6. DISCUSSION

Although it was in the late 1980s, when AZT-treated patients developed myopathy, mitochondrial involvement in causing NRTI related abnormalities was identified, subsequently, many other clinical and pathological adverse effects of NRTI therapy have been associated with acquired mitochondrial injury. As these toxicities become apparent only at the post licensing stage, a combination of human, animal, and in vitro studies were carried out to define the underlying histological and molecular defects [Gardner K. et al., 2014].

Mitochondria being the producer of >90% of endogenous ROS, an active organelle of intrinsic pathway of apoptosis, its role in the formation of inflammasomes involved in pyroptosis, hosts certain steps of viral replication including many others yet to be unraveled. Thus, they are now much explored in chronic inflammatory diseases such as HIV infection. This is evident from the growing body of literature that is available recently within a short span.

There is lack of data on mitochondrial toxicity of the NRTI being tested from the pre-licensing preclinical studies, and was first observed only in post licensing studies. Establishing mitochondrial toxicity, as per FDA is not a requirement for drug approval [Sims K., 2010]. Thus, a greater effort may be required to specifically identify potential mitochondrial damage during NRTI drug development [Gardner K. et al., 2014]. Moreover, evaluating newer treatment strategies for primary mitochondrial disorders has been hampered due to lack of appropriate biomarker to monitor disease status or treatment response [Enns GM., 2014]. In the settings of HIV infection both the etiology and its treatment can influence mitochondria that
results in a prolonged mitochondrial damage which is complicated with aging as mitochondrial activities deteriorate with aging.

In the current study in order to estimate mitochondrial dysfunction caused by NRTI and HIV per se we have chosen the easily accessible PBMCs as the specimen from which the mitochondrial dysfunction was measured in a multifaceted way so that the measured parameters could depict the wider aspects of mitochondrial activity deficits.

Mitochondrial parameters included in the current study were quantification of mtDNA content which is known to be depleted due to the inhibition of pol $\gamma$ by NRTI, pol $\gamma$ is the sole enzyme involved in the replication of mtDNA, mitochondrial dysfunction causes oxidative stress which is further expected to influence the dissipation of mitochondrial membrane potential which subsequently induces apoptosis of blood lymphocytes. Besides, ROS could cause mtDNA mutations hence a representative gene of mtDNA, the ND1 gene was sequenced to analyse the impact of oxidative stress in causing mtDNA mutations. However, oxidative stress which is the basic pathological phenomenon of mitochondrial dysfunction was estimated by measuring the lipid peroxidation product, the plasma F2- IsoP. Moreover, all the mitochondrial parameters were correlated with the clinical parameters known to be associated with mitochondrial abnormalities. It is known from various studies that there is ethnic influence of mitochondrial adverse events attributed to mtDNA haplogroups hence we have estimated all the mitochondrial and clinical parameters in the LoRHC that would facilitate in gauging the mitochondrial abnormalities in the HIV infected.
With the change in guidelines for the implementation of early initiation of ART, understanding the pathophysiology involved in mitochondria functions at a higher CD4 T-cell count would help in devising strategies for better management of HIV infected patients. Hence, in the current study we have analysed the impact of HIV on mitochondrial activities at a higher CD4 T-cell count, which is 624 (481-738) cells/µL at study entry.

NRTI induced mitochondrial dysfunction usually sets in at a longer exposure to NRTI and many studies have prospectively followed the NRTI treated only for a shorter duration, whereas in our study the median duration of ART treatment was 85(69-93) months at the final visit, which is an ideal cohort to study the NRTI induced mitochondrial dysfunction.

Moreover, in order to appreciate the link between the mitochondrial parameters and the mitochondrial dysfunction related adverse events among the NRTI treated we have analysed the mitochondrial parameters in those with NRTI related adverse events who were identified based on clinical symptoms and laboratory abnormalities that were graded as per ACTG toxicity grading.

