

# Blood mitochondrial DNA mutations in HIV-infected women and their infants exposed to HAART during pregnancy

Marissa H.J. Jitratkosol<sup>a</sup>, Beheroze Sattha<sup>a</sup>, Evelyn J. Maan<sup>b</sup>,  
Izabelle Gadawski<sup>a</sup>, P. Richard Harrigan<sup>c</sup>, John C. Forbes<sup>b</sup>,  
Ariane Alimenti<sup>b</sup>, Julie van Schalkwyk<sup>d</sup>, Deborah M. Money<sup>d,e</sup>,  
Hélène C.F. Côté<sup>a,e</sup>, the CIHR Emerging Team Grant on  
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**Objectives:** Nucleo(s)tide reverse transcriptase inhibitors given to HIV-infected pregnant women to prevent vertical transmission may adversely affect mitochondrial DNA (mtDNA). We hypothesized that HAART-exposed/HIV-uninfected infants may show higher blood mtDNA mutation burden than controls born to HIV-uninfected mothers.

**Methods:** Blood was collected from in-utero HIV/HAART-exposed infants and controls, as well as from a subset of their mothers. The presence of mtDNA A→C/T→G (AC/TG) mutations was measured by cloning and sequencing D-loop PCR amplicons. Relationships with maternal characteristics were examined.

**Results:** No significant difference was found between the percentage of HIV/HAART-exposed infants with AC/TG mutations ( $N=15/57$ , 26.3%) and controls ( $N=10/70$ , 14.3%) before ( $P=0.090$ ) or after controlling for covariates ( $P=0.058$ ), although a tendency was observed. However, significantly more HIV/HAART-exposed mothers ( $N=18/42$ , 42.9%) harboured AC/TG mutations compared with controls ( $N=7/39$ , 17.9%) before ( $P=0.015$ ) and after ( $P=0.012$ ) controlling for covariates. AC/TG mutations were more prevalent in HIV/HAART-exposed mothers than in their infants ( $N=42$ , 42.9 vs. 23.8%,  $P=0.033$ ), however, this difference disappeared after controlling for covariates. No difference was seen between control mothers and their infants ( $N=39$ , both 17.9%). In HIV/HAART-exposed mothers, only a detectable HIV plasma viral load near delivery predicted AC/TG mutations.

**Conclusion:** Our results suggest that HIV and/or HAART exposure are associated with increased prevalence of AC/TG mtDNA mutations in mothers and show a similar tendency in infants exposed during pregnancy. As accumulation of mtDNA mutations has been linked with aging and age-associated diseases, this may raise concerns in the long term for HIV and HAART-exposed populations.

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<sup>a</sup>Department of Pathology and Laboratory Medicine, University of British Columbia, <sup>b</sup>British Columbia Women's Hospital, Oak Tree Clinic, <sup>c</sup>British Columbia Centre for Excellence in HIV/AIDS, <sup>d</sup>British Columbia Women's Hospital, and <sup>e</sup>Women's Health Research Institute, Vancouver, British Columbia, Canada.

Correspondence to Hélène C.F. Côté, Department of Pathology and Laboratory Medicine, University of British Columbia, G227-2211 Wesbrook Mall, Vancouver, BC, V6T 2B5, Canada.

Tel: +1 604 822 9777; fax: +1 604 822 7635; e-mail: helene.cote@ubc.ca

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## Introduction

Approximately 3 million HIV-infected women become pregnant every year [1] and as many as 90% of HIV-infected children acquire HIV through mother-to-child transmission [2]. To prevent transmission and/or for their own health, HIV-infected women can receive HAART during pregnancy. This greatly decreases the risk of antepartum mother-to-child transmission, from 20–25% to less than 2% [3,4].

