

Perinatal outcomes, mitochondrial toxicity and apoptosis in HIV-treated pregnant women and in-utero-exposed newborn

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Objective: Highly active antiretroviral therapy (HAART) has decreased the risk of HIV mother-to-child transmission. However, HIV and HAART have been associated with adverse perinatal outcome. HAART has been associated with mitochondrial dysfunction in nonpregnant adults, and HIV, additionally, to apoptosis. We determined whether mitochondrial toxicity and apoptosis are present in HIV-pregnant women and their newborns and could be the basis of adverse pregnancy outcome.

Design: Single-site, cross-sectional, controlled observational study without intervention.

Methods: We studied mitochondrial and apoptotic parameters in mononuclear cells from maternal peripheral blood and infant cord blood at delivery in 27 HIV-infected and treated pregnant women, and 35 uninfected controls and their infants, to correlate clinical outcome with experimental findings: mitochondrial number (CS), mtDNA content (ND2/18SrRNA), mitochondrial protein synthesis (COX-II/V-DAC), mitochondrial function (enzymatic activities) and apoptotic rate (caspase-3/ β -actin).

Results: Global adverse perinatal outcome, preterm births and small newborn for gestational age were significantly increased in HIV pregnancies [odds ratio (OR) 7.33, 5.77 and 9.71]. Mitochondrial number was unaltered. The remaining mitochondrial parameters were reduced in HIV mothers and their newborn; especially newborn mtDNA levels, maternal and fetal mitochondrial protein synthesis and maternal glycerol-3-phosphate + complex III function (38.6, 25.8, 13.6 and 31.2% reduced, respectively, $P < 0.05$). All materno-fetal mitochondrial parameters significantly correlated, except mtDNA content. Apoptosis was exclusively increased in infected pregnant women, but not in their newborn. However, adverse perinatal outcome did not correlate mitochondrial or apoptotic findings.

Conclusions: Transplacental HAART toxicity may cause subclinical mitochondrial damage in HIV-pregnant women and their newborn. Trends to increased maternal apoptosis may be due to maternal-restricted HIV infection. However, we could not demonstrate mitochondrial or apoptotic implication in adverse perinatal outcome.

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Introduction

Antiretroviral therapy (ART) during pregnancy is critical to prevent mother-to-child transmission (MTCT) of human immunodeficiency virus (HIV) infection and to delay disease progression [1]. Widespread use of antiretrovirals has been accepted for the prevention of MTCT, despite the lack of safety data related to human pregnancies [2–4].

The potential clinical risks associated with antiretroviral exposure in HIV-pregnant women, fetus and infants have been reported by observational studies with varying strengths of evidence and conflicting results [5–9]. Antiretroviral drugs have been associated with adverse pregnancy outcomes such as preeclampsia, fetal death, preterm birth and low birth weight, although controversial results have been published [10–15].

Several physiopathological mechanisms have been proposed to explain the adverse clinical effects in HIV pregnancies. The negative mitochondrial and clinical effects of nucleoside reverse transcriptase inhibitors (NRTIs) used in highly active antiretroviral therapy (HAART) have been firmly established in HIV-infected nonpregnant adults [16–18]. These negative effects depend on the capacity of the NRTI to inhibit DNA γ -polymerase, the only enzyme devoted to the replication (and to a lesser extent, the repair) of the mitochondrial DNA (mtDNA) genome, thus leading to a decrease in mtDNA copy number and quality, which, in turn, may finally cause mitochondrial dysfunction [16].

Such mitochondrial abnormalities may be magnified by the effects of HIV itself, since it has been demonstrated to cause diffuse mitochondrial alterations, either *in vivo* or *in vitro*, probably through the activation of apoptotic mechanisms triggered by HIV proteins [19–21]. Additionally, increased apoptosis rates have also been demonstrated after *in-vitro* exposure to NRTI and in tissue from patients using antiretroviral drugs [22].

The known patterns of mitochondrial and apoptotic toxicity in HIV patients receiving NRTI therapy suggest that some children, though HIV-uninfected, may be at risk of developing sequels from *in-utero* HIV and NRTI exposure [5–15,23–32]. Mitochondrial toxicity along pregnancy has been studied in animal models including uninfected pregnant monkeys exposed to ART. This model demonstrates the association between *in-utero* NRTI exposure and fetal tissue mtDNA depletion, altered mitochondrial respiratory chain (MRC) function and mitochondrial dysmorphism [23].

Blanche *et al.* [24] associated, for the first time, mitochondrial dysfunction with the manifestation of perinatal hyperlactaemia and neurological and developmental sequels in NRTI-exposed children from HIV

mothers. Although few abnormal clinical findings are usually found in NRTI-exposed infants, the incidence of mitochondrial dysfunction is increased in these children by 26% [25]. However, conflicting experimental results have been reported in the few studies currently available analysing mitochondrial toxicity in cord blood mononuclear cells (CBMCs), peripheral blood mononuclear cells (PBMCs) or placenta of asymptomatic antiretroviral-exposed newborn [24–32]. Consequently, no concern has been raised about the obstetric and perinatal safety of NRTIs in human pregnancies. Some studies have shown mtDNA depletion compared to controls [26–29], whereas others have reported no significant changes [25,30] and the remaining have even described increased content [31,32]. Additionally, most of these studies did not assess mitochondrial number, function, translation efficiency or apoptosis development, which would bring light into the cell consequences of mtDNA depletion.

Considering the controversial results regarding mtDNA depletion in newborn from HIV-infected mothers and the lack of assessment of alternative markers of mitochondrial lesion we measured the real impact of mitochondrial dysfunction on CBMCs of antiretroviral-exposed HIV-uninfected children. Additionally, to our knowledge, no study has evaluated mitochondrial function and apoptosis in HIV-pregnant women or has correlated these data with pregnancy outcome.

