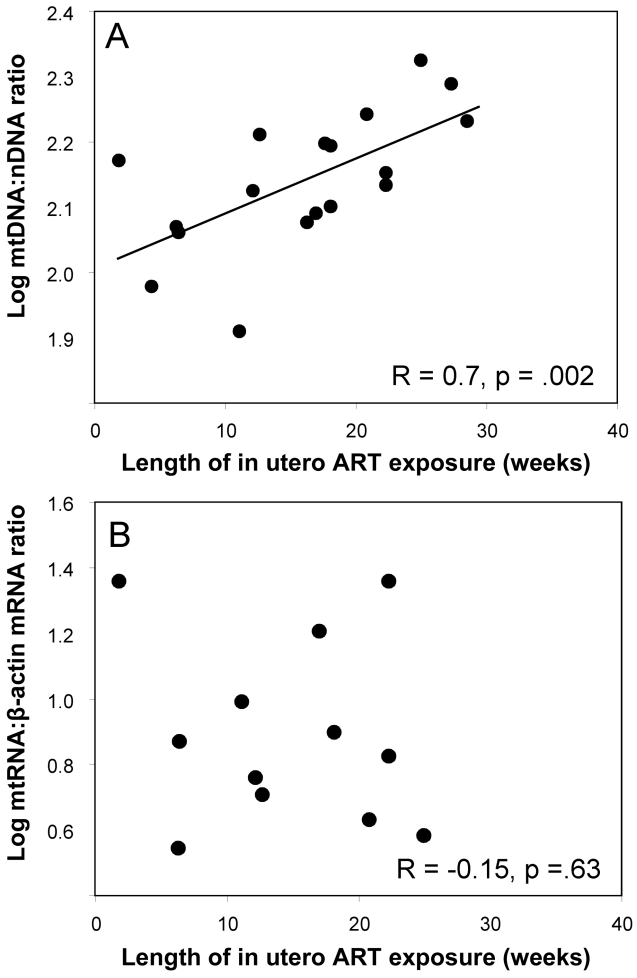

Figure 3. Relationship between the length of in utero ART exposure and the mtDNA:nDNA ratio *(A)* or mtRNA:β-actin ratio *(B)* at birth (0–3 days), for infants who were born to HIV-infected women who started ART after becoming pregnant and received a ZDV+3TC-based regimen.

change in mtDNA level versus the change in mtRNA level over time (data not shown).

DISCUSSION

This study showed that during the early neonatal period, blood mtDNA levels were higher and blood mtRNA levels were lower in infants exposed to ART, compared with infants born to HIV-uninfected mothers. These higher mtDNA levels were maintained through the end of the study period, whereas the difference in mtRNA content seemed to dissipate over time. The increase in mtDNA was independent of all risk factors tested, namely the use of drugs of addiction during pregnancy, overall length of in utero ART exposure, maternal infection with HCV or HBV, birth weight, gestational age at delivery, and smoking during pregnancy.

The peripheral blood mtDNA results we observed are in agreement with those obtained with peripheral blood mononuclear cells (PBMCs) from a large US cohort of HIV-uninfected infants born to HIV-infected mothers [31]. Our findings appear to contradict several previously published smaller studies that reported significantly lower mtDNA content in cord blood from HIV-infected women treated with ZDV+3TC–based ART during pregnancy, compared with the levels observed in HIV-uninfected control subjects [14, 29, 30]. However, some of the mtDNA levels reported in one of these studies [30] would appear to be insufficient to support life and may reflect technical assay limitations. In addition, cord blood and peripheral blood may differ with respect to their mtDNA content. The results from this study also suggest that d4T-containing or ddI-containing regimens may affect the mitochondria differently from ZDV-based regimens, especially with respect to mtRNA; however, studies with larger sample sizes would be required to confirm this observation.

