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Elevated frequencies of micronucleated erythrocytes in infants exposed to zidovudine in utero and postpartum to prevent mother-to-child transmission of HIV

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Abstract

Zidovudine-based antiretroviral therapies (ART) for treatment of HIV-infected pregnant women have markedly reduced mother-to-child transmission of the human immunodeficiency virus (HIV-1) from ~25% to <1%. However, zidovudine (ZDV; AZT), a nucleoside analogue, induces chromosomal damage, gene mutations, and cancer in animals following direct or transplacental exposures. To determine if chromosomal damage is induced by ZDV in infants exposed transplacentally, we evaluated micronucleated reticulocyte frequencies (%MN-RET) in 16 HIV-infected ART-treated mother-infant pairs. Thirteen women received prenatal ART containing ZDV; 3 received ART without ZDV. All infants received ZDV for 6 weeks postpartum. Venous blood was obtained from women at delivery, and from infants at 1–3 days, 4–6 weeks, and 4–6 months of life; cord blood was collected immediately after delivery. Ten cord blood samples (controls) were obtained from infants of HIV-uninfected women who did not receive ART. %MN-RET was measured using a single laser 3-color flow cytometric system. Ten-fold increases in %MN-RET were seen in women and infants who received ZDV-containing ART prenatally; no increases were detected in 3 women and infants who received prenatal ART without ZDV. Specifically, mean %MN-RET in cord blood of ZDV-exposed infants was 1.67±0.34 compared with 0.16±0.06 in non-ZDV ART-exposed infants (P=0.006) and 0.12±0.02 in control cord bloods (p<0.0001). %MN-RET in ZDV-exposed newborns decreased over the first 6 months of life to levels comparable to cord blood controls. These results demonstrate that transplacental ZDV exposure is genotoxic in humans. Long-term monitoring of HIV-uninfected ZDV-exposed infants is recommended to ensure their continued health.

Keywords

AZT; antiretroviral; chromosome damage; transplacental exposure; nucleoside analogues; mutagenicity

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Conflict of interest statement

S Dertinger is employed by Litron Laboratories, which holds patents pertaining to the flow cytometric analysis of micronucleated erythrocytes.

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INTRODUCTION

The nucleoside analogue zidovudine (ZDV) is widely used in combination antiretroviral therapy (ART) regimens and these regimens are highly effective in reducing mother-to-child transmission of HIV from an estimated rate of 25% to less than 1% (Dorenbaum et al., 2002). In the United States, to protect against vertical transmission, HIV-infected pregnant women typically receive ART as standard care during pregnancy and their infants receive prophylactic ZDV for 6 weeks postpartum (CDC, 2005). Thus, HIV-uninfected infants born to mothers with HIV may be exposed to ZDV both transplacentally during gestation, and directly during the postpartum treatment period. Although ZDV is tremendously effective in preventing vertical transmission of HIV, ZDV has been shown to be carcinogenic in animals (Ayers et al., 1996;National Toxicology Program, 1999,2006;Olivero et al., 1997;Diwan et al., 1999), genotoxic in numerous in vitro and in vivo test systems (Phillips et al., 1991;Sussman et al., 1999;Poirier et al., 2004;Chan et al., in press;von Tungeln et al., 2004;Meng et al., 2002), and incorporated into DNA of primates and humans after transplacental or direct exposure (Poirier et al., 2004,1999;Olivero et al., 2000,2002).