We hypothesized that mitochondrial toxicity and apoptosis caused by HIV and ART could be the underlying pathophysiological mechanism of adverse perinatal outcome in HIV pregnancy. To address this question we compared mitochondrial and apoptotic parameters of HIV-infected women and their children with respect to healthy controls to correlate experimental results with immunovirological, therapeutic and obstetric data.

Patients and methods

Design

We performed a single-site, cross-sectional, controlled observational study without intervention.

Study population

Sixty-two pregnant women were prospectively and consecutively included in the present study during their routine prenatal care at first trimester of gestation, in the Materno-Fetal Medicine Department of the Hospital Clinic of Barcelona (Barcelona, Spain).

Mitochondrial and apoptotic studies of 27 asymptomatic HIV-1-infected pregnant women, 35 uninfected pregnant controls and their newborns were performed at delivery.

Controls and cases were matched by age and parity. The inclusion criteria for pregnant women were: age at least 18 years, single pregnancy and delivery after at least 22 weeks of gestation and, in case of HIV-infected patients, previous diagnosis of HIV-1 infection.

All individuals were informed and signed written consent was obtained for inclusion in this protocol and approved by the Ethical Committee of our hospital.

In order to avoid confounders of mitochondrial toxicity, patients taking other potentially toxic drugs for mitochondria were excluded from the study as were patients with familial history of mitochondrial disease.

An electronic database was created to collect epidemiological, obstetric, immunovirological and therapeutic data, perinatal outcome and experimental results.

Maternal epidemiological and obstetric parameters included information on maternal age, race, parity, illicit substance abuse and mode of delivery.

Immunovirological parameters for HIV-1-infected women consisted in measuring CD4⁺ T-cell count (by flow cytometry) and plasmatic viral load by HIV-1 RNA copy quantification (Amplicor HIV Monitor; Roche Diagnostic Systems, Branchburg, New Jersey, USA) along pregnancy.

Antiretroviral therapy was administered to all HIV-infected pregnant women following international guidelines. HIV women were stratified into different categories of therapeutic care according to the use of antiretroviral drugs during pregnancy. HAART always consisted of a double-NRTI schedule and either one protease inhibitor or one non-NRTI drug (NNRTI).

Information regarding perinatal outcome for both HIV-infected pregnant women and controls included: gestational diabetes mellitus, preeclampsia (new onset of hypertension of >140 mmHg systolic or >90 mmHg diastolic pressure and >300 mg proteins/24 h of urine), fetal death (>22 weeks of pregnancy), gestational age at delivery, preterm birth (<37 weeks of gestation), birth weight, newborn small for gestational age (<10th percentile), 5-min Apgar score below 7, neonatal admission to intensive care unit and global adverse perinatal outcome.

Finally, experimental data included the collection of maternal and fetal mitochondrial and apoptotic measures.

Sample collection and processing

Immediately after delivery, 20 ml of peripheral blood was collected from women by antecubital vein puncture and 20 ml of cord blood was collected from their infants in EDTA tubes. This method of infant blood extraction was

designed to prevent invasive sample collection and increase study participation. In both samples mononuclear cells were isolated by Ficoll density gradient centrifugation, divided into aliquots and stored at -80°C until analysis.

Platelet count was confirmed below 25 per lymphocyte/monocyte, suggesting negligible platelet contamination for the mtDNA depletion assay.

Protein content was measured according to the Bradford protein-dye binding-based method [33].

Mitochondrial studies in maternal peripheral blood mononuclear cells and newborn cord blood mononuclear cells

Mitochondrial DNA quantification

To evaluate mtDNA depletion, total DNA was obtained by standard phenol-chloroform extraction procedure. A fragment of the mitochondrial-encoded *ND2* gene and the nuclear-encoded *18S rRNA* gene were amplified in triplicate and separately by quantitative rtPCR using the Roche Lightcycler thermocycler [18]. The relative content of mtDNA was expressed as the ratio between mitochondrial to nuclear DNA amount (*ND2* mtDNA/*18S rRNA* nDNA content).

Mitochondrial protein synthesis

To assess mitochondrial translation efficiency, we performed Western blot analysis of 20 μg of total cell protein through 7/13% SDS-PAGE electrophoresis and posterior immunoquantification of the mitochondrial-encoded and located COX-II subunit (25.6 kDa) with respect to the nuclear-encoded and mitochondrially located voltage-dependent anion channel protein (V-DAC; 31 kDa) to establish the relative COX-II abundance per mitochondria (COX-II/V-DAC) [18].

Mitochondrial respiratory chain complex II, glycerol-3-phosphate dehydrogenase + complex III (G3Pdh + III) and complex IV (COX) enzyme activity

To evaluate mitochondrial function, all mitochondrial enzymatic activities were measured by spectrophotometry according to the Rustin *et al.* [34] and Miró *et al.* [35] methodology. Enzymatic activity of MRC complex II was used as the control parameter supposedly unaffected by antiretroviral drugs because it is completely encoded, transcribed and translated by cytoplasmic machinery independent of mitochondria, whereas MRC complex III (CIII) and complex IV (CIV) are partially encoded, transcribed and translated by mitochondrial means. Specific enzymatic activities were expressed in absolute values as nanomols of synthesized substrate or consumed product per minute and milligram of protein (nmols/min/mg protein).

Mitochondrial mass

To assess mitochondrial number we performed the spectrophotometric measurement of citrate synthase

activity [citrate synthase (CS): EC 4.1.3.7], a mitochondrial enzyme of the Krebs's cycle widely considered as a reliable marker of mitochondrial content [34].