6.1 mtDNA content of PBMC among the study groups:

Significant reduction in mtDNA content among HIV infected than the healthy controls was observed in all the follow-up visits and the total visits (except for the 12th month of ART-treated), which reiterate the observation of many previously published studies [Maagaard A. et al., 2006; Ribera E.et al., 2008] signifying the impact of NRTI and the virus per se on the replicative mechanism of mtDNA
thereby causing mtDNA depletion. A number of possible mechanisms suggested are inhibition of pol γ, direct inhibition of OXPHOS, inhibition of endogenous nucleotide kinase and ROS generation. There are studies [Morse CG. et al., 2012; Shikuma CM. et al., 2008] which even failed to document a significant difference between controls and the HIV infected especially in the PBMCs. Although few studies [Lopez S. et al., 2004; Ribera E. et al., 2008] have seen a significant difference in mtDNA content between ART-treated and untreated, we did not find any in any of the visits, which was also documented in some of the previous studies [Maagaard A. et al., 2006; Morse CG. et al., 2012; Kampira E. et al., 2014].

Mitochondrial dysfunction does not always depict mtDNA depletion rather increased mtDNA has also been reported [Cote HCF. et al., 2008; Casula M. et al., 2005] and also witnessed in some of the HIV infected, both treated and the untreated patients of our study population as well. This fluctuation in mtDNA content signifies the fact that increase in mtDNA content can also occur although the factors paving way for this is not yet identified. Some studies have claimed that this could be a homeostatic mechanism to increase the protein production and thereby to increase the ATP generation by the affected mitochondria [Payne BA. et al., 2011], in contrary to this there are studies which has found that mitochondria with reduced mtDNA would tune up the downstream components of the central dogma by either increasing the transcription or the translational machinery to maintain the normal levels of the proteins needed for the optimal mitochondrial functioning thereby the cellular activity [Kim JM. et al., 2008].
Mitochondria being the most dynamic organelle performing vital functions of the cell, mechanisms of mitochondrial regulation have evolved to avoid cellular damage and to maintain the overall fitness of the cell. Mitophagy has emerged as a key regulatory mechanism, responsible for the elimination of damaged mitochondria [Kanki T. & Klionsky. DJ., 2010]. In line with this it may be contemplated that dysfunctional mitochondria may be removed by mitophagy which eventually mitigates the pathological situation created by such mitochondria. Thus there is still mtDNA depletion but the general health is replenished as seen in the asymptomatic patients of the present study which is also supported by the finding that fragmented mitochondria can fuse together if they have normal membrane potential, but loss of membrane potential prevents fusion and leads to mitochondrial segregation and subsequent degradation by mitophagy [Song Z. et al., 2007]. Thus further research on the mechanism of mitophagy must be taken up to understand the phenomenon which might open up newer treatment strategies.

6.2 Correlation between mtDNA content of PBMC and Immuno-virological parameters:

mtDNA content not only correlated positively with CD4 T-cell count and CD4% but also negatively with the plasma viral load with significance among the ART-naïve but such a correlation was not achieved in the ART-treated. Significant immuno-virologic correlation in many of the previous studies was not documented, the reason could be that those studies were cross-sectional however we have found a significant correlation in all the visits, Miura T. et al., 2003, in a longitudinal study design had shown a significant correlation between mtDNA content and the immuno-virologic parameters whereas Peraire J. et al., 2007 in a cross-sectional study has shown such a correlation albeit a mild correlation with viral load alone.
As mtDNA is dynamic and found to vary among the individuals, longitudinal study design will be ideal to appreciate the changes in mtDNA content and its association with the covariables. *In vivo* and *in vitro* studies suggest that mitochondria-mediated apoptosis may play an important role in CD4 T-cell depletion in HIV-1 infection [Miura T. *et al*., 2003] which is also evident from the present study. Mitochondrial dysfunction in the form of mtDNA depletion could have triggered the intrinsic pathway of apoptosis of the CD4 T-cells and hence the significant positive correlation. However, mtDNA depletion in PBMCs may be related to but would not be completely explained by CD4 T-cell apoptosis per se because of the rapidly expanding CD8 T-cells [Miura T. *et al*., 2003] and the other subsets of PBMCs but these subsets may have an impact only at a lower CD4 T-cell counts.