Nucleo(s)ide reverse transcriptase inhibitor (NRTI)-containing HAART can interfere with mitochondrial DNA (mtDNA) integrity, either inhibiting polymerase- $\gamma$  (POLG) [5] or decreasing its fidelity, leading to mitochondrial dysfunction and oxidative stress [5–7]. In adults, reports have suggested alterations in mtDNA heteroplasmy over time in individuals receiving HAART [8,9]. As NRTIs can cross the placenta [10], these may affect mtDNA in developing fetuses. A 2009 study in umbilical cord tissue suggested that zidovudine (ZDV)-based therapy induced mtDNA tRNA gene mutations [11], whereas more recently, the clonal expansion of somatic mtDNA mutations in NRTI-treated HIV-infected individuals was suggested as a plausible mechanism for accelerated aging seen in HIV [12]. Given the high rates of mtDNA replication during embryogenesis and organogenesis, the unborn child could be at increased risk for mtDNA damage. Neonates exposed to NRTIs perinatally show mitochondrial toxicity with abnormal haematological findings, although the long-term clinical significance of this is unclear [13,14]. Several groups have also reported increased blood mtDNA levels in uninfected infants perinatally exposed to antiretrovirals compared with infants born to HIV-infected untreated [15] or HIV-uninfected mothers [16,17]. This may represent compensatory mitochondria proliferation to offset mtDNA damage or mitochondrial dysfunction.

The benefits of HAART in pregnancy clearly outweigh the risks; however, the full long-term impact of these drugs is largely unknown. This study aimed to investigate random mtDNA point mutations in HIV-uninfected children and their mothers, who were either HIV-infected HAART-treated in pregnancy or HIV-uninfected.

## Materials and methods

### Study design and population

Samples were from two prospective cohorts which enrolled infants born between 2003 and 2006 and mothers and their infants born between 2005 and 2009, at British Columbia's Women's Hospital. Infants were eligible for inclusion if they were born to HIV-infected

mothers treated with HAART during pregnancy (HIV/HAART-exposed infants) or to HIV-uninfected mothers (unexposed controls), and if a blood sample was collected between 0 and 6 days of life. Maternal blood samples were collected at last prenatal visit, approximately 32–36 weeks of gestation. Although none of the HAART-exposed infants were infected with HIV, they are herein referred to as 'HIV/HAART-exposed', as they have potentially been exposed to the effects of their mothers' circulating virus. Consent was obtained from mothers and/or guardians. The study was approved by the University of British Columbia Research Ethics Board and the Children's and Women's Health Centre of British Columbia Research Review Committee (H03-70356 and H04-70540).

### Sample, clinical and demographic data collection

For infants, heel prick blood (0.5 ml) was collected at the time of routine newborn screening or HIV testing. For mothers, samples were collected through venipuncture into EDTA or acid citrate dextrose and frozen at  $-80^{\circ}\text{C}$  without processing.

For both cohorts, baseline information included maternal demographics, pregnancy history, maternal antiretroviral therapy (ART) history and other drug/toxic exposures, as well as delivery and infant birth information, including antenatal and postnatal antiretroviral use. Maternal CD4<sup>+</sup> cell count and HIV plasma viral load (pVL) 1–4 weeks prior to delivery are hereafter referred to as 'near delivery'.

### Mitochondrial DNA mutation burden assay

This assay was developed to estimate the frequency of random mtDNA mutations. Total genomic DNA was extracted from 0.1 ml of whole blood using QIAamp DNA Mini Kit (Qiagen, Toronto, Canada). A 509-bp fragment in the mtDNA genome D-loop region was amplified with MT16535F (5'-GCCACACGTTCC CCTTAAATAAGA-3') and MT474R (5'-AGTATGGG AGTGRGAGGGRAAAA-3'). The 25  $\mu\text{l}$  PCR reaction contained 1.5 mmol/l  $\text{MgCl}_2$ , 200  $\mu\text{mol/l}$  deoxyribonucleotide triphosphate, 0.4  $\mu\text{mol/l}$  of each primer, 2.5  $\mu\text{l}$  of DNA extract and 0.5 U of Expand High Fidelity<sup>PLUS</sup> Enzyme Blend (Roche Applied Science, Laval, Quebec, Canada). Amplification conditions were  $1 \times 94^{\circ}\text{C}/30\text{ s}$ ,  $35 \times 94^{\circ}\text{C}/15\text{ s}$ ,  $60^{\circ}\text{C}/30\text{ s}$ ,  $72^{\circ}\text{C}/30\text{ s}$ ,  $1 \times 72^{\circ}\text{C}/7\text{ min}$ .

PCR products were ligated into pCR2.1-TOPO (Invitrogen, Carlsbad, California, USA), transformed into TOP10 *Escherichia coli* cells (Invitrogen) with colour selection. Inserts from 93 white clones/colonies were individually PCR amplified using plasmid-specific primers and sequenced with universal primer M13R. For some participants with longer D310 C-tracts (i.e. C<sub>6–12</sub>T<sub>1</sub>C<sub>6</sub>), an 'out of phase' sequence (i.e. a mixed sequence) was observed downstream of the C-tract.

