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Mitochondrial DNA content of peripheral blood mononuclear cells in ART untreated & stavudine/zidovudine treated HIV-1-infected patients

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***Background & objectives*: Nucleoside reverse transcriptase inhibitors (NRTIs) are known to cause mitochondrial toxicity. This study was done to estimate mitochondrial DNA (mtDNA) content of peripheral blood mononuclear cells (PBMCs) among human immunodeficiency virus (HIV) infected, NRTI treated and antiretroviral therapy (ART)-naïve patients and evaluate the utility of mtDNA content as a biomarker of mitochondrial toxicity.**

***Methods*: mtDNA content in PBMCs of 57 HIV-infected ART untreated and 30 ART treated with stavudine (d4T) or zidovudine (AZT) containing regimen were compared against 24 low-risk healthy controls (LoRHC).**

***Results*: There was a significant (*P*=0.01) reduction in mtDNA content among HIV-infected (104; 80-135) compared to LoRHC (127; 110-167), and it was the same in both the treated (104.8; 88-130) and untreated patients (104.7; 78-142). mtDNA significantly (*P*=0.014) declined in ART treated patients symptomatic for toxicity (97; 74-111) than the asymptomatic patients (128; 103- 153).**

***Interpretation & conclusions*: mtDNA depletion in PBMCs was evident among HIV-infected individuals on ART. Moreover, as mtDNA content was reduced among the patients symptomatic for toxicity than the asymptomatic in both the HIV-infected groups, the current study supports mtDNA content of PBMCs to serve as a biomarker of mitochondrial dysfunction induced by NRTI and HIV. Longitudinal studies with a large sample need to be done to confirm these findings.**

Key words ART naïve - HIV - mitochondrial dysfunction - mitochondrial DNA - nucleoside reverse transcriptase inhibitors - peripheral blood mononuclear cells - toxicity

Nucleoside reverse transcriptase inhibitors (NRTI) occupy a vital position in antiretroviral (ARV) regimen of both first and second line highly active antiretroviral therapy (HAART) but due to their long-term toxicity profile there are attempts such as, NRTI sparing regimens to simplify therapy, mitigate toxicity and to avoid resistance issues, but to date none of such studies have resulted in better efficacy and safety

than the standardized first-line treatment[1] containing NRTI, which underscores the importance of NRTI in the treatment of HIV for years to come. Among the nucleoside/nucleotide RT inhibitors, stavudine (d4T) is known to cause increased mitochondrial dysfunction followed by lamivudine (3TC), tenofovir (TDF), zidovudine (AZT) and carbovir (CBV)[2]. Although phasing out of d4T was implemented by WHO in 2010, the other mitochondrial toxic agents are still in use, moreover, d4T is recommended in conditions where AZT, TDF and CBV cannot be used albeit for a shorter period with close monitoring[3].

As the occurrence of NRTI associated adverse events is one of the common reasons for modifying therapy, more studies are needed for the early detection of toxicity. NRTI, due to their low cost is the most commonly used ARVs especially in resource-limited settings (RLS); the mechanism of NRTI-induced mitochondrial dysfunction includes DNA *pol* γ inhibition, mitochondrial oxidative stress and mitochondrial DNA (mtDNA) mutation[4]. mtDNA in peripheral blood mononuclear cells (PBMCs) as a biomarker of mitochondrial toxicity has been proposed[5]. The interrelationships between mtDNA depletion, NRTIs and adverse effects associated with mitochondrial dysfunction have been studied by several research groups. Although many studies[6,7] have documented depletion of mtDNA of PBMC, yet some have reported mtDNA to increase in patients receiving NRTI[8] in comparison to controls. As factors such as persistent immune activation and cell proliferation in HIV infection might influence the various cell subtypes within PBMCs differently, that are reflected on relative mtDNA content in each cell subtype, it is more appropriate to study PBMC subsets for NRTI toxicity markers.

Research to unfold the molecular mechanisms involved in the changes of kinetics of mtDNA, mitochondrial dysfunction and NRTI associated adverse events has expanded in the past few years. P53R2, the ribosomal protein regulated by p53, is found to be involved in the replication of mtDNA[9], decreased P53R2 expression parallels mtDNA loss and over expression of TK2 accelerates the process of mitochondrial toxicity. Telomerase reverse transcriptase's catalytic activity at the mitochondria may protect mtDNA against oxidative stress[10] and prevent mitochondrial dysfunction[11] in a telomere synthesis independent manner and NRTIs exhibit inhibitory potencies on telomerase enzyme[12] AZT and d4T were found to impair the synthesis of all the immunoglobulin classes with concomitant depletion of mtDNA in primary human B lymphocytes, a finding that could contribute to incomplete recovery of B cell responses under HAART[13]. *In vitro* experiments aimed at validating mitochondrial proteomic showed that downregulation of mitochondrial chaperon prohibitin was a causative event in NRTI-induced mitochondrial damage[14].