Apoptotic studies

To evaluate apoptotic cell death we performed Western blot analysis of 20 µg of total cell protein by 7/13% SDS-PAGE electrophoresis and posterior immunoblot analysis of active (cleaved) caspase-3 pro-apoptotic protein expression (17–19 KDa) normalized by the content on β-actin protein signal (47 KDa) as a cell loading control. Chemiluminescence results were expressed as caspase-3/β-actin relative content and were interpreted as a marker of advanced apoptotic events.

Statistical analysis

Clinical and epidemiological parameters were expressed as means and range interval and experimental results as means and standard error of the mean (SEM) or as a percentage of increase/decrease with respect to healthy controls.

Adverse perinatal outcome, mitochondrial and apoptotic results of HIV-infected women and newborn were compared to those of uninfected women and their children to assess the presence of obstetric/perinatal problems and mitochondrial or apoptotic damage due to HIV infection and ART. Additionally, different correlations were sought between: molecular and functional mitochondrial parameters (to ascertain dependence of proper mitochondrial function on mitochondrial genome depletion); mother-to-child mitochondrial or apoptotic parameters (to determine maternal influence on newborn cellular status); and clinical and experimental data (to assess mitochondrial or apoptotic basis of obstetric problems and perinatal outcome).

Differences between cases and controls and correlations among quantitative parameters were assessed using nonparametric tests: Mann–Whitney analysis to search for independent sample differences, chi-squared test to calculate odds ratio (OR) values [OR; 95% confidence interval (CI); significance] and the Spearman's rank coefficient for parameter correlation (R^2 and significance). The level of significance was set at 0.05 for all the statistical tests.

Results

Clinical data

The clinical and epidemiological characteristics of the study participants and immunovirological and therapeutic data of HIV mothers are summarized in Table 1.

All pregnant women were mainly of Caucasian origin, with an age ranging from 25 to 42 years. Nonsignificant

differences were observed in maternal epidemiological data between HIV-positive and HIV-negative women.

For HIV participants, the mean time of HIV infection and HAART treatment prior to delivery was 84 and 48 months, respectively.

Most HIV women were under HAART before pregnancy (85%) and only four cases (15%) were naive for ART and started HAART at the second trimester of gestation. HAART was given to all patients at the time of delivery to avoid MTCT and consisted of two NRTI and either one protease inhibitor or one NNRTI (see Table 1 for percentages of treatment assignment).

At delivery, all women had received at least 6 months of double-NRTI treatment and the mean CD4⁺ T-cell count was 560 cells/µl with an undetectable viral load.

Perinatal outcomes

The obstetric and neonatal outcomes of the study cohort are detailed in Table 2.

All infants were HIV-uninfected with no clinical symptoms compatible with mitochondrial toxicity. Gestational diabetes mellitus, gestational age at delivery and neonatal intensive care unit admission tended to be increased, albeit not significantly, among HIV pregnancies, together with the reduction of birth weight at delivery.

Additionally, global adverse perinatal outcomes were significantly more frequent among HIV-positive pregnancies (11/27 vs. 3/35, OR 7.33; 95% CI 1.8–30.1; $P=0.048$). The overall prematurity rate was also significantly increased for newborns to HIV mothers (7/27 vs. 2/35, OR 5.77; 95% CI 1.1–30.6; $P=0.034$) with 57% (4/7) of the premature deliveries being at less than 35 weeks, 50% of which (2/4) were at less than 32 weeks. Finally, the number of infants who were small for gestational age was also significantly increased among HIV pregnancies (6/27 vs. 1/35, OR 9.71; 95% CI 1.1–86.5; $P=0.036$).

Mitochondrial and apoptotic maternal peripheral blood mononuclear cell analysis

Compared with uninfected controls, HIV-pregnant women showed a marked but nonsignificant trend to a decreased mtDNA content of 26.3% ($P=NS$, and Table 1a).

HIV-pregnant women also exhibited a significant decrease in the mitochondrial protein synthesis rate (25.9% COX-II/V-DAC content reduction, $P=0.042$; and Table 1a and b) and all MRC enzyme activities (18.5% for CIV and 31.2% for G3Pdh + CIII function diminution; $P=NS$ and $P=0.049$, respectively; and Table 1a). The only mitochondrial enzymatic activity

Table 1. Clinic, epidemiologic, immunovirologic and therapeutic characteristics of HIV-infected and uninfected pregnant women.

	HIV-positive (n = 27)	HIV-negative (n = 35)	P
Maternal age at delivery ^a	34.7 (27–42)	33.7 (25–41)	NS
HCV infection [N (%)]	3 (11.1)	1 (2.6)	NS
Illicit drug use [N (%)]	0 (0)	0 (0)	–
Alcohol use [N (%)]	0 (0)	0 (0)	–
HIV RNA copies per ml at delivery ^a	62.3 (49–250)	–	–
CD4 ⁺ T-cell count per µl at delivery ^a	560.2 (97–1242)	–	–
NRTI prior pregnancy (months) ^a	48 (0–106)	–	–
NNRTI prior pregnancy (months) ^a	3 (0–86)	–	–
PI prior pregnancy (months) ^a	12 (0–97)	–	–
Time from diagnosis of HIV infection to delivery (months) ^a	84 (4–228)	–	–
HAART second and third trimesters			
2NRTI + 1PI [No. (%)]	4 (15)	–	–
HAART all trimesters			
2 NRTI + 1 PI [No. (%)]	15 (55.5)	–	–
2 NRTI + 1 NNRTI [N (%)]	8 (29.5)	–	–

HCV, hepatitis C virus; HIV, human immunodeficiency virus; NRTI, nucleoside-analogue reverse transcriptase inhibitor; NNRTI, non-NRTI reverse transcriptase inhibitor; NS, not significant; PI, protease inhibitors; RNA, ribonucleic acid.