When this sequencing polymerase ‘slippage’ [18] occurred, the clones were also sequenced from the opposite direction using the universal primer T7. Apart from C-tract slippage, the sequencing polymerase did not induce mutations (data not shown).

Sequences were aligned against the revised Cambridge reference sequence for human mtDNA [19]. Only 448 bp between positions nt16560–451 were analysed, excluding the primers and D310 C-tract (nt303–315) regions. As per Wilding *et al.* [20], insertions and deletions (indels) with at least two mutations were considered a single event. Furthermore, if more than three clones from one sample had identical mutations at the same position, this was defined as mtDNA heteroplasmy [20] and these mutations were excluded from the analyses. For each sample, 93 clones were sequenced and the first 80 readable sequences were used for the analyses.

To determine the background error rate of the assay, DNA from 14 individual clonal suspensions were subjected to the assay as described above. As the starting material was clonal (i.e. not heteroplasmic), any mutation detected represented PCR error introduced as part of the assay background. All study samples were assayed randomized and blinded. To minimize assay variability between mother/infant pairs, these were assayed on the same plate.

Given the high total mutation background rate observed, only the presence of A→C and T→G (AC/TG) mutations was considered for the statistical analyses, as these were not observed during background determination experiments (see Results section).

### Statistical analyses

For infant analyses, samples from both cohorts were considered. When comparing between mothers or between mother/infant pairs, only the cohort with maternal samples was used. Statistical analyses were performed using  $\chi$ LSTAT (Addinsoft, Paris, France) and SPSS (SPSS, IBM Corporation, Armonk, New-York, USA). A *P* value of less than 0.05 was defined as significant.

The AC/TG mutations were analysed in terms of their absence or presence in a sample by  $\chi^2$ -test between groups and Wilcoxon signed-rank test within groups. Logistic regression was used to examine the relationship between HIV/HAART exposure during pregnancy and AC/TG mutations, while controlling for potential covariates. These included maternal age at delivery as mtDNA mutations accumulate with age [21] and if occurring in oocytes may be transmitted to progeny [22]. Smoking cigarettes or marijuana, use of drugs of addiction (see Table 1) and/or methadone ever in pregnancy were included as they have been associated with oxidative stress

**Table 1. Demographic characteristics, clinical characteristics and laboratory values for HIV/HAART-exposed infants and their mothers as well as unexposed control infants and their mothers.**

	HIV/HAART-exposed <sup>a</sup>	Unexposed controls <sup>b</sup>	<i>P</i>
Infant characteristics			
Male sex	31 (54)	38 (54)	0.991
	23 (55)	23 (59)	0.702
Birth weight (kg)	3.05 [2.73–3.41] (1.62–4.05)	3.30 [2.81–3.64] (1.40–4.57)	<b>0.026</b>
	3.05 [2.73–3.38] (1.62–4.05)	3.13 [2.67–3.50] (1.40–4.54)	0.698
Gestational age at delivery (weeks)	38.3 [37.7–39.9] (31.3–41.6)	39.1 [38.3–40.1] (28.9–41.9)	0.070
	38.3 [37.5–39.6] (31.3–41.1)	39.0 [38.1–39.7] (28.9–41.9)	0.542
Delivery method, vaginal birth <sup>c</sup>	31 (54)	51 (73)	<b>0.030</b>
	21 (50)	28 (72)	<b>0.045</b>
Apgar score at 5 min	9 [9–9] (7–10)	9 [9–9] (5–10)	0.593
	9 [9–9] (7–10)	9 [9–9] (5–9)	0.451
Maternal characteristics			
Maternal age (years)	30.6 [25.1–35.6] (17.4–41.5)	32.3 [29.4–35.6] (22.2–43.0)	<b>0.049</b>
	31.0 [25.1–34.7] (18.0–41.5)	31.4 [26.9–35.2] (22.2–43.0)	0.479
Active HCV co-infection <sup>d</sup>	8 (19) (N=42)	1 (6.7) (N=15)	0.420 <sup>e</sup>
	8 (30) (N=27)	1 (6.7) (N=15)	0.082 <sup>e</sup>
Maternal ethnicity			
Aboriginal, First Nations, Métis or Inuit	17 (30)	6 (8.6)	
	14 (33)	6 (15)	
White	22 (39)	38 (54)	
	15 (36)	24 (62)	
Black African Canadian	12 (21)	0 (0)	
	8 (19)	0 (0)	
Hispanic	0 (0)	1 (1.4)	
	0 (0)	0 (0)	
Asian and other	6 (10)	18 (26)	
	5 (12)	9 (23)	
Missing information	0 (0)	7 (10)	
	0 (0)	0 (0)	