The contribution of CD4⁺ T-cells to mtDNA levels in whole PBMCs should be small in patients with advanced disease who have very low CD4 T-cell counts. Therefore, additional cell types such as CD8 T-cells should be involved. CD8 T-cell expansion is increased in HIV-1 infection [Hellerstein M. *et al*., 1999]; therefore, the mechanism for mtDNA depletion in PBMCs may be complicated [Miura T. *et al*., 2003]. In addition in our own finding that mtDNA content is generally high in CD4⁺ T-cells than in the non-CD4⁺ T-cells was evidenced by the significantly elevated mtDNA content in CD4⁺ T-cells both in the treated and the untreated though not confirmed in the healthy controls. Having said that, significant loss of CD4 T-cells must have been triggered by mitochondrial dysfunction as evidenced by the significant negative correlation between TLA (%) and CD4 T-cell count simultaneously with mtDNA depletion among the ART-naïve patients with overall increased oxidant stress in 6th month and in the enrollment visit implying the greater
role of oxidative stress in bringing out the potential links that exist between mtDNA depletion, TLA (%) and CD4 T-cell count in ART-naïve.

Though it is also evident that in HIV infection there is a higher expansion of CD8 T-cells than the CD4 T-cells, probably it occurs at a lower CD4 T-cell counts, but the CD4 T-cell count of the present study was quite high (>500 cells/µL). Hence would not have had much CD8 T-cell expansion which is why we could demonstrate a significant correlation between TLA (%) and CD4 T-cell count. Thus owing to the fact that there is a potentially higher amount of mtDNA content among the CD4 T-cells than the non-CD4 T-cells, its depletion and the probable induction of CD4 T-cell apoptosis has been reflected in the positive correlation between the CD4 T-cell count and the PBMC mtDNA. Taken together these findings implies that CD4 T-cells occupy a prominent proportion among the PBMCs at least at a higher CD4 T-cell count and the proportion of CD4 T-cell reduction is reflected in PBMC mtDNA content in ART-naïve.

In contrast to the above correlation that was documented in the ART-naïve, we could not associate mtDNA content with CD4 T-cell count in any of the visits of the ART-treated (except for Enrl. with CD4%) so as been reported by many of the previous studies [Peraire J. et al., 2009]. Moreover we could not identify a correlation with CD4 T-cell count even in situations of oxidative stress. The above observation evidently shows that in spite of the homeostatic mechanisms and the factors such as cellular expansion kinetics and the mitochondrial dynamics complicated by the diverse subsets of PBMCs, mtDNA depletion induced by NRTI including various viral and host factors that interplay with one another has resulted
in not only causing CD4 T-cell depletion but also of other subsets. Thus the lack of correlation between PBMC mtDNA and CD4 T-cell count among the ART- treated in contrast to the existence of correlation at many instances among the ART-naïve signifies the uniform impact of the NRTI induced mtDNA depletion in all the PBMC subsets.

Many HIV-1 proteins have been proposed as being responsible for HIV-1-related mtDNA depletion and mitochondrial impairment in PBMC of untreated asymptomatic patients. Among them, gp120, Tat, Nef and Vpr have been shown to cause dysregulation of the Bcl-2 family member’s expression, increase in mitochondrial membrane permeabilization, cytochrome C release, and loss of mitochondrial transmembrane potential, all of which lead to cell apoptosis [Alimonti JB. et al., 2003]. Though there are studies that have found the impact of HIV viral proteins and mitochondrial mediated apoptosis, there is a lack of experimental data to identify the direct effect of HIV on mtDNA depletion. Most of the previous studies on mt DNA depletion by HIV have concluded saying a probable link through viral proteins and mitochondrial components of apoptosis which is explained by the association between viral load and mtDNA depletion.