^aData are presented as means and range interval.

which was preserved in HIV women was MRC complex II (increased 13.7% compared to controls, $P=NS$), used as a control parameter because it is independent to mtDNA depletion.

No differences were found in mitochondrial mass (CS activity) between cases and controls and, consequently, mtDNA content, mitochondrial protein synthesis or MRC enzymatic activities maintained the same trends when normalized to mitochondrial mass (data not shown).

HIV-infected pregnant women presented a marked, albeit nonsignificantly increased apoptotic rate of 100% compared with healthy controls ($P=NS$; and Table 1a and b).

Maternal mtDNA levels and mitochondrial function (G3Pdh + CIII activity) were positively and significantly correlated (Fig. 2).

No significant correlation was, however, found between maternal experimental (mitochondrial or apoptotic) parameters and clinical results (including perinatal

outcome, immunological status, ART or maternal age), except on considering time on NRTI treatment before pregnancy. Women exposed to NRTI for over 100 months presented a lower mtDNA content with respect to those under-exposed (88 vs. 55% remaining mtDNA content compared to controls, $P=0.048$).

Mitochondrial and apoptotic newborn cord blood mononuclear cell analysis

Infants born from HIV women exposed *in utero* to HIV and ART showed a significant decrease in mtDNA content of 38.6% compared with uninfected controls ($P=0.006$; and Table 1a).

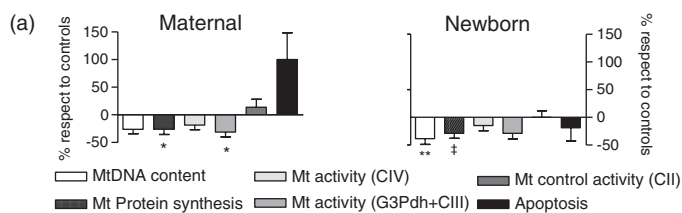
Similar to their mothers, infants born to HIV women also exhibited a significant decrease in mitochondrial protein synthesis (28.9% COX-II/V-DAC content reduction, $P=0.045$; and Table 1a and b) and a trend towards a decrease in all MRC enzyme activities compared to controls (14.8% for CIV and 28.9% for G3Pdh + CIII activity diminution, $P=NS$ in both cases; and Table 1a). According to its control function, the only mitochondrial enzymatic activity which remained unchanged between

Table 2. Obstetric and neonatal outcomes of the study cohorts.

	HIV-positive (n = 27)	HIV-negative (n = 35)	OR [95% CI]
Gestational diabetes mellitus [N (%)]	2 (7.4)	1 (2.5)	2.72 [0.2–31.7]
Preeclampsia [N (%)]	0 (0)	0 (0)	–
Fetal death [N (%)]	0 (0)	0 (0)	–
Gestational age at delivery (weeks) ^a	37.5 (32.2–41.2)	39.2 (38.5–40.4)	–
Preterm birth (<37 weeks of gestation) ^a	7 (25.9)	2 (5.7)	5.77 [1.1–30.6]
Birth weight (g) ^a	2879 (1940–4040)	3165 (3130–3350)	–
Small for gestational age (<10th percentile) [N (%)]	6 (22.2)	1 (2.8)	9.71 [1.1–86.5]
5-min Apgar score <7 [N (%)]	0 (0)	0 (0)	–
Neonatal intensive care unit admission [N (%)]	3 (11.1)	1 (2.8)	4.25 [0.4–43.4]
Global adverse perinatal outcome [N (%)]	11 (40.7)	3 (8.5)	7.33 [1.8–30.1]

95% CI, 95% confidence interval of the mean; No., number; NS, not significant; OR, odds ratio.

^aData are presented as means and range interval.



	Pregnant women		Newborn	
	Control	HIV-infected	Control	HIV-mothers
MtDNA (ND2mtDNA/18SrRNA nDNA)	4.45±0.59	3.28±0.35	3.06±0.26	1.88±0.32
	-26.3%, p=NS		-38.6%, p=0.006	
Mt Protein Synthesis (COX-II/V-DAC relative units)	1.43±0.23	1.06±0.14	1.80±0.34	1.28±0.16
	-25.9%, p=0.042		-28.9%, p=0.045	
Mt activity CIV (nmol/min.mg prot)	47.72±5.91	38.89±3.90	43.69±5.54	37.24±4.10
	-18.5%, p=NS		-14.8%, p=NS	
Mt activity G3Pdh+CIII (nmol/min.mg prot)	27.89±3.58	19.19±2.41	21.84±4.75	15.52±2.26
	-31.2%, p=0.049		-28.9%, p=NS	
Mt control activity CII (nmol/min.mg prot)	22.24±2.33	25.28±3.22	28.38±3.41	28.36±3.36
	+13.7%, p=NS		-0.1%, p=NS	
Apoptosis (Caspase-3/β-Actine relative units)	0.18±0.05	0.36±0.09	0.56±0.13	0.46±0.13
	+100%, p=NS		-18.9%, p=NS	

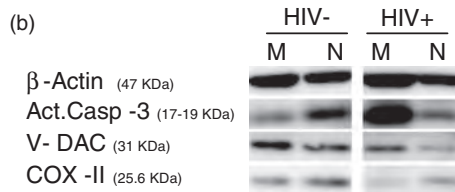


Fig. 1. Mitochondrial and apoptotic parameters. (a) Percentage of increase/decrease of mitochondrial parameters in HIV participants with respect to mean values of uninfected controls. MtDNA, mitochondrial DNA; Mt Protein synthesis, mitochondrial protein synthesis (COX-II/V-DAC); CIV, G3Pdh + CIII or CII enzymatic activity, mitochondrial respiratory chain complex IV, glycerol-3-phosphate dehydrogenase and complex III or complex II enzymatic function, respectively; apoptosis, caspase cleavage and activation (caspase-3/β-Actin). (*) $P = 0.042$; (●) $P = 0.049$; (†) $P = 0.006$; (‡) 0.045. (b) Western blot for mitochondrial protein synthesis and apoptosis quantification. HIV±: human immunodeficiency virus infected/not-infected pregnant-women; M/N: mothers/newborn.