Table 1 (continued)

	HIV/HAART-exposed <sup>a</sup>	Unexposed controls <sup>b</sup>	<i>P</i>
Maternal use ever during pregnancy			
Alcohol	19 (35) ( <i>N</i> = 54)	16 (23)	0.131
	16 (41) ( <i>N</i> = 39)	15 (39)	0.817
Smoking <sup>f</sup>	30 (55) ( <i>N</i> = 55)	20 (29)	<b>0.003</b>
	25 (63) ( <i>N</i> = 40)	18 (46)	0.145
Drugs of addiction <sup>g</sup> and/or methadone	20 (36) ( <i>N</i> = 55)	16 (23)	0.097
	17 (42) ( <i>N</i> = 41)	14 (36)	0.610
Maternal HAART			
In pregnancy (weeks)	19.3 [13.6–28.6] (0.6–41.1)	N/A	
	17.9 [13.9–24.3] (0.6–41.1)		
HAART started		N/A	
Before conception	12 (21)		
	8 (19)		
In first trimester	5 (9)		
	3 (7.1)		
In second trimester	31 (54)		
	26 (62)		
In third trimester	9 (16)		
	5 (12)		
N(t)RTI (ZDV/3TC/d4T/ddI/ABC/FTC/TDF) <sup>h</sup>	51/54/31/2/2/7	N/A	
	38/40/1/0/1/2/6		
Pre-pregnancy (weeks)	41.1 [19.7–96.1] (0.1–604) ( <i>N</i> = 33 <sup>i</sup> )	N/A	
	33.0 [19.4–84.6] (0.1–604) ( <i>N</i> = 23 <sup>i</sup> )		
Maternal HIV clinical data			
Duration of HIV infection at delivery (year) <sup>j</sup>	3.8 [0.9–6.0] (0.1–15.3)	N/A	
	3.9 [1.2–5.7] (0.1–15.3)		
Detectable HIV pVL <sup>k</sup> 1–4 weeks prior to delivery <sup>l</sup>	8 (14)	N/A	
	7 (17)		
Detectable HIV pVL, HIV RNA (copies/ml)	432 [264–648] (53–1280)	N/A	
	414 [231–621] (53–1280)		
CD4 <sup>+</sup> cell count 1–4 weeks prior to delivery (cells/ $\mu$ l)	450 [300–630] (90–1200)	N/A	
	425 [300–630] (90–1150)		

Data are number (%) of individuals or median [interquartile range] (range) and *N*, number of individuals with available data (if not available for entire group) unless indicated otherwise. For each category, the data for all infants (2003–2006) are presented above that for the mother/infant pairs (2005–2009), which are italicized. Significant *P* values are bolded. N/A, not applicable.

<sup>a</sup>All infants (*N* = 57); mother/infant pairs (*N* = 42).

<sup>b</sup>All infants (*N* = 70); mother/infant pairs (*N* = 39).

<sup>c</sup>Delivery method: vaginal or caesarean section.

<sup>d</sup>On the basis of hepatitis C virus (HCV) RNA PCR testing.

<sup>e</sup>Fisher's exact test used if less than five individuals in a given category.

<sup>f</sup>Smoking includes cigarettes and/or marijuana.

<sup>g</sup>Drugs of addiction included but were not limited to heroin, cocaine, crack, crystal methamphetamine, ecstasy, benzodiazepine and opioids.

<sup>h</sup>Nucleo(s)tide reverse transcriptase inhibitors [N(t)RTIs], zidovudine (ZDV), lamivudine (3TC), stavudine (d4T), didanosine (ddI), abacavir (ABC), emtricitabine (FTC), tenofovir (TDF).

<sup>i</sup>Other individuals had no prior exposure to antiretroviral therapy (ART) or HAART.