This cross-sectional study was intended to evaluate the changes in mtDNA content in mitochondrial toxicity induced by NRTI, eventually to determine the utility of mtDNA content as a novel biomarker for NRTI toxicity. For this, mtDNA levels from PBMCs of HIV-infected ART treated, ART untreated and low-risk healthy controls (LoRHC) were compared with the routine markers of toxicity monitoring.

Material & Methods

The study was carried out from 2011 to 2014 at YRG Centre for AIDS Research and Education (YRG CARE), Chennai, India. Patient recruitment for the present study was done at YRG CARE hospital between February 2012 and April 2013. The protocol was approved by Institutional Review Board of YRG CARE, Chennai, and written informed consent and questionnaire for the demographic details of the participants including their ART regimen were obtained.

The sample size was calculated with 90 per cent confidence level, margin of error of 5 per cent with the expected NRTI-induced lipodystrophy (3.2%) reported from the previous study[15] for which the recommended sample size was 34. We restricted the sample size to 30 for ART treated and to 57 for ART- naïve group. Consecutive HIV-1-infected patients fulfilling the study criteria were enrolled. A total of 30 HIV-infected participants who were treated with non-nucleoside analogue reverse transcriptase inhibitor (NNRTI)-based first-line HAART containing two NRTI (d4T or AZT) backbone for more than six months were enrolled under the ART-treated group; 57 HIV-infected ART naïve participants who were expected to have a CD4 T cell count of >500 cells/µl were enrolled in the ART naïve group. Further, 24 low-risk healthy controls (volunteers from laboratory staff) were also enrolled. Patients with opportunistic infections, neoplasm, pregnancy, allergic drug hypersensitivity, with a known history

of mitochondrial disease, those receiving other mitochondrial toxic drugs such as aminoglycosides and statins, habitual smokers and alcoholics were excluded from the study.

Blood specimen (20 ml) was obtained in the morning after a 12 h fasting state. For lactate estimation blood was collected without tourniquet in sodium fluoride (grey-top) tubes and plasma was separated within 30 min. Complete blood count in Sysmex XT-1800i (Kobe, Japan), CD4 T cell count by Pan Leukocyte Gating in Cytomics FC500, Beckman Coulter (Miami Florida Inc., USA), and HIV-1 RNA viral load testing by Abbott m2000rt (Abbott Molecular Inc., IL, USA) real-time polymerase chain reaction (PCR) were performed. All the biochemistry testings were analyzed in Olympus AU 400 Clinical Chemistry Autoanalyser (Olympus optical, Japan). PBMCs were isolated from fresh blood by Ficoll overlay method[16]. Live cells were thawed and checked for platelet contamination, PBMCs with platelets >1 per cent were washed twice with phosphate buffered saline (PBS) to remove platelets.

In the ART-treated group, based on clinical and laboratory findings, 18 patients were symptomatic [AIDS clinical trials group (ACTG) toxicity grade 1 and above] for NRTI toxicity, and 12 were asymptomatic. The symptomatic patients were identified with the help of clinicians and were discerned that the symptomatic clinical condition was not because of any opportunistic infection or due to other comorbidities. Among the symptomatic ART treated patients, four had lipodystrophy (body shape changes (subcutaneous lipoatrophy and fat accumulation) assessed on the basis of patient's self-report and direct physical examination. Weight and body mass index (BMI) were routinely determined, and two had peripheral neuropathy (bilateral, distal sensory axonal neuropathy with burning sensation and painful dysesthesias), five had pancreatitis [elevated pancreatic amylase and lipase, >1.0 × upper limit of normal (ULN)] and four had hepatotoxicity [elevated aspartate aminotransferase (AST) and alanine transaminase (ALT), >1.25 × ULN], six had hyperlactatemia (lactate value >2.2 mmol/l) and nine had dyslipidaemia [triglycerides: >400 mg/dl, low-density lipoprotein (LDL): >130 mg/dl and total cholesterol (TC): >200 mg/dl]. ART naïve group was also subdivided into symptomatic (n=17) and asymptomatic (n=40) patients based on biochemistry parameters related to mitochondrial toxicity according to ACTG toxicity grading[17].