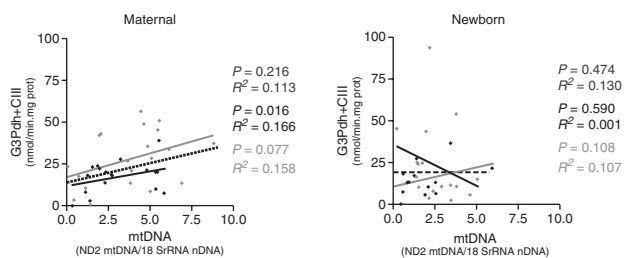


Fig. 2. Correlation between genetic and functional mitochondrial parameters. Association between genetic and functional parameters is shown in the graph for HIV-pregnant women or newborn from HIV pregnancies (black line and significance), for control women and newborn (grey line and significance) and for all included women or children (discontinued line and significance in bold).

cases and controls was complex II (0.1% decreased in HIV and HAART-exposed infants, $P = NS$).

Again, no differences were found in mitochondrial mass (CS activity) between cases and controls and, consequently, mtDNA content, mitochondrial protein synthesis or MRC function preserved identical trends when normalized to mitochondrial mass (data not shown).

Contrarily to their mothers, however, newborn mtDNA levels and mitochondrial function (G3Pdh + CIII activity) were not correlated (Fig. 2) and seronegative infants born from HIV women presented an unaltered apoptotic rate (18.9% decreased) with respect to controls ($P = NS$; and Table 1a and b).

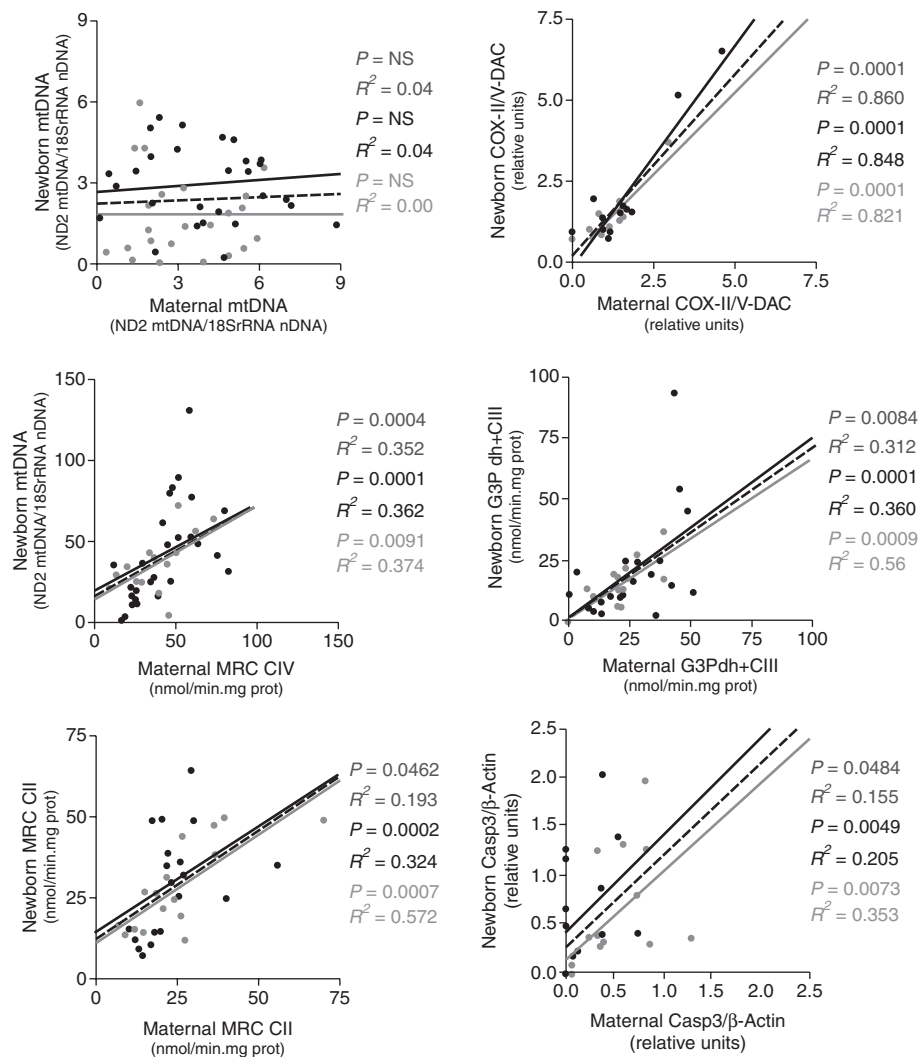


Fig. 3. Correlation between materno-fetal mitochondrial or apoptotic parameters. Association between quantitative materno-fetal parameters are shown in the graphs for HIV patients (grey line and significance), uninfected controls (black line and significance) or for both patients and controls (discontinued line and significance in bold). MtDNA, mitochondrial DNA; Mt protein synthesis, mitochondrial protein synthesis (COX-II/V-DAC); MRC CIV, G3Pdh + CIII or CII enzymatic activity, mitochondrial respiratory chain complex IV, glycerol-3-phosphate dehydrogenase and complex III or complex II enzymatic function; Casp3/β-actin, apoptotic development (caspase-3/β-actin).