Correlation analysis among the ART-naïve with a viral load of >4 log copies/mL showed the impact of the viral replication and its proteins on the mitochondria witnessed as negative correlation between mtDNA and TL \( \Delta \Psi_m^{\text{low}} \) (%). Besides, at uncontrolled viral replication there was a significant correlation between
HDL-C, LDL-C, TLC and HB with mtDNA. Thus as the lipid and RBC parameters were found to be impacted by oxidant stress it can be speculated that the general oxidant stress might have catalysed the association between mtDNA content and the aforementioned biochemical parameters that was observed to be higher in those with higher viral load in two of the three visits in the present study. The possible role of mitochondria in the regulation of HDL-C and TGL is mainly concerned with ROS production [Flaquer A. et al., 2015]. An excess production of ROS in mitochondria, accumulation of mtDNA damage, and progressive respiratory chain dysfunction have been associated with atherosclerosis [Mercer JR. et al., 2010]. These findings highlight the important role of mtDNA among the factors that contribute to the balance of the lipid profile [Flaquer A. et al., 2015]. The negative correlation between mtDNA and TLC is attributed to the increase in non-CD4 T-cells, especially the CD8+ T-cells which is expected to expand proportionally to the viral load which was >4 log copies/mL. This was evidenced by an increased TLC (2.7, 2.0-3.3 x10^9/L vs 2.4, 2.1-2.7 x10^9/L) among those with > and < 4 log copies/mL of viral load, respectively in the enrollment visit.

Thus in the ART-naïve the significant correlation between PBMC mtDNA and the immune-virological parameters signifies the probable utility of PBMC mtDNA content as a marker of disease progression. This finding warrants further research on mtDNA depletion among the ART naïve which would perhaps unravel the intricate mechanisms involved in CD4 T cell depletion.

6.3 PBMC mtDNA content in oxidant stress and adverse events:

Studies on oxidative stress and its impact on cardiovascular diseases have concluded that increased oxidative stress can directly cause mtDNA damage thereby leading to mtDNA depletion. Even though the mechanisms controlling the mtDNA
copy number are still poorly understood, mtDNA depletion may result from a ROS-induced mutation at the origins of replication and also outside the origins of replication is capable of disturbing the mtDNA content [Moraes CT. et al., 1991]. Lack of complex chromatin organization consisting of histone proteins, the limited repair activities of mtDNA, being a major producer of ROS and the vicinity of mtDNA to the MRC are the reasons for it to be a major target for ROS mediated damage. There are also studies that have found mitochondrial toxicity without mtDNA depletion characterized by the pro-oxidative effects of NRTI such as AZT [Kline ER. et al., 2009].

Although, there was an association between mtDNA content and AST/ALT increase among the ART-treated; significant increase in ALT in conditions of oxidative stress, a significant decrease in mtDNA during oxidant stress in condition of adverse events was not achieved. This indicates that more than the ROS induced inhibitory effect on mtDNA, the inhibitory effect of NRTI on pol $\gamma$ might have triggered mtDNA depletion in general in the enrollment visit, that leads to energy deprivation due to the lack of adequate proteins that are essential for OXPHOS. The overall decrease in ATP production cause leakage of electrons from the ETC that eventually leads to the increased generation of ROS [Wallace DC., 1999] that can further deplete mtDNA. The above explanation is supported by the significant negative correlation between mtDNA and plasma F2-IsoP among those with mtDNA depletion. However it cannot be denied that during oxidant stress there is mtDNA depletion induced mitochondrial dysfunction of the liver cells that has caused liver cell damage evidenced as increased ALT due to the existence of a significant correlation between mtDNA and AST/ALT ratio in the enrollment visit.
Thus the status of mitochondrial dysfunction of the liver has been reflected in the mtDNA content of the PBMCs only to an extent.

NRTI may cause mtDNA depletion of the hepatocytes with the eventual mitochondrial respiratory chain impairment leading to inhibition of fatty acid oxidation of mitochondria which can induce accumulation of free fatty acids and triglycerides. This results in macrovesicular steatosis, reduced ATP synthesis and decreased production of ketone bodies which is responsible for major energy deficiency in extra-hepatic tissues [Le Bras P. et al., 1994]. Alternatively due to mitochondrial dysfunction there can be decreased hepatic production of VLDL and thus low levels of TGL and VLDL in the blood. Prolonged episodes of mitochondrial damage of the liver cells, stimulates for cell death by apoptosis or necrosis which is seen as elevated ALT in steatohepatitis. It might also be added that liver being the pivotal organ for the generation of glutathione [DeLeve LD. & Kaplowitz N., 1991], the major anti oxidant, liver damage might aggravate the oxidant stress that might result in a vicious cycle eventually causing adverse events related to NRTI drugs. Moreover, in the process of detoxification of the superoxide anion radical, mitochondrial manganese superoxide dismutase (MnSOD) dismutates superoxide into hydrogen peroxide ($H_2O_2$), which is detoxified into water by the mitochondrial glutathione peroxidase (GPx) that uses reduced glutathione (GSH) as a cofactor. Thus in normal state most of the ROS produced by MRC are detoxified by mitochondrial antioxidant defenses.