<sup>j</sup>Data based on HIV clinical diagnosis date.

<sup>k</sup>Detectable HIV plasma viral load (pVL) signifies more than 50 copies/ml.

<sup>l</sup>One to 4 weeks prior to delivery refers to 'near delivery' herein.

[23–27] which may induce mtDNA mutations [28]. Because it varied, amount of DNA template in the PCR was also considered as a possible confounder. Of note, experiments performed after statistical analyses showed that the latter had no influence on the results.

Hierarchical logistic regressions were used to examine predictive models of the presence of AC/TG mutations within the HIV/HAART-exposed group. For the infants, possible predictors included the following: duration of mother's ART/HAART pre-pregnancy, duration of infant in-utero HAART exposure and detectable maternal HIV pVL near delivery. For the mothers, possible predictors also included total lifetime exposure to ART/HAART and CD4<sup>+</sup> cell count near delivery.

Comparisons of demographic characteristics, clinical and laboratory values for the groups were done by two-sample Student's *t*-test (two-tailed) or  $\chi^2$ -test.

## Results

### Study populations

#### All infants

Fifty-seven HIV/HAART-exposed and 70 unexposed control infants were studied. Their demographic and clinical characteristics, as well as laboratory values, are shown in Table 1 (top line). Both groups were similar except that control infants had a higher birth weight, their

mothers had fewer caesarean sections, were slightly older and of different ethnicity and fewer smoked during their pregnancy.

#### Mother/infant pairs

For 42 of the HIV/HAART-exposed and 39 of the unexposed controls, a blood sample was collected from the mother near delivery. Within the mother/infant pairs, the groups were similar with respect to all demographic and clinical parameters except ethnicity and delivery method (Table 1, bottom italic line).

Overall, 80% ( $N=8/10$  in both groups) of all mothers receiving methadone during their pregnancy also reported using drugs of addiction. Active hepatitis C virus (HCV) co-infection based on RNA PCR was more common in HIV/HAART-exposed mothers than in unexposed controls; however, this information was unavailable for the majority of control mothers.

#### HIV/HAART-exposed group

None of the infants acquired HIV. Of the 57 exposed women, 12 (21%) conceived while on HAART and these were more likely to be on non-ZDV/lamivudine (3TC) regimens ( $N=8/12$ , 67%). Of those who initiated HAART in pregnancy, the majority did so in the second trimester, as per standard care. All women continued HAART through the remainder of their pregnancy. Thirty-three women (58%) had received HAART prior to their pregnancy. The  $CD4^+$  cell count was above 250 cells/ $\mu$ l for 51 (89%) of HIV-infected mothers, whereas eight (14%) women had detectable virus near delivery.

#### Mitochondrial DNA mutation burden

When comparing the background mutation rate due to PCR enzyme errors to the total mutation rate in clinical samples, a high noise-to-signal ratio was observed. The assay's background was measured by subjecting clonal (plasmid) DNA containing a single sequence to the assay 14 independent times. Further tests confirmed that the high background was due to PCR errors introduced

during the initial PCR reaction (data not shown). We then further examined the prevalence of each type of mutation individually and determined that A→C and T→G (AC/TG) substitutions were the only mutations never introduced by HiFi Taq under our assay conditions. We therefore elected to only consider AC/TG mutations despite their rarity and these were statistically analysed in terms of their presence or absence in a given sample. Among all the participants assayed in this study ( $N=208$ ), 55 had one AC/TG mutation in the 80 sequences analysed and five had two (three HIV/HAART-exposed mothers, one HIV/HAART-exposed infant and one unexposed infant), none had more than two such that no AC/TG mutations were excluded due to heteroplasmy. Mutations within the D310 C-tract region were excluded from the analyses because our control background experiments showed that the PCR polymerase induced high levels of mutations (mostly indels) in this region compared with regions outside the C-tract (data not shown).

Although a higher percentage of HIV/HAART-exposed infants (26.3%) had AC/TG mutations compared with the unexposed control infants (14.3%), this difference did not reach statistical significance ( $P=0.090$ ). However, this difference approached significance ( $P=0.058$ ) after controlling for the following covariates: amount of DNA in initial PCR, maternal age at delivery, smoking ever in pregnancy as well as drugs of addiction and/or methadone use ever in pregnancy (Table 2). In contrast, a significantly higher percentage of HIV/HAART-exposed mothers (42.9%) harboured these mutations compared with unexposed control mothers (17.9%,  $P=0.015$ ). This effect persisted after controlling for the above covariates ( $P=0.012$ ).