Additionally, there was a positive and significant correlation between all maternal and fetal mitochondrial and apoptotic parameters except in mtDNA content, either considering exclusively HIV cases, controls or both (Fig. 3).

No significant correlation was, however, found between newborn mitochondrial parameters and maternal immunological status, ART regimen, age or adverse perinatal outcome.

Discussion

Animal models have shown evidence of mitochondrial toxicity from in-utero NRTI exposure [23]. However,

clinical evidence of mitochondrial toxicity has largely been lacking in HIV mothers and their infants and there is conflicting evidence regarding the association of in-utero HIV and ART exposure with mortality and morbidity due to mitochondrial dysfunction [24–32]. We observed a reduced mtDNA content and derived protein synthesis and MRC function in both PBMC of HIV-pregnant women at delivery receiving ART as well as in CBMC of their in-utero-exposed newborns. Although not all mitochondrial parameters showed statistically significant differences between cases and controls, all measures were reduced in HIV-pregnant women and their newborn, suggesting NRTI or HIV-mediated mitochondrial lesion. Our results are in concordance with the studies reporting mtDNA depletion in newborn from HIV-infected and treated mothers [26–29]. However, none of these studies

parallelly evaluated mitochondrial number, mtDNA-encoded MRC protein expression and mitochondrial function in HIV and HAART-exposed newborn. The present work strengthens the mitochondrial toxicity hypothesis by confirming that fetal mtDNA depletion is not an isolated finding and subclinical mitochondrial lesion is present in several markers of mitochondrial function downstream from mitochondrial genetics.

Although previously reported in nonpregnant adults [16–18], this is the first time that similar results have been reported in HIV-infected and treated pregnant women. Mitochondrial lesion in oocytes of HIV-infected and treated women has previously been suggested to play an etiopathological role in the infertility of these women [17] and could, potentially, be the cause of their associated obstetric problems.

Whether mitochondrial lesion in both infected mothers and their newborn is due to HIV or ART is still a matter of doubt because, in our study, all HIV-pregnant women received ART prior delivery following international guidelines. Interestingly, we found increased mtDNA depletion in HIV-pregnant women with NRTI administration extended over 100 months, suggesting NRTI-mediated injury. However, HIV implication in mitochondrial lesion can not be discarded, especially because apoptosis was increased in HIV-infected women. HIV-derived mitochondrial toxicity has been reported to be indirectly caused by the activation of apoptotic pathways [19–21]. To our knowledge, apoptotic studies have never previously been performed in HIV mothers and their infants. We observed that apoptotic events tend to be increased in HIV-pregnant women but remain unaltered in their newborn. This finding, although not significant, suggests that HIV itself could trigger the apoptotic phenomenon exclusively in HIV-infected pregnant women, whereas their infants, which are uninfected because of the therapeutic activity of HAART along pregnancy, show an unaltered apoptotic rate. Further studies are required to elucidate if apoptotic lesion is also present in naive pregnant women and in HIV-infected newborns. If apoptotic lesions are increased in these populations, obstetric problems of HIV pregnancies could be attributed, at least in part, to apoptotic means, thereby confirming the prevention of not only MTCT of HIV infection but also HIV-mediated apoptosis in newborn from HIV-infected women with the use of HAART.

Despite studying maternal and newborn mitochondrial and apoptotic parameters in different kinds of tissues (PBMCs and CBMCs, respectively), all were positively and significantly correlated, except for mtDNA content. We attribute this concordance to the maternal heritage of mitochondria organelles, exclusively provided by the oocyte along the fecundation process [17].

In our opinion, different theories may explain materno-fetal correlation of mitochondrial parameters and strengthen the hypothesis of NRTI-mediated mitochondrial toxicity, in detriment of HIV-induced mitochondrial damage. First, mitochondrial injury in maternal blood cells could limit its transplacental function once maternal blood is in the fetus. Second, mitochondrial toxicity may be nonexclusively restricted to maternal cells and could also be present in tissues more related to fetal development (including placenta) [29]. Finally, NRTIs cross the placenta [36], becoming direct fetal-damaging agents.

Interestingly, maternal mtDNA levels and function positively correlated, demonstrating the dependence of mitochondrial functionalism on mitochondrial genetics. However, mtDNA levels and function were independent parameters in their newborn, with no trend towards association, especially in infant born from HIV pregnancies. This finding may explain why maternal and fetal mtDNA levels were the only parameters not associated between mother and child; mtDNA levels seem to be independently regulated in maternal and newborn compartments, perhaps due to distinct biological needs or capacities of response to toxic exposure. In our HIV cohort, global adverse perinatal outcome, preterm birth and frequency of small for gestational age newborns were significantly increased. However, in these women and children, mitochondrial and/or apoptotic compromise was not increased compared to uninfected controls or the remaining HIV cohort. Although all these adverse outcomes are unspecific of HIV and HAART and could be associated with other pathological situations, with respect to HIV pregnancies, our findings failed to demonstrate that mitochondria or apoptosis disturbances played a role in infant outcome. This assertion does not rule out the possibility that mitochondrial dysfunction in newborns could contribute to the future development of metabolic problems in adulthood, independently of its subclinical perinatal consequences [37].

The study may have some limitations. First, the analysis of mononuclear cells could show different mitochondrial and apoptotic results compared to those present in other tissues directly related to pregnancy development. Second, the small size of our sample could hinder the testing of our hypothesis (mitochondrial or apoptotic basis of adverse perinatal outcome), independently of its veracity. Third, lack of pregnant HIV women naive for HAART or HIV-infected children could limit the assessment of HIV-implication in adverse perinatal outcome. Fourth, lack of newborn symptomatic of mitochondrial toxicity at delivery could reduce the presence of molecular lesion in our infant population. Fifth, although all treated women had received at least 6 months of double-NRTI schedules, previous and current treatment options were not completely uniform and the results could therefore differ in alternative treatment interventions. Finally, we cannot discard the possibility that adverse effects of HIV or

HAART, alternative to mitochondrial dysfunction or apoptosis, may play a role.