Of greatest concern to our group were results of recent experiments we conducted in mice that revealed 10-fold increases in micronucleated reticulocytes (immature erythrocytes) in peripheral blood of newborn pups exposed to a human equivalent dose of ZDV throughout gestation and for the first 3 weeks of life; the treatment regimen in these mouse studies modeled that used in humans to prevent mother-to-child transmission of HIV (Bishop et al., 2004;Witt et al., 2004). Micronuclei (MN) are well-characterized biomarkers of structural or numerical chromosomal damage; they arise from acentric chromosome fragments or lagging whole chromosome(s) that fail to incorporate into daughter nuclei after nuclear division (Heddle et al., 1991), and they are commonly evaluated in lymphocytes and erythrocytes, due to the ease of obtaining blood samples. Prompted by the observations of increased frequencies of micronucleated reticulocytes (MN-RET) in mouse pups exposed to ZDV, we designed a pilot study to determine if similar effects were detectable in human infants exposed to ZDV in utero and postnatally. We used a recently developed single-laser three-color flow cytometric system for enumerating MN-RET in human blood (Dertinger et al., 2004). The advent of this new technology was important for our study design because, although the mouse peripheral blood erythrocyte micronucleus test has been used routinely for decades to evaluate genotoxicity (Heddle et al., 1991;OECD, 1997; MacGregor et al., 1990; Albertini et al., 2000;Witt et al., 2000;Hayashi et al., 1994), there has not been a corresponding human assay because the human spleen, unlike the mouse spleen, rapidly removes damaged erythrocytes from circulation. Thus, until recently, experimental evidence of genotoxicity obtained through use of routine rodent erythrocyte MN studies could not be confirmed in the same cell type in humans unless the human subject had undergone splenectomy (MacGregor et al., 1997). However, this new flow cytometry-based test method enables the identification, selection, and evaluation of the youngest fraction of reticulocytes (<48 hr old), representing approximately10% of all circulating reticulocytes, before the effects of splenic selection have much impact on the frequency of MN-RET. Identification of this fraction of reticulocytes is based on expression of a specific cell surface marker, the transferrin (CD71) receptor, which is active for only a few hours after the young reticulocyte enters the peripheral circulation. Thus, using this new technology, we were able to minimize the action of the spleen on frequency of MN-RET and directly compare a well-characterized biomarker of genotoxicity (i.e., MN) in the same cell type (reticulocytes) in the animal model system and in humans.

MATERIALS AND METHODS

Study Subjects

All pregnant women in this study were patients at one of the two participating clinical sites, Duke University Medical Center (DUMC) or University of North Carolina (UNC). Because this was a pilot study, few restrictions were placed on subject eligibility. Cases were broadly defined as pregnant women who were HIV-infected, receiving prenatal antiretroviral therapy (ART); inclusion of ZDV in the ART was not specifically required. Detailed demographic information was collected for each study subject, but there were no exclusions based on race, ethnicity, age, HIV RNA, CD4 counts, or method of delivery (vaginal or caesarean). Controls were defined as HIV-uninfected women with uncomplicated, full-term, singleton pregnancies. The study protocol was approved by the Institutional Review Boards of the National Institute of Environmental Health Sciences (NIEHS), DUMC, and UNC. All women provided written informed consent.

Cases consisted of 16 HIV-infected pregnant women who received prenatal antiretroviral therapy (ART) as prescribed by their HIV providers. Thirteen of these women received prenatal ART that included ZDV as a component and 3 women received ART without ZDV (Tables I and II). All 16 women received intravenous ZDV during labor and delivery, and all 16 infants born to these women received prophylactic ZDV orally for 6 weeks postpartum, at standard doses. All infants were HIV-DNA PCR negative at birth and at 6 months of age.

Blood Sampling Schedule

All blood samples were obtained at routine, scheduled blood draws; no blood samples were requested solely for this study. Therefore, maternal venous blood was collected at the time of delivery and infant cord blood was obtained immediately after delivery. Venous blood samples were obtained from infants at 1–3 days, 4–6 weeks, and 4–6 months of life, in conjunction with routine, scheduled blood draws. Cord blood samples were also obtained from infants born to 10 HIV-uninfected women who were not receiving ART, and these served as controls.