In conditions of GSH depletion within liver mitochondria, their capability to detoxify $H_2O_2$ is greatly affected since liver mitochondria do not have catalase [Mari
Depletion of mitochondrial GSH below a critical threshold thus favors \( \text{H}_2\text{O}_2 \) accumulation by impairing its detoxification. This in turn triggers mitochondrial dysfunction, mitochondrial permeability transition pore opening, activation of c-Jun-N-terminal kinase (JNK), and cell death [Jones DP. et al., 2010].

As we could demonstrate a correlation between mtDNA and ALT among the ART-treated, we further explored to see if PBMC mtDNA content could reflect the mitochondrial dysfunction induced pathophysiology of macrovesicular steatohepatitis which is characterized by inhibition of fatty acid oxidation, increased hepatic \textit{de novo} lipid synthesis and decreased synthesis of VLDL associated TGL. Eventually when we analysed those with elevated ALT levels, there was concurrent decrease in TGL \((p= 0.005)\) and VLDL \((p= 0.012)\) and increased plasma F2-IsoP \((162 \text{ pg/mL}, 135-294 \text{ pg/mL} \text{ vs } 149 \text{ pg/mL}, 116-223 \text{ pg/mL})\) but mtDNA content \((97,76-102 \text{ vs } 96, 69-126)\) did not reflect the condition of steatohepatitis. Finally, although an inverse correlation exists between mtDNA content and the AST/ALT ratio, a significant elevation in ALT occurs during oxidative stress and in adverse events, PBMC mtDNA does not reflect the intricate pathophysiology such as macrovesicular steatosis which underscores the tissue specificity of mitochondrial pathologies [Kohler JJ. et al., 2009].

The National Cholesterol Education Program’s Adult Treatment Panel III report (ATP III) identified 6 components of the metabolic syndrome that relate to CVD: they are abdominal obesity, atherogenic dyslipidemia, raised blood pressure, insulin resistance ± glucose intolerance, proinflammatory state and prothrombotic state. In the 6\textsuperscript{th} month visit of the ART-treated with adverse events, some of these
components are interconnected that could bring out the pathophysiology involving mitochondrial dysfunction in metabolic syndrome increasing the risk of atherosclerosis. There is an inverse relationship between plasma high-density lipoprotein (HDL)-cholesterol and the incidence of coronary artery disease. HDL-C, owing to its ability to transport cholesterol from cells in the arterial wall to the liver referred to as reverse cholesterol transport is atheroprotective in nature. Its decrease is seen in atherogenic dyslipidemia.

Platelets are involved in both the initiation and progression of atherosclerotic lesions. Platelet aggregation called the thrombi over the ruptured atherosclerotic plaques results in the narrowing or complete occlusion of coronary arteries. Recent research has identified that HDL may exert antiplatelet effects and that may counteract the development of atherothrombotic vascular disease. In vitro studies also show that HDL inhibits agonist-stimulated platelet aggregation, fibrinogen binding, granule secretion and liberation of thromboxane A (2). Thus HDL-C is highly anti-platelet in nature.

In addition, neutrophils have been reported to be increasingly recognized for their role in atherosclerosis. Hyperlipidemia and inflammation represent the two main phenomena in the pathophysiology of atherosclerosis. Recent advances point at a contributory role of neutrophils during atherogenesis and plaque destabilization. In addition, the contribution of immune cells such as T-lymphocytes, mast cells, dendritic cells, and platelets to atherosclerosis has been firmly established in the past (Galkina E. et al., 2009).
Atherosclerosis is the result of the oxidative modification of LDL-C in the arterial wall by ROS. Recent studies exploring the mechanisms linking ROS and inflammation found that ROS derived from mitochondria act as signal-transducing molecules that provoke endothelial dysfunction associated with uncoupling of nitric oxide synthase, induce the infiltration and activation of inflammatory cells, and increase apoptosis of endothelial and vascular smooth muscle cells. The increase in the neutrophils inspite of the bone marrow suppression might stimulate the atherosclerotic events which also increased the overall WBC count as neutrophil is the abundant subset of WBCs. All these components correlated with mtDNA content of PBMCs reflecting the atherosclerotic picture of the blood.