Despite these limitations, we can, however, conclude that ART administration along pregnancy may cause the transplacental subclinical mitochondrial lesions observed in both pregnant women and their in-utero-exposed newborn. This finding is confirmed by materno-fetal correlated mitochondrial protein synthesis and enzymatic function. On the contrary, antiretroviral drugs may prevent newborn apoptotic lesions, thereby leading to beneficial effects independent of MTCT prevention.

In conclusion, no relationship was found between mitochondrial and apoptotic abnormalities and adverse perinatal outcome. However, mitochondrial toxicity associated with NRTI administration and newborn morbidity should be further investigated in tissues directly related to pregnancy in larger cohorts of patients including naive pregnant HIV women or women under NRTI-sparing regimens, as well as HIV-infected newborn and newborn with symptomatic manifestation of mitochondrial or apoptotic lesion.

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S.H., M.L. and O.C. performed patient inclusion and follow-up and collected samples and all the clinical information of the study participants. C.M., F.C., O.M. and G.G. processed samples and performed the experimental analysis of experimental parameters. All authors participated in the study design, statistical and result analyses and manuscript production.

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Conflicts of interest

None of the authors has any financial, consultant, institutional and other relationship that might lead to bias or a conflict of interest for the present manuscript.

References

- Volmink J, Siegfried NL, van der Merwe L, Brocklehurst P. **Antiretrovirals for reducing the risk of mother-to-child transmission of HIV infection.** *Cochrane Database Syst Rev* 2007; CD003510.
- Watts DH. **Management of human immunodeficiency virus infection in pregnancy.** *N Engl J Med* 2002; **346**:1879–1891.
- Public Health Service Task Force. **Recommendations for the use of antiretroviral drugs in pregnant HIV-1 infected women for maternal health and interventions to reduce perinatal HIV-1 transmission in the United States.** *MWWR* September 22, 2003.
- Sturt AS, Dokubo EK, Sint TT. **Antiretroviral therapy for treating HIV infection in ART-eligible pregnant women.** *Cochrane Database Syst Rev* 2010; CD0008440.
- Brocklehurst P, French R. **The association between maternal HIV infection and perinatal outcome: a systematic review of the literature and metaanalysis.** *Br J Obstet Gynaecol* 1998; **105**:836–848.
- Thorne C, Patel D, Newell ML. **Increased risk of adverse pregnancy outcomes in HIV-infected women treated with highly active antiretroviral therapy in Europe.** *AIDS* 2004; **18**:2337–2339.
- Tuomala RE, Shapiro DE, Mofenson LM, Bryson Y, Culnane M, Hughes MD, et al. **Antiretroviral therapy during pregnancy and the risk of an adverse outcome.** *N Engl J Med* 2002; **346**:1863–1870.
- Tuomala RE, Watts DH, Li D, Vajaranant M, Pitt J, Hammill H, et al. **Improved obstetric outcomes and few maternal toxicities are associated with antiretroviral therapy, including highly active antiretroviral therapy during pregnancy.** *J Acquir Immune Defic Syndr* 2005; **38**:449–473.
- Lambert JS, Watts DH, Mofenson L, Stiehm ER, Harris DR, Bethel J, et al. **Risk factors for preterm birth, low birth weight, and intrauterine growth retardation in infants born to HIV-infected pregnant women receiving zidovudine.** *Pediatric AIDS Clinical Trials Group 185 Team.* *AIDS* 2000; **14**:1389–1399.
- Suy A, Martinez E, Coll O, Lonca M, Palacio M, de Lazzari E, et al. **Increased risk of preeclampsia and fetal death in HIV-infected pregnant women receiving highly active antiretroviral therapy.** *AIDS* 2006; **20**:59–66.
- Wimalasundera RC, Larbalestier N, Smith JH, de Ruiter A, McG Thom SA, Hughes AD, et al. **Preeclampsia, antiretroviral therapy, and immune reconstitution.** *Lancet* 2002; **360**:1152–1154.
- Rudin C, Spaenhauer A, Keiser O, Rickenbach M, Kind C, Aebi-Popp K, et al. **Antiretroviral therapy during pregnancy and preterm birth: analysis of Swiss data.** *HIV Med* 2011; **12**:228–235.
- Haeri S, Shauer M, Dale M, Leslie J, Baker AM, Saddlemire S, Boggess K. **Obstetric and newborn infant outcomes in human immunodeficiency virus-infected women who receive highly active antiretroviral therapy.** *Am J Obstet Gynecol* 2009; **201**:315.e1–315.e5.
- Townsend CL, Cortina-Borja M, Peckham CS, Tookey PA. **Antiretroviral therapy and premature delivery in diagnosed HIV-infected women in the United Kingdom and Ireland.** *AIDS* 2007; **21**:1019–1026.
- Szyld EG, Warley EM, Freimanis L, Gonin R, Cahn PE, Calvet GA, et al. **Pregnancy outcome in women infected with HIV-1 receiving combination antiretroviral therapy before versus after conception.** *AIDS* 2006; **20**:2345–2353.
- Lewis W, Dalakas MC. **Mitochondrial toxicity of antiviral drugs.** *Nat Med* 1995; **1**:417–422.
- Lopez S, Coll O, Durban M, Hernández S, Vidal R, Suy A, et al. **Mitochondrial DNA depletion in oocytes of HIV-infected antiretroviral-treated infertile women.** *Antivir Ther* 2008; **13**:833–838.
- Garrabou G, Morén C, Gallego-Escudero JM, Milinkovic A, Villarroya F, Negro E, et al. **Genetic and functional mitochondrial assessment of HIV-infected patients developing HAART-related hyperlactatemia.** *J Acquir Immune Defic Syndr* 2009; **52**:443–451.
- Miró O, López S, Martínez E, Pedrol E, Milinkovic A, Deig E, et al. **Mitochondrial effects of HIV infection on the peripheral blood mononuclear cells of HIV-infected patients who were never treated with antiretrovirals.** *Clin Infect Dis* 2004; **39**:710–716.
- Yao XJ, Moulard AJ, Subbramanian RA, Forget J, Rougeau N, Bergeron D, Cohen EA. **Vpr stimulates viral expression and induces cell killing in human immunodeficiency virus type 1-infected dividing Jurkat T cells.** *J Virol* 1998; **72**:4686–4693.
- Wan ZT, Chen XL. **Mechanisms of HIV envelope-induced T lymphocyte apoptosis.** *Virol Sin* 2010; **25**:307–315.
- Hooker DJ, Gorry PR, Ellett AM, Wesselingh SL, Cherry CL. **Measuring and monitoring apoptosis and drug toxicity in HIV patients by ligation-mediated polymerase chain reaction.** *J Cell Mol Med* 2009; **13**:948–958.