MN-RET Measurements

MN-RET $^{CD71+}$ frequencies (%MN-RET) were measured in heparinized venous blood samples obtained from women and infants at the time points outlined above. Samples were processed using flow cytometric micronucleus analysis kits (Prototype Human MicroFlow® kits, Litron Laboratories, Rochester NY). Details of the collection, processing, and analysis of blood samples using this method have been previously described (Dertinger et al., 2004). Briefly, a 60–120 μL sample of whole blood (venous or cord blood) was diluted with 350μL of sodium heparin solution. Aliquots of the heparinized blood samples were then forcefully injected into tubes containing ultracold (−80°C) methanol at the clinical site. These fixed blood samples were allowed to accumulate in a −80°C freezer and then were bulk-shipped overnight on dry ice to Litron Laboratories for flow cytometric analysis. Approximately 20,000 young reticulocytes (i.e., CD71-positive) were evaluated per blood sample for %MN-RET.

Statistical Analysis

Because %MN-RET values were often not normally distributed and because some of the sample sizes in subgroups were small (i.e., the 3 non-ZDV-exposed women and infants), the nonparametric one-sided Mann-Whitney test was used to compare two groups, and Kruskal-Wallis analysis of variance was used to compare three or more groups. Spearman correlation coefficients were used to relate % MN-RET values at each of the five sample times to continuous variables such as maternal age, weight, CD4 counts, and HIV RNA.

RESULTS

A detailed description of the study population is presented in Table I, while the complete % MN-RET data set for women and infants is shown in Table II. A few blood specimens were not amenable to flow cytometric analysis and, thus, the numbers of samples analyzed in the specific study groups discussed below do not always equal the number of subjects sampled. Nevertheless, the positive genotoxic outcomes in ART-exposed patients described below remained independent and significant when correlated to other variables. %MN-RET was unrelated to maternal age, height, weight, body mass index, CD4 counts, or HIV RNA values ($p \geq 0.10$); in addition, delivery method (vaginal or caesarean), baby's sex, location of clinic, duration of prenatal ART, birth weight, and gestational age were also unrelated to %MN-RET ($p \geq 0.10$) (Table III). No correlation was seen between %MN-RET and any of the non-ZDV medications (nucleosides as well as protease inhibitors) included in the prenatal ART regimens represented among the 16 cases.

The mean MN-RET frequencies observed in the women and newborns (data from the cord and day 1–3 infant bloods) who received prenatal ART that included ZDV were increased 10-fold compared with the frequency in the control cord blood samples; the three mother-infant pairs who received prenatal ART without ZDV did not show significant increases in MN-RET (Fig. 1). In cord blood (Fig. 1; Table II), the mean %MN-RET in newborns exposed to ZDV in utero (N=12) was 1.67 ± 0.34 ($p<0.0001$, compared with controls). Mean %MN-RET in newborns not exposed to ZDV (N=3) in utero was 0.16 ± 0.06 (significantly lower than the ZDV-exposed group, p=0.006; but not different from controls, p=0.17). The mean %MN-RET in cord blood control samples (N=10) was 0.12 ± 0.02, which closely matches the normal values for %MN-RET in a sample of 50 healthy male and female adults (0.12±0.009%) (Dertinger et al.).

Women who received ZDV as part of their prenatal ART therapy (N=11) had a mean %MN-RET of 1.51 ± 0.62, which was significantly elevated (p=0.015) over the mean (0.12±0.02) for women who did not receive prenatal ZDV (N=3). The mean %MN-RET for the women who did not receive prenatal ZDV closely matches the mean %MN-RET reported for the sample of 50 (25 males, 25 females) healthy adults referenced above (Dertinger et al.).

The markedly elevated %MN-RET in newborns exposed to ZDV in utero decreased during the postpartum ZDV treatment period (Fig. 1). At the 4–6 week sampling time, the mean % MN-RET in infants transplacentally exposed to ZDV had decreased to 0.45 ± 0.15, a value that was still significantly elevated over the cord blood control frequency ($p = 0.025$). This decline is consistent with the marked reduction in erythropoiesis that normally occurs in newborns at birth and persists for approximately 6–8 weeks after birth (Palis and Segel, 1998). By age 6 months, %MN-RET (N=7) declined to levels comparable to control cord bloods (p=0.41) (Fig. 1; Table II); this observation is consistent with the completion of ZDV prophylaxis well before the final postnatal blood sampling.