For 81 of the infants, a maternal sample was available and the following analyses are restricted to mother/infant pairs (Table 3). Within the HIV/HAART-exposed group, the percentage of infants with the AC/TG mutations (23.8%) was significantly lower than that of their mothers (42.9%,  $P=0.033$ ). However, this difference disappeared

**Table 2. Comparison of the percentage of HIV/HAART-exposed and unexposed control infants and mothers with the AC/TG mutations.**

	Infants		Mothers	
	HIV/HAART exposed ( $N=57$ )	Unexposed controls ( $N=70$ )	HIV/HAART exposed ( $N=42$ )	Unexposed controls ( $N=39$ )
Individuals with AC/TG mutations, $N$ (%)	15 (26.3)	10 (14.3)	18 (42.9)	7 (17.9)
Ratio between groups	1.84		2.39	
$\chi^2$	2.88		5.88	
$P$ value	0.090		<b>0.015</b>	
Controlling for covariates				
Odds ratio (95% CI)	2.5 (0.97–6.46)		4.7 (1.4–15.6)	
$P$ value	0.058		<b>0.012</b>	

A→C and T→G (AC/TG). Covariates included amount of DNA in initial PCR, maternal age at delivery, smoking ever in pregnancy as well as drugs of addiction and/or methadone use ever in pregnancy. Significant  $P$  values are bolded. None of the covariates were significantly independently correlated to the outcome. CI, confidence interval.

**Table 3. Comparison of the percentage of infants and mothers with the AC/TG mutations with and without HAART-exposure.**

	HIV/HAART exposed (N = 42)		Unexposed controls (N = 39)	
	Infants	Mothers	Infants	Mothers
Individuals with AC/TG mutations, N (%)	10 (23.8)	18 (42.9)	7 (17.9)	7 (17.9)
Ratio within groups		1.80		1.00
Wilcoxon, Z		-2.1		0.0
P value		<b>0.033</b>		>0.999
P value controlling for covariates		0.777		0.283

A→C and T→G (AC/TG). Covariates included amount of DNA in initial PCR, maternal age at delivery, smoking ever in pregnancy as well as drugs of addiction and/or methadone use ever in pregnancy. Significant *P* values are bolded. None of the covariates were significantly independently correlated to the outcome.

after controlling for covariates ( $P=0.777$ ). Within the control group, there was no difference between percentage of infants and mothers with these mutations (both 17.9%) either prior to or after controlling for covariates ( $P=0.283$ ).

For both the all infants and the mother/infant pair analyses, none of the covariates were independently associated with the presence of AC/TG mutations. Length of gestation was negatively correlated with smoking ( $N=125$ ,  $r=-0.33$ ,  $P<0.001$ ), alcohol consumption ( $N=124$ ,  $r=-0.24$ ,  $P=0.007$ ), drugs of addiction and/or methadone use ( $N=125$ ,  $r=-0.31$ ,  $P<0.001$ ) as well as HCV co-infection ( $N=57$ ,  $r=-0.34$ ,  $P=0.011$ ). However, smoking was highly correlated with using drugs of addiction and/or methadone ( $N=124$ ,  $r=0.78$ ,  $P<0.001$ ) and mothers who smoked tended to be younger ( $N=125$ ,  $r=-0.42$ ,  $P<0.001$ ).

### Other covariates in the HIV/HAART-exposed group

Using hierarchical logistic regression, in addition to the above covariates, the following predictors were considered: a detectable maternal HIV pVL near delivery, duration of mother's HAART pre-pregnancy and duration of infant in-utero HAART exposure. None predicted the presence of AC/TG mutations in HIV/HAART-exposed infants (all  $P>0.5$ ), nor did total lifetime exposure to HAART or CD4<sup>+</sup> cell count near delivery for the mothers (both  $P>0.1$ ). However, a detectable HIV pVL near delivery was associated with increased odds of having these mutations {six of seven HIV/HAART-exposed women with a detectable HIV pVL had AC/TG mutations, odds ratio [95% confidence interval (CI)]: 14.0 (1.4–137.3),  $P=0.024$ }, albeit the CI was wide. Drugs of addiction and/or methadone use was not associated with a detectable HIV pVL ( $N=55$ ,  $r=0.22$ ,  $P=0.10$ ).