A parallel could be drawn between our study's findings and observations made in vitro. In HeLa cells exposed to high concentrations of ZDV, mtDNA content increased, oxidative phosphorylation genes were upregulated, and mitochondrial membrane potential increased [37]. Over the longer term, however, mtDNA depletion, along with mitochondrial morphological abnormalities, altered expression of oxidative phosphorylation genes, and severe loss of mitochondrial transmembrane potential occurred. Other recent studies involving HepG2 cells also observed increased mtDNA levels on short-term exposure to ZDV, especially if used in combination with 3TC, but decreased mtDNA levels in response to ddI or d4T [38, 39]. The initial increase may be an attempt to compensate for mitochondrial stress or dysfunction, which may also explain some of the discrepancies in the NRTI mitochondrial toxicity literature with respect to adult HIV populations, for which results have been mixed [40]. Although studies of long-term NRTI exposure in therapy-experienced patients tend to show decreased blood cell mtDNA levels [33, 41, 42], initial increases in blood cell mtDNA level have been observed in d4T-naive adults initiating HAART [43] and in children switching to ZDV-containing HAART [44]. It has also been suggested that early increases in mtDNA may represent a restorative phenomenon, countering the mtDNA-decreasing effect of HIV itself [43]. Longer exposure would presumably lead to deleterious mitochondrial effects, loss of the initial beneficial effect of ART, and overall mtDNA depletion.

The increase in mtDNA content observed here is likely the result of an adaptive mitochondrial biogenesis process in response to drug-induced mitochondrial dysfunction. This would also be consistent with the observation that longer in utero exposure to ZDV and 3TC, when started during pregnancy, was positively correlated with higher mtDNA levels at birth. Others have observed a correlation between length of in utero ART exposure and the degree of morphological mitochondrial damage in umbilical cord endothelial cells [29].

There have been few reports about the effect of NRTI exposure on the expression level of mtDNA-encoded genes (mtRNA). One study associated the initiation of HAART with increased PBMC mtRNA levels (along with increased mtDNA levels) [43], but another study reported decreased blood cell mtRNA levels in patients on ZDV and 3TC [45]. In other tissues, significant decreases in mtRNA have been associated with NRTI exposure [46–48]. We observed a decrease in mtRNA levels in ART-exposed infants at birth and during early infancy, in the face of increased mtDNA levels. This may suggest that exposure to ART is associated with perturbations in the regulation of mitochondrial gene expression or in the mtRNA's half-life. Low mtRNA levels could also contribute to triggering a response through increased biogenesis and mitochondrial mass, hence the increased mtDNA levels.

In this longitudinal study, hematological toxicities and hyperlactatemia in ART-exposed infants were mild to moderate, but persistent, in agreement with observations published by others [18, 20]. No infant had long-term clinical symptoms suggestive of mitochondrial dysfunction, suggesting that mitochondrial damage may exist at the molecular level, in the absence of clinical phenotype [29].

Our study had several important limitations. Our control group did not include ART-unexposed infants born to HIV-infected mothers, as it would be unethical to recruit these infants. Considering that HIV infection by itself (in the absence of therapy) has been reported to decrease mtDNA levels [33, 49, 50], our control group could be conservatively biased. Similarly, because some have reported decreased platelets in children who received ART [18, 20, 51, 52], the increase in mtDNA observed in the study group could be underestimated, representing another potential conservative bias. Serial blood samples were not obtained from control infants. Multiple samples from these control subjects would have reduced the scatter associated with interindividual variability; however, it was deemed unethical to request additional blood samples from healthy infants who would not otherwise have had blood samples obtained. The infants in the control group were having blood work done for various reasons and may not be the best representatives of a healthy population. In addition, the mothers of ART-exposed and control infants differed with respect to their ethnicity, smoking habits, use of drugs of addiction, and coinfections. Finally, whether changes in neutrophil count may influence the ratio of mtDNA to nDNA in whole blood is unknown. Although blood mtDNA content may be appropriate as a surrogate marker for mitochondrial toxicity in blood cells, it may not be such for other tissues.

Our observations suggest that significant changes in mitochondrial proliferation and gene expression take place during and after perinatal ART exposure. Although the relative influence of in utero exposure versus postnatal ART exposure, and that of HIV infection itself, remain unclear, it is postulated that these changes are a consequence of the effects of the antiretroviral drugs on the cells and their mitochondria. As the first HIV-uninfected, ART-exposed children are now reaching adolescence, we believe the long-term significance of these alterations should be studied into reproductive age.

Although HAART for HIV-infected pregnant women is clearly beneficial, more research is needed to evaluate the safety and efficacy of potentially less toxic antiretroviral regimens to maximize the prevention of MTCT while minimizing short- and long-term mitochondrial toxicity. This is especially relevant given the rapidly increasing access to HIV therapy in women of childbearing age throughout the world.

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