%MN-RET values obtained with cord blood and with infant venous blood sampled at 1–3 days of age were essentially identical (Table II), suggesting that either sample type could be selected for evaluation in future studies.

DISCUSSION

The nucleoside analogue ZDV is highly effective at reducing mother-to-child transmission of HIV (Dorenbaum et al., 2002). As such, it is a critical component of antiretroviral treatment regimens. However, data on adverse genetic effects induced by ZDV in a variety of test systems are accumulating. Recently, increasing emphasis has been placed on examining the effects in infants of transplacental exposures to ZDV due to the increasing number of children born to HIV-infected women who receive ART during pregnancy. For example, experiments in mice

have shown that transplacental exposure to ZDV results in an increased frequency of cancer in exposed mice at maturity (NTP, 2006;Olivero et al., 1997), as well as increases in mitochondrial DNA mutations (Chan et al., in press) and elevated mutation frequencies in lymphocytes (von Tungeln et al., 2002,2004,2007;Torres et al., 2007). Furthermore, the incidences of K-*ras* and *p53* mutations were found to be elevated in lung tumors arising in adult mice transplacentally exposed to ZDV, compared with the incidences of these mutations in spontaneous tumors arising in unexposed mice, suggesting that these mutations were the result of chemical-specific events leading to increases in tumor formation later in life (Hong et al., in press). In humans, ZDV has been shown to incorporate into DNA of cord blood cells and maternal lymphocytes after prenatal and perinatal exposures (Olivero et al., 2000).

Our previous experiments in mice investigating the induction of chromosomal damage following transplacental exposure to human equivalent doses of ZDV, alone or in combination with other nucleosides, showed that ZDV induced extraordinary, 10-fold increases in micronucleated erythrocytes (Bishop et al., 2004;Witt et al., 2004), implying a strong potential for ZDV-induced genetic damage in all exposed dividing cell populations. To determine whether similar effects could be detected in infants exposed to ZDV in utero as a consequence of maternal ART, we designed and conducted the study reported here. We included an extended monitoring period so that any effects that might be noted in infants at birth could be followed during the 6-week postpartum treatment period and further out to 6 months of age, after all exposure to ZDV had ceased. The results in infants following transplacental exposure are in accord with those obtained in the mouse model (10-fold increases in MN-RET were noted in both infants and mouse pups) and suggest that, although we are monitoring a biomarker of chromosomal damage in a surrogate population of terminally differentiated cells, other dividing cell populations and stem cells that are exposed to ZDV may be at risk for acquiring similar damage. The health consequences of this damage are not yet evident, and will depend in large part on whether the ZDV-induced damage is eliminated by repair enzymes or cell death, or converted to a stable, persisting alteration in a continuously propagating tissue. In addition, since ZDV is a chain terminator (Zidovudine, IARC Monograph vol. 76, 2000) and affects telomere length (Olivero et al., 1997), it is possible that consequences involving accelerated aging or related effects may result from ZDV-induced chromosomal damage.

The flow cytometric system used to evaluate %MN-RET in this study has been validated in mice (Torous et al., 2005) and is accepted by regulatory agencies as a method of measuring genotoxicity. This study in HIV-infected women and their infants is one of the first to extend this evaluation procedure to human blood samples. Results of similar studies in humans have recently been reported (Stopper et al., 2005;Dertinger et al.;Harrod et al.) and all, including the study reported here, have shown remarkable consistency in baseline MN-RET frequencies in adult and pediatric control populations (~0.12%) as well as a clear ability to detect and quantify changes from baseline levels following exposures to known clastogens such as chemotherapeutic agents or radiotherapy. In our study, we achieved remarkable consistency between mean maternal and infant cord blood MN-RET frequencies, as well as highly similar values in instances where duplicate samples were evaluated from a single blood draw (data not shown), demonstrating that our measurements were reproducible.