Mitochondrial DNA (mtDNA) quantification: From live PBMCs, total DNA was extracted using QIAamp DNA Blood Kit (Qiagen®, Hilden Germany) as per the manufacturer's instructions. Extracted DNA was quantified using Qubit dsDNA BR assay kit in Qubit fluorometer (Life Technologies, Carlsbad, USA) and the target concentration was optimized to 5 ng/µl. mtDNA content was quantified by real time PCR-based relative quantification method performed in ABI 7500 Fast DX (Applied Biosystems, USA) real-time PCR instrument. For consistency, all samples were run in duplicate with TaqMan® Gene Expression Assay (part No. 4331182, Life Technologies, Carlsbad, USA) targeting mitochondrially encoded NADH dehydrogenase subunit 2 (ND2) primer probe (Hs02596874_g1) with FAM™ reporter dye at the 5' end of the TaqMan® MGB probe, for the target and copy number reference assay (part No. 4403326, Life Technologies, Carlsbad, USA) for the endogenous control, Ribonuclease P (RNaseP), which had VIC® dye-labelled TAMRA™ probe. Briefly, the reaction mixture consisted of 10 µl, TaqMan Fast Universal Master mix, 1 µl each of ND2 and RNaseP primer probe and 4 µl of nuclease-free water. Finally, for 16 µl of the master mix, 4 µl of 20 ng DNA target was added. In every PCR performed throughout the study, a DNA reference sample was always included to assess inter-experiment variation. All samples were tested in duplicates, and the mean cycle threshold (Ct) value was taken for calculating mtDNA content. 2-EXP (Ct mtDNA- Ct nDNA)[18] formula for relative quantification was used to calculate mtDNA content where Ct mtDNA was the Ct of *ND2* gene and Ct nDNA, was that of RNase P.

Statistical analysis: Non-parametric tests were used throughout the study. Data are presented as median values and interquartile ranges (IQRs) unless stated otherwise. Mann-Whiney U-test was used for group-wise comparison. Spearman rank test and linear regression were used to analyze the association between two variables. For more than two group comparisons Kruskal-Wallis test was used. Prism version 5.00 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses.

Results

Table I shows the demographic details of the study participants. The median (IQR) age of the HIV-infected group was 35 (31 to 41) years and the male/female ratio was 0.4:1 among the HIV-infected and 1.6:1 among the LoRHC, median duration of HIV infection was 36 months in ART untreated and 83 months in ART treated

Table I. Demographic details the study participants

Characteristics	Healthy controls (n=24)	ART naïve (n=57)	ART treated (n=30)
Age, median yr (range)	26 (19-39)	33 (22-55)	40 (20-52)
Gender, n (%)			
Male	63	33	30
Female	38	67	70
Duration on cART in months, median (range)	-	-	46 (6-140)
Patients on d4T (%)	-	-	47
Patients on AZT (%)	-	-	53
BMI (kg/m^2) (IQR)	22.75 (20.55-24.65)	25.62 (20.83-27.41)	21.22 (18.41-24.82)
Baseline CD4 T-cell count (cells/µl), median (IQR)	-	-	165 (83-235)
Duration of infection in months, median (IQR)	NA	36 (12-61)	83 (32-123)
HIV RNA level (log copies/ml), median (IQR)	NA	4.0 (3.0-6.5)	1.6 (1.6-1.61)
CD4 T cell count (cells/µl), median (IQR)	-	642 (481-738)	504 (347-621)
CD4%, median (IQR)	-	25 (20.5-31.5)	21.5 (19-27)

Values are median (IQR) unless otherwise stated. BMI, body mass index; d4T, stavudine; AZT, zidovudine. IQR, interquartile range; cART, combination antiretroviral therapy

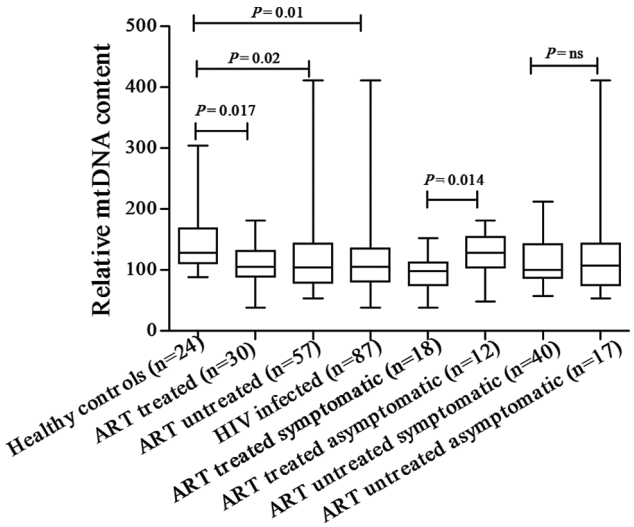

Fig. 1. mtDNA content represented in median (IQR) values of the study groups. ns, not significant.