23. Gerschenson M, Nguyen V, Ewings EL, Ceresa A, Shaw JA, St Claire MC, *et al.* **Mitochondrial toxicity in fetal Erythrocebus patas monkeys exposed transplacentally to zidovudine plus lamivudine.** *AIDS Res Hum Retroviruses* 2004; **20**:91–100.
24. Blanche S, Tardieu M, Rustin P, Slama A, Barret B, Firton G, *et al.* **Persistent mitochondrial dysfunction and perinatal exposure to antiretroviral nucleoside analogues.** *Lancet* 1999; **354**:1084–1089.
25. Barret B, Tardieu M, Rustin P, Lacroix C, Chabrol B, Desguerre I, *et al.* **Persistent mitochondrial dysfunction in HIV-1-exposed but uninfected infants: clinical screening in a large prospective cohort.** *AIDS* 2003; **17**:1769–1785.
26. Divi RL, Walker VE, Wade NA, Nagashima K, Seilkop SK, Adams ME, *et al.* **Mitochondrial damage and DNA depletion in cord blood and umbilical cord from infants exposed in utero to combivir.** *AIDS* 2004; **18**:1013–1021.
27. Aldrovandi GM, Chu C, Shearer WT, Li D, Walter J, Thompson B, *et al.* **Antiretroviral exposure and lymphocyte mtDNA content among uninfected infants of HIV-1-infected women.** *Pediatrics* 2009; **124**:e1189–e1197.
28. Poirier MC, Divi RL, Al-Harhi L, Olivero OA, Nguyen V, Walker B, *et al.* **Long-term mitochondrial toxicity in HIV-uninfected infants born to HIV-infected mothers.** *J Acquir Immune Defic Syndr* 2003; **33**:175–183.
29. Shiramizu B, Shikuma KM, Kamemoto L, Gerschenson M, Erdem G, Pinti M, *et al.* **Placenta and cord blood mitochondrial DNA toxicity in HIV-infected women receiving nucleoside reverse transcriptase inhibitors during pregnancy.** *J Acquir Immune Defic Syndr* 2003; **32**:370–374.
30. Vigano A, Bianchi R, Schneider L, Cafarelli L, Tornaghi R, Pinti M, *et al.* **Lack of hyperlactatemia and impaired mitochondrial DNA content in CD4+ cells of HIV-uninfected infants exposed to perinatal antiretroviral therapy.** In: *11th Conference on Retroviruses and Opportunistic Infections*. San Francisco, CA; 2004.
31. Cote HC, Raboud J, Bitnun A, Alimenti A, Money DM, Maan E, *et al.* **Perinatal exposure to antiretroviral therapy is associated with increased blood mitochondrial DNA levels and decreased mitochondrial gene expression in infants.** *J Infect Dis* 2008; **198**:851–859.
32. McComsey GA, Kang M, Ross AC, Lebrecht D, Livingston E, Melvin A, *et al.* **Increased mtDNA levels without change in mitochondrial enzymes in peripheral blood mononuclear cells of infants born to HIV-infected mothers on antiretroviral therapy.** *HIV Clin Trials* 2008; **9**:126–136.
33. Bradford MM. **A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding.** *Anal Biochem* 1976; **72**:248–254.
34. Rustin P, Chretien D, Bourgeron T, Gérard B, Rötig A, Saudubray JM, Munnich A. **Biochemical and molecular investigations in respiratory chain deficiencies.** *Clin Chim Acta* 1994; **228**:35–42.
35. Miró Ò, Cardellach F, Barrientos A, Casademont J, Rötig A, Rustin P. **Cytochrome c oxidase assay in minute amounts of human skeletal muscle using single wavelength spectrophotometers.** *J Neurosci Methods* 1998; **80**:107–111.
36. Poirier MC, Patterson TA, Slikker W Jr, Olivero OA. **Incorporation of 3'-azido-3'-deoxythymidine (AZT) into fetal DNA and fetal tissue distribution of drug after infusion of pregnant late-term rhesus macaques with a human-equivalent AZT dose.** *J Acquir Immune Defic Syndr* 1999; **22**:477–483.
37. Gemma C, Sookoian S, Alvariñas J, García SI, Quintana L, Kanevsky D, *et al.* **Mitochondrial DNA depletion in small- and large-for-gestational-age newborns.** *Obesity (Silver Spring)* 2006; **14**:2193–2199.