Studies in human populations have shown that elevated lymphocyte chromosome aberration frequencies, indicating exposure to a genotoxicant, are associated with an increased risk of chronic diseases, particularly cancer, for that population (Albertini et al., 2000;Rossner et al, 2005;Bonassi et al., 2005). The frequency of micronucleated lymphocytes in healthy individuals has also been directly correlated with an increased risk of future cancer (Bonassi et al., in press). However, the degree of correlation between MN frequencies in lymphocytes and in reticulocytes has only been partially characterized in a recent pilot study (Stopper et al., 2005). Therefore, we cannot conclude with certainty that the elevated reticulocyte

micronucleus frequencies observed in ZDV-exposed infants in our study will be associated with a higher risk of future disease in these infants compared with healthy, non-exposed individuals. However, we are concerned about the long-term health implications for these infants because the MN increases noted in this study add to the growing body of evidence that ZDV readily induces genetic damage (mutational and clastogenic) in both nuclear and mitochondrial DNA in a variety of in vitro and in vivo test systems (Sussman et al., 1999;Poirier et al., 2004;Chan et al., in press;Von Tunglen et al., 2004;Meng et al., 2002;Olivero et al., 2002;Bishop et al., 2004;Witt et al., 2004;Meng et al., 2000; Hong et al., in press; Olivero, in press).

Two observations may help to explain the seeming contradiction of the declining %MN-RET observed in infants during postpartum ZDV prophylaxis. First, DNA incorporation of ZDV and resulting genetic effects may be enhanced by the presence of additional nucleoside analogues such as lamivudine or didanosine (Meng et al., 2002;Bishop et al., 2004;Witt et al, 2004;Meng et al., 2000), although neither of these two nucleosides alone induces micronuclei in mice (Phillips et al., 1991;Von Tungeln et al., 2004;Witt et al., 2004;Von Tungeln et al., 2002; Von Tungeln et al., in press). All women in this study who received ZDV during the prenatal period also received lamivudine, whereas infants received ZDV alone during postpartum treatment. Thus, transplacental exposure to multiple antiretroviral nucleosides may have contributed to the high levels of micronucleated reticulocytes observed in neonates immediately after birth compared with the lower levels seen 4–6 weeks after birth. However, it is more likely that the marked reduction in erythropoiesis that occurs in the newborn at birth and persists for ~6–8 weeks (Palis and Segel, 1998) was largely responsible for the drop in MN-RET frequencies observed during postpartum ZDV treatment. In the absence of cell division, no induction of micronuclei can occur and, because the human spleen rapidly sequesters and destroys damaged erythrocytes, the existing pool of micronucleated reticulocytes would be quickly depleted. Indeed, flow cytometric determinations of erythropoietic index showed marked decreases in most of the 4–6 week infant blood samples (data not shown). Therefore, the declining MN-RET frequencies observed in the infants in this study during ZDV postpartum treatment should not be interpreted as indicating reduced risk to other exposed cell populations that continue to undergo rapid division in the neonate.

We recognize that our results are derived from a small number of study subjects, especially those in the group that did not receive ZDV prenatally (only 3 subjects). However, the consistency in response among both women and infants within the two subgroups (+ZDV, −ZDV) provides strong evidence that prenatal exposure to ZDV is indeed genotoxic to erythrocyte precursor cells, as evidenced by the markedly elevated frequencies of MN-RET^{CD71+} observed in HIV-uninfected infants born to women who received ZDV-based prenatal ART. Furthermore, prenatal exposure to the nucleosides tenofovir, emtricitabine, didanosine, or lamivudine, in the absence of ZDV, did not result in elevated frequencies of MN-RET in the three infants or their mothers enrolled in this study.