A possible association between mtDNA mutations in mothers and their infants was explored. Within the HIV/HAART-exposed group, presence of AC/TG mutations in infants and their mothers was positively correlated ( $N=42$ ,  $r=0.31$ ,  $P=0.048$ ), whereas no correlation was

seen in the control groups ( $N=39$ ,  $r=-0.045$ ,  $P=0.787$ ). Finally, maternal age was mildly but not significantly positively correlated with the presence of AC/TG mutations in mothers ( $N=81$ ,  $r=0.178$ ,  $P=0.112$ ). No relationship was seen between maternal age at delivery and the presence of AC/TG mutations in infants ( $N=127$ ,  $r=0.011$ ,  $P=0.899$ ).

## Discussion

mtDNA mutations are believed to accumulate with age and exposure to oxidative stresses. As NRTIs can cause mitochondrial dysfunction and exert pressure on POLG, we investigated whether infants born to HIV-infected women treated with HAART during pregnancy harboured increased blood mtDNA mutations. To do so, an mtDNA D-loop 'mtDNA mutation burden' assay was developed. The D-loop region was chosen because it is not subject to deletions and is the most variable region of the mitochondrial genome. Blood was used because of its convenient availability, although as a high turnover tissue, blood cells are less likely to show mtDNA mutations than other tissues [29].

Not unexpectedly, a high and variable error rate was observed within the D310 C-tract, likely due to polymerase 'slippage' [18]. Mutations arising within the C-tract were, therefore, excluded. Because of the high PCR error mutation rate, our analyses were restricted to AC/TG transversions, two mutations that were not induced by HiFi Taq under the assay conditions. AC/TG can arise from the oxidation of nucleotide pool deoxyguanine triphosphate (dGTP) into 8-oxo-dGTP. During mtDNA replication, the oxidized nucleotide can pair with deoxycytidine triphosphate and deoxyadenosine triphosphate (dATP) with almost equal affinity, leading to AC/TG transversions [30]. As dATP is also the pairing site for thymidine analogues ZDV and d4T within elongating DNA, AC/TG mutations could also be linked to decreased POLG fidelity under drug pressure.

A direct mtDNA fragment cloning/sequencing strategy into plasmid or phage may have introduced fewer

artificial mutations; however, this would require mitochondria isolation prior to DNA extraction, something that was not feasible in the context of our study [31]. The amount of fresh starting material would greatly exceed the small amount of blood that could be obtained from neonates. Use of a PCR enzyme with higher fidelity or of ultra-deep sequencing could be considered in future studies.

This study's principal finding is that a significantly larger proportion of pregnant women within the group exposed to HIV/HAART harboured AC/TG mutations compared with unexposed controls, suggesting an association between HIV/HAART exposure during pregnancy and AC/TG mtDNA mutations. After controlling for relevant covariates, in-utero HIV/HAART-exposed infants showed marginally significantly more AC/TG mutations than control infants. No covariate was independently associated with AC/TG mutations. Although the infants tended in the same direction, it is reassuring that they did not show higher prevalence of mutations than their own mothers. Furthermore, had a statistically significant increase in mutations been observed in infants, it would remain unclear whether these were inherited from their mothers or acquired through in-utero exposures. As our study could not include an HIV-infected HAART-untreated group, it could not address the possible relative contribution of HIV vs. HAART exposure. However, a 2003 study reported an accumulation of mtDNA mutations in HAART-treated individuals, something that was not seen in HIV-infected untreated individuals followed for the same length of time, suggesting an association with HAART rather than HIV [8]. Our approach does not distinguish between mutations arising from increased somatic mtDNA mutations or from clonal expansion of the latter as suggested by Payne *et al.* [12], although both likely share the same long-term biological consequences.

In the HIV/HAART-exposed group but not the controls, AC/TG mutations were more often detected in mothers than in their infants; however, this difference disappeared after controlling for covariates. If HAART were the determining factor for mtDNA mutation accumulation, one may expect more mtDNA mutations in the HIV/HAART-exposed infants compared with controls, especially considering that concentrations of ZDV, 3TC and stavudine (d4T) in the amniotic fluid can be equivalent or higher than maternal circulating levels [10,32]. Our finding in the infants showed an odds ratio of 2.5 for the occurrence of AC/TG in exposed infants which raises some concern. Because sample size and duration of exposure may have affected our ability to show low-level differences, this warrants further study with a larger sample size and more sensitive mtDNA mutation quantification, as with deep sequencing.