who were exposed to ART for a median of 46 months and had a median baseline CD4 T cell count of 165 cells/µl at ART initiation. At the time of enrolment, median CD4T cell count was 642 cells/µl in ART untreated and 504 cells/µl in ART treated. Viral load was at a median of 4.0 log copies/ml in ART untreated; 47 per cent of the ART treated patients were on d4T and 53 per cent on AZT.

mtDNA relative quantification

Median Ct value of endogenous control, RNAse P of nuclear DNA in the LoRHC [25.22 (24.71-25.32)] and in HIV infected [25.23 (25.13-25.38)] were not significantly different, whereas the median Ct values of *ND2* showed a significant difference [*P*=0.03, LoRHC: 18.24 (17.67-18.7); HIV infected: 18.51 (18.13-18.92)]. There was a significant (*P*=0.01) reduction in mtDNA content among HIV infected (104.7; 80.56-135.3) compared to the LoRHC (127.5; 110-167.7), but it was the same in both the treated (104.8; 88-130) and untreated (104.7; 78-142) (Fig. 1). Significant (*P*=0.01) reduction in mtDNA among ART-treated patients, symptomatic for toxicity (97.99; 74.22-111.9) was observed than among the asymptomatic patients (128.3; 103.7-153.4), and among the ART untreated symptomatic patients, mtDNA was reduced (99.6; 86.46-141.9) compared to the asymptomatic (106.4; 74.67-142.9), (*P*=0.807). A CD4 T cell count cut-off, 700 cells/µl was identified among the ART untreated below which a significant (*P*=0.04) decrease in mtDNA content was observed (Table II). mtDNA content was not associated with stratified patient characteristics such as gender, age, body mass index and duration on ART. Similarly, there was no difference in mtDNA content of the individuals on d4T [102 (81-131)] and AZT [106 (88-135)].

Among the symptomatic ART treated, mtDNA content correlated positively with lactate (*P*=0.005, *r*=0.675), and TC/high-density lipoprotein (*P*=0.033, *r*=0.55), in the ART untreated group, significant association between mtDNA content and CD4 per cent [*P*=0.04, odds ratio (OR): 1.039; 95% confidence interval (CI) 1.00-1.08] (Fig. 2) and plasma

Table II. Mitochondrial DNA content of antiretroviral therapy (ART) naïve and treated stratified on demographic and clinical parameters

Characteristics	ART untreated (n=57)	*P*	ART treated (n=30)	*P*
Gender				
Female	101.9 (76.77-151.1)	0.925	104.7 (82.08-128.3)	0.587
Male	110.3 (88.89-135.3)		123.0 (90.56-143.0)	
Age (months)				
<30	112.8 (83.52-159.2)	0.248		
31-40	91.95 (75.29-130.3)			
≥30	130.5 (89.46-146.2)			
<40			104.6 (74.22-131.1)	0.787
≥40			104.8 (93.54-132.3)	
BMI (kg/m^2)				
Underweight (<18)	112.4 (90.64-135.3)	0.689	92.12 (78.55-114.3)	0.344
Normal (18-25)	113.1 (72.02-155.5)		117.0 (91.52-134.7)	
Overweight (>25)	94.03 (76.57-138.4)		104.4 (75.8-126.2)	
CD4 T-cell count (cells/µl)				
<700	93.64 (76.77-130.8)	0.0427*		
≥700	133.4 (97.31-151.1)			
<500			105.5 (84.3-127.6)	0.723
≥500			103.8 (89.63-135.2)	
d4T	NA	NA	102 (81-131)	0.719
AZT			106 (88-135)	
Viral load (copies/ml)				
<1000	111.00 (77.83-142.9)	0.755	NA	NA
≥1000	98.36 (81.49-142.3)			
Symptomatic*	99.66 (86.46-141.9)	0.807	97.99 (74.22-111.9)	0.014*
Asymptomatic**	106.4 (74.67-142.9)		128.3 (103.7-153.4)	
Duration on ART (months)				
<24	NA	NA	100.9 (59.89-126)	0.495
24-72			108.2 (92.12-132.5)	
>72			102.5 (66.94-146.9)	
Duration of infection (months)				
<48	94 (78-132)	0.304		
≥48	115 (79-159)			
<72			117.0 (97.47-133.9)	0.362
≥72			102.6 (84.3-128.6)	