Given the results of the study reported here, we recommend long-term monitoring of ZDV-exposed HIV-uninfected infants to ensure their continued health. We must emphasize that we do not advocate eliminating the use of ZDV in the treatment of HIV infection: ZDV-based ART is highly effective in preventing mother-to-child transmission of a devastating disease. However, we do hope that the results of this study will stimulate a search for and acceptance of non-ZDV-based antiretroviral therapies that are equally effective in preventing maternal transmission of the virus, while securing maternal health, but that have less potential for genotoxicity in healthy non-infected infants.

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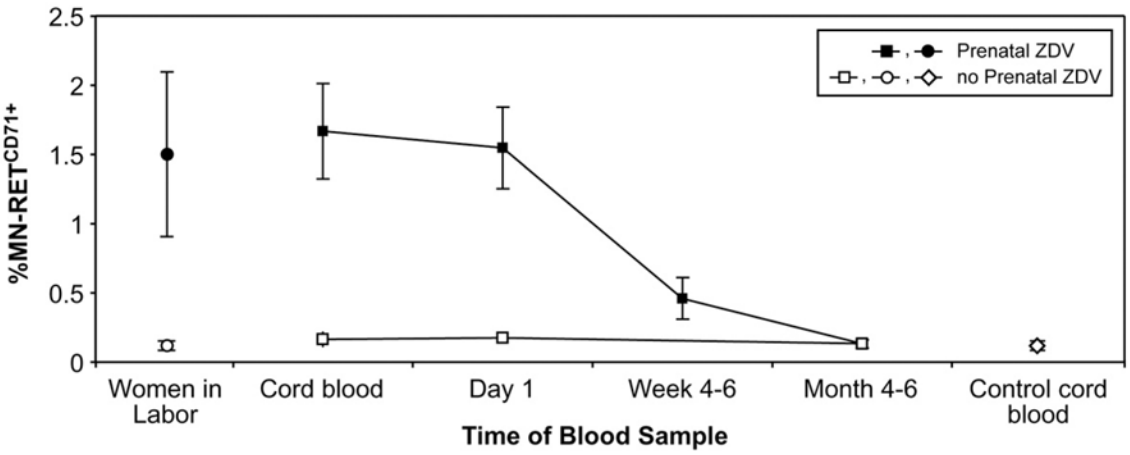

Fig 1.
%MN-RET^{CD71+} by time and prenatal ZDV exposure.

TABLE I

Case Demographics

Mother	
Clinic	**n (%) or range (median)**
Duke	11 (69%)
UNC	5 (31%)
Race	
Black	12 (75%)
Hispanic	4 (25%)
Age (yrs)	14 to 39 (29)
Height (in)	59 to 69 (65)
Weight (kg)	61 to 126 (87)
Body mass index	20.9 to 46.7 (32.0)
Delivery mode	
c-section	8 (50%)
vaginal	8 (50%)
Prenatal ART	
> 3 months	11 (69%)
≤ 3 months	5 (31%)
Type of ART	
with AZT	13 (81%)
without AZT	3 (19%)
Maternal CD4	0.02 to 1,081 (477)
Maternal HIV RNA	50 to 222,000 (6,590)
Infant	
Infant sex	**n (%) or range (median)**
Female	11 (69%)
Male	5 (31%)
Birth weight (kg)	1.05 to 3.48 (2.85)
Gestational age (weeks)	32 to 41 (38)