Although only seven of 42 (17%) of the HIV/HAART-exposed mothers had a detectable HIV pVL near delivery, the latter predicted the presence of maternal AC/TG mutations. As HIV itself is a possible source of oxidative stress through inflammation, it may be associated with increased mtDNA mutations. In an in-vitro cell model of HIV infection, HIV RNA transcripts were found in mitochondria at higher levels than in the cytoplasm and nucleus. Mitochondria 'viability' or function decreased as mitochondrial HIV RNA density increased, leading the authors to postulate that HIV RNA transcripts compromise mitochondrial function [33]. In another study, HIV Tat-expressing transgenic mice showed decreased expression of antioxidant genes involved in controlling reactive oxygen species (ROS) levels and concomitantly increased oxidative stress levels [34]. Mitochondrial dysfunction, which is known to increase ROS, ultimately leads to oxidative mtDNA damage. In addition, in this study, length of HAART exposure, either in mothers or infants, did not predict AC/TG mutations. This would again not support HAART as the main factor for mtDNA mutation induction. However, in mothers, HAART exposure length is highly correlated to HIV infection duration, possibly confounding the effect.

Because fibroblast mtDNA mutations accumulate with age [21], we hypothesized a higher mtDNA mutation burden in mothers than infants, especially in controls. However, no such difference was observed. Our study population may be too young to detect such difference, or blood cells may not accumulate mutations as readily as other cells.

mtDNA mutations have implications on aging and disease, and their effects may be even more pronounced if they are acquired early in life. Mice models with proofreading deficient POLG generated independently by two research groups showed signs of accelerated aging with an approximately three to five times rise in somatic mtDNA point mutations in solid organs compared with wild-type animals [35–37].

### Limitations

Although we demonstrated statistical differences in terms of the presence or absence of AC/TG mutations, it remains difficult to gauge the biological and clinical significance of our results. To put this in perspective, in the 42 HIV/HAART-exposed mothers, 21 AC/TG mutations were observed. If we were to extrapolate this mutation rate for all mutation types (substitutions and indels) throughout the mtDNA genome, this would amount to an overall mutation rate of 4.6 mutations per mtDNA genome. In contrast, the control mothers had 1.6 mutations per mtDNA genome. That said, we cannot presume all mutations are equal everywhere. As the D-loop is noncoding, it may be more permissive to mutations than coding regions. Conversely, given that the D-loop is a regulatory region, a mutation here may affect

replication and transcription exerting broader consequences than would a single gene mutation.

Although blood was used in this study, it is not the most sensitive tissue to mitochondrial toxicity. In mitochondrial disease, it is common to detect mutations in skeletal muscle that are absent in blood cells from the same individual [29]. Also, NRTIs affect various tissues differently within an individual. For example, although treatment with d4T and didanosine (ddI) were both strongly associated with adipocyte mtDNA depletion, only ddI did so in blood [38]. Similar findings were reported when muscle was compared with blood cells [39]. Therefore, tissues other than blood should be investigated to assess whether HIV/HAART exposure hastens the accumulation of mtDNA mutations. However, obtaining solid tissue samples for research may not be an option in young paediatric populations. Finally, although duration of smoking and use of drugs of addiction prior to the pregnancy would have been relevant, these data were not available.

There are several limitations to this study, but the results suggest that HIV and/or HAART are associated with AC/TG mtDNA mutations in mothers and may show a similar tendency in their infants. In addition, a detectable HIV pVL near delivery predicted the presence of AC/TG mutations in the mothers. Given that mtDNA mutations have been associated with aging and age-associated diseases [21,40–42] and in light of recent epidemiological studies, suggesting an earlier incidence of pathologies in HIV-infected individuals compared with the general population [43], including a high relative risk of cancer in young adults [44], it raises possible concerns for the HIV-infected and exposed population. Although the benefits of HAART in decreasing morbidity and mortality in HIV-infected individuals and in preventing mother-to-child transmission of HIV are undeniable, further investigations are warranted, especially in HAART-naïve individuals and those on long-term HAART.

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## Conflicts of interest

There are no conflicts of interest.

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