*Those who had clinical and laboratory abnormalities indicative of mitochondrial dysfunction; **Those who did not have clinical and laboratory abnormalities indicative of mitochondrial dysfunction. Values are median (IQR). BMI, body mass index; d4T, stavudine; AZT, zidovudine; IQR, interquartile range; NA, not applicable

viral load (*P*=0.52, OR: 0.018; 95% CI 0.003-0.096) (Fig. 3) and TC (*P*=0.04, *r*=0.27) was observed.

Discussion

Lack of significance in the endogenous control, RNase P median Ct value and a significant difference in median Ct values of the target, mtDNA-ND2 among the three groups implied appropriate normalization and variation in the mtDNA levels, respectively. Reduction in mtDNA content among HIV infected than the LoRHC reiterated the observation of earlier studies[19] signifying the impact of NRTI and the virus

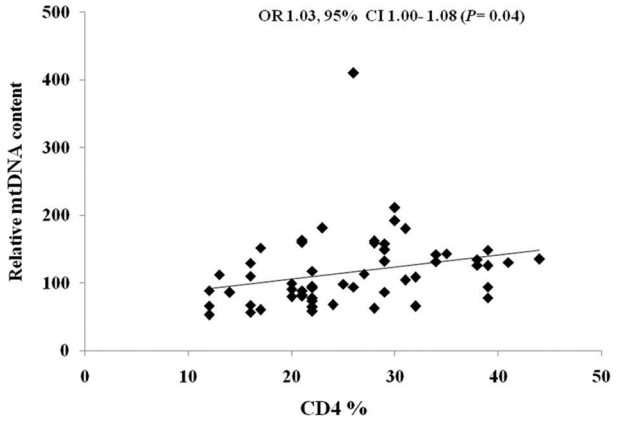

Fig. 2. Association between mtDNA content and CD4 per cent in ART untreated by linear regression model. The graph shows a significant positive association between mtDNA content and CD4 per cent.

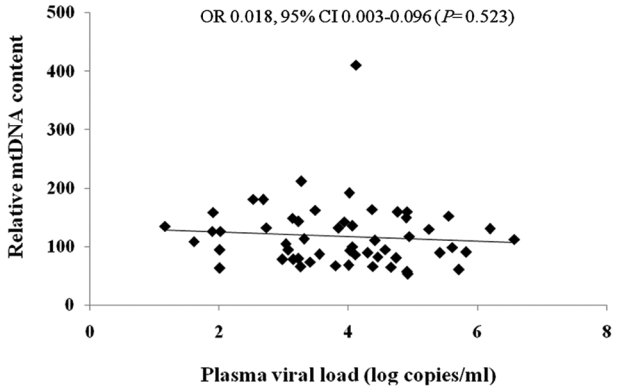

Fig. 3. Association between mtDNA content and plasma viral load (log copies/ml) in ART untreated by linear regression model. The graph shows a trend of negative association between mtDNA content and plasma viral load (log copies/ml).

per se on mtDNA depletion. There are studies[20], which failed to document a significant difference between controls and the HIV infected especially in the PBMCs. Although Ribera *et al*[7] reported a significant difference in mtDNA content between ART treated and untreated, we did not find any, which was similar to that reported by others[20].

Mitochondrial dysfunction does not always depict mtDNA depletion rather increased mtDNA has also been reported[8]. In line with the previous report[19], in the present study, the ART treated symptomatic patients had a significant mtDNA depletion than the asymptomatic patients indicating the co-occurrence of mtDNA depletion and the existence of symptomatic toxicity. This signifies that mtDNA has the potential to act as an indicator of mitochondrial toxicity.

Further, in this study among the ART untreated, there was a significant direct mild correlation between mtDNA content and CD4 per cent and a trend of negative correlation with viral load which marks the virus-induced mtDNA depletion. Such a finding in the past has been recorded by Miura *et al*[21] who observed mtDNA to correlate moderately with CD4 T-cell count and viral load, respectively and Peraire *et al*[22] observed a mild correlation for viral load alone. Our results might have important clinical implications that we were able to set a CD4 T-cell count threshold of 700 cells/µl below which there was a significant decrease in mtDNA and *vice versa* among the ART untreated, though this count of CD4 T-cell is considered normal in HIV uninfected. This finding signifies the underlying influence of HIV on mitochondria at a high CD4 T-cell count having not known about the actual physiology of the cells. Thus, this finding indicates early initiation of ART that would preserve the mitochondrial parameters which is likely to be worsened with the long-term NRTI containing regimen.