TABLE II

Prenatal Antiretroviral Treatment Regimens and %MN-RET Data

Subj. Code	Prenatal Treatment, [12]	Treatment Duration	Del. Mode[4]	Gestational Age (wks)	Birth Weight (kg)	Infant Sex	Maternal	Cord	%MN-RET Day 0–3[3]	Wk 4–6	Mo 4–6
Prenatal ART without ZDV:											
05	FTC, TNF; NFV	2mo	c	39	2.6	f	0.17	0.10	0.19	NA	0.07
03	FTC, TNF; KAL	>3mo	c	40	3.0	m	0.11	0.28	0.20	x	0.21
23	3TC, ddI, KAL	>3mo	v	40	3.1	f	0.09	0.11	0.13	(0.09)[5]	0.11
Mean values[6]**:**							0.12±0.02	0.16±0.06	0.17±0.02	NA	0.13±0.04
Prenatal ART with ZDV:											
22	ZDV, 3TC; KAL	4da	c	32	2.1	f	0.45	0.76	0.86	0.12	0.15
26	ZDV, 3TC, ABC; KAL	3mo	c	32	1.1	m	6.84	0.91	1.15	0.52	0.03
02	ZDV, 3TC; KAL	3mo	c	41	2.8	f	0.62	3.80	2.55	0.21	0.18
04	ZDV, 3TC; KAL	3mo	c	39	2.7	f	0.33	1.00	0.87	0.10	0.25
21	ZDV, 3TC; KAL	>3mo	v	38	2.9	f	0.22	3.54	3.30	1.54	0.10
24	ZDV, 3TC; ATZ	>3mo	v	39	3.1	f	3.84	2.95	3.28	(0.14)[5]	0.10
25	ZDV, 3TC; NFV	>3mo	v	34	2.1	f	0.10	NA	NA	1.03	NA
27	ZDV, 3TC; KAL	>3mo	c	37	2.3	m	0.96	0.59	0.56	NA	0.09
28	ZDV, 3TC, ABC; TNF	>3mo	c	38	3.5	f	NA	0.61	0.57	0.13	NA
29	ZDV, 3TC; KAL	>3mo	v	38	3.3	f	NA	0.40	0.43	0.51	0.18
30	ZDV, 3TC; NFV	>3mo	v	36	2.4	m	0.70	1.96	1.55	0.32	0.02
31	ZDV, 3TC; NFV	>3mo	v	40	3.5	m	0.61	1.70	1.78	0.04	0.13
01	ZDV, 3TC; NFV	>3mo	v	38	3.0	f	1.92	1.77	1.66	NA	NA
Mean values:							1.51 ± 0.62	1.67 ± 0.34	1.55 ± 0.29	0.45 ± 0.15	0.12 ± 0.02
Mean cord blood control (10 samples):							0.12 ± 0.02				
Mean adult[7] **control (50 samples):**							0.12 ± 0.009				

[1] Nucleosides: FTC = emtricitabine, TNF = tenofovir (a nucleotide), 3TC = lamividine. ddI = didanosine. ZDV = zidovudine, ABC = abacavir. Protease inhibitors: KAL= lopinavir and ritonavir as a fixed dose combination, NFV = nelfinavir, ATZ = atazanavir.

[2] All patients who received FTC and TNF, ZDV and 3TC, or ZDV, 3TC and ABC received the medication as a fixed dose combination.

[3] NA = Non-analyzable; x = unable to obtain blood sample.

[4] c = cesarean section, v = vaginal.

[5] Infant postpartum ZDV therapy was completed 1 (0.09) or 3 (0.14) weeks prior to blood sampling, and therefore, these 2 data points were excluded from analysis. All other 4–6 wk samples were collected during postpartum treatment.

[6] Mean ± standard error.

[7] 25 males and 25 females (Dertinger et al.).

TABLE III

Mann-Whitney Two-Sided P-values for Comparisons among Subgroups

	%MN-RET $^{CD71+}$				
	Women	**Cord blood**	**Day 1**	**Week 4–6**	**Month 4–6**
Race, Black vs. Hispanic	0.04	0.22	0.13	0.92	0.13
Baby's sex, M vs. F	0.29	0.85	0.95	0.92	0.27
Duration of treatment, ≤ 3 mo. vs. > 3 mo.	0.79	1.00	0.95	0.78	0.77
Location, UNC vs. Duke	0.69	0.95	0.76	0.56	0.12
Delivery, vaginal vs. c-section	0.90	0.27	0.22	0.65	0.57