Significant depletion of mtDNA in symptomatic ART patients indicates the influence of mtDNA decline on the biochemical abnormalities as mitochondrial damage seems to be the common pathway for these complications[23]. Others[5,24] have reported the decline of mtDNA to precede the increase in random venous lactate. We have found mtDNA depletion in some of the asymptomatic patients which speculates the prior changes at mtDNA level that is expected to be followed by the biochemical abnormalities, this characteristic feature if proved with longitudinal follow up of a larger cohort would make mtDNA as an early indicator of mitochondrial toxicity.

Maagaard *et al*[6] concluded that neither mtDNA in PBMCs nor lactate to pyruvate (L/P) ratio was a good marker of NRTI-associated mitochondrial toxicity as there was no significant difference in mtDNA between ART treated and untreated groups which was in line with the results of the current study. Later, the same group of researchers found a decrease in mtDNA levels in CD8 T cells among the ART-naïve and mtDNA decrease in both CD8 and CD4 T cells in NRTI-exposed patients[25]. This difference in mtDNA levels in T cell subsets could imply that quantification of mtDNA in CD4 T cells, rather than PBMCs, may better reflect NRTI-associated mitochondrial toxicity[25].

The decrease in mtDNA was found to precede the rise in venous lactate levels[5] this could be the reason for the significant positive correlation between mtDNA and

lactate among the symptomatic ART treated. There was a positive moderate level of correlations of mtDNA with the lipid profile which was consistent with Cossarizza *et al*[26] who observed a significant positive correlation to exist between mtDNA and cholesterolemia but Maggiolo *et al*[27] showed a negative correlation with cholesterolemia. However, due to the small number of patients in each category of symptomatic toxicity strong conclusions could not be made.

The significant positive correlation between mtDNA and CD4 per cent, trend of negative correlation with viral load observed in ART untreated implies the probable impact of virus induced mitochondrial mediated apoptosis of CD4 T cells as many of the previous studies[28] have found the potential link between HIV proteins and mitochondrial components of apoptosis. In contrast, the lack of such correlation among ART treated shows the uniform impact of NRTI among the subsets of PBMCs. Thus, further studies involving PBMC subsets would throw more light on HIV and NRTI-mediated mitochondrial dysfunction and would open up new avenues of finding precise novel biomarkers of mitochondrial toxicity.

Some of the studies on mitochondrial dysfunction induced by NRTI have concluded mtDNA as a poor marker probably owing to factors such as small sample size, platelet contamination[6], improper patient selection, differences in duration of HIV infection and duration of exposure to NRTI. Moreover, determination of mtDNA content in purified cells should be estimated with well defined, validated methodology[26] and cross-validation of methods being developed by different laboratories for quantifying mtDNA from patient samples will become increasingly important to allow comparison of results from different studies. However, in the current study, isolated PBMCs were washed additionally to remove platelet contamination thereby PBMCs were 99 per cent pure. Small sample size and heterogeneity in characteristics of the study participants, cross-sectional study design, lack of an adequate number of patients in each category of symptomatic mitochondrial toxicity and lack of other direct mitochondrial derangement parameters were the potential limitations of our study.

Although currently, there is no treatment regimen involving stavudine except in conditions where AZT/TDF cannot be used, NRTI class of ARVs are very promising in RLS. Studying mitochondrial toxicity caused by stavudine would be a standard to estimate and compare the relatively less toxic new generation NRTI such as tenofovir.

In conclusion, our results showed mtDNA depletion from mtDNA content of PBMCs which was suggestive of mitochondrial dysfunction induced by NRTI and HIV *per se*. Moreover, the outcome of mtDNA depletion is substantiated by symptomatic toxicity that is associated with mitochondrial dysfunction. In the settings of HIV/AIDS, as both HIV and NRTI are toxic to mitochondria early initiation of ART could be made mandatory especially in RLS to preserve the mitochondrial functionality that is worsened by long-term NRTI treatment. With limited resources for HIV-1 treatment in developing countries, low-cost treatment options such as d4T still need to be pursued, if safety can be improved by dose optimization with regular monitoring strategies involving early indicators of toxicity.

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***Conflicts of Interest*:** None.

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