Atherogenic Index of Plasma (AIP) values of -0.3 to 0.1 are associated with low, 0.1 to 0.24 with medium and above 0.24 with high cardiovascular risk [Bhardwaj S. et al., 2013]. Among the naïve, in the 12th month visit, mtDNA content correlated negatively, although mildly with significance. The AIP in this visit was at an average of 0.646 which is considered to be under the category of high risk for cardiovascular disease.

In the ART-naïve, correlations between PBMC mtDNA content and CD4 T-cell count and TLA (%) are found to be pronounced in oxidative stress which might address the implication of viral protein induced generation of ROS causing mtDNA depletion of the CD4 T-cells. This is in contrast to the observation that the association between mtDNA content and plasma F2-IsoP was much pronounced in mtDNA depletion, signifying the initial effect of the pol \( \gamma \) inhibitory effect of the NRTI with the subsequent generation of ROS among the ART-treated.
6.4 PBMC mtDNA dynamics:

Prospective studies have recorded mtDNA depletion to occur in 48 weeks of initiation of dual NRTI of which one is AZT and the other one is the phased out NRTIs – ddI/ ddC, surprisingly they have documented the depletion to occur as early as in 4\textsuperscript{th} week of ART initiation [Miura \textit{et al.}, 2003]. In a previously conducted larger prospective study, though that PBMC mtDNA did not associate with lipoatrophy, have found duration of NRTI therapy to associate significantly with mtDNA content and have noticed that almost all patients with increased mtDNA content had been on ARV therapy for longer than 5 years [Mc comsey G. \textit{et al.}, 2005]. Our results were very much consistent with the above finding that those who had increased mtDNA content of more than 127.5 were treated with ART for a median of 5.8, 4.1- 9.3 years but it was in 1000 days as reported by Miura T. \textit{et al.}, 2003, this discrepancy could be probably ascribed to the treatment regimen difference in Miura T. \textit{et al.}, 2003 study which also had PIs along with NRTIs, in addition to ddC whereas it was all most the same regimen as of the current study except that few of the patients were also treated with ddI in Mc Comsey G. \textit{et al.}, (2005) study.

Those with increased mtDNA were followed to estimate the mtDNA kinetics and found that there was a 6.2% increase in mtDNA subsequent to -42% decreases. After the increase there was again a fluctuation with alternate decrease and increase that was documented as -40% decreases subsequent to the first increase of probable compensatory amplification of mtDNA in response to mitochondrial dysfunction which seemed to vary among different individuals [Bai RK. \textit{et al.}, 2004]. The above
finding would potentially explain the absence of correlation between mtDNA depletion and clinical / laboratory toxicity in majority of the visits.

6.5 **PBMC mtDNA content as a biomarker of nucleoside related adverse events:**

One of the initial aims of this study was to investigate the possibility of using quantification of mtDNA of PBMCs as a biomarker for adverse events occurring during NRTI therapy. We looked for 2 criteria to answer this hypothesis 1) whether there is a significant reduction in mtDNA in conditions of adverse events associated with mitochondrial dysfunction caused by NRTI drugs. 2) The correlation between mtDNA content and the biochemical/hematological parameters reported to be associated with mitochondrial dysfunction.

To answer the first criteria, we in the current study has found a significant decrease in mtDNA between those with adverse events and those without any in enrollment but in subsequent visits mtDNA depletion was noticed in both the above said groups thus questioning the utility of mtDNA as a marker of NRTI mediated mitochondrial toxicity. Taking the above mentioned findings with ALT and AST/ALT ratio into consideration we again looked for the percentage of patients with mtDNA depletion among those with elevated ALT as per ACTG grading and found that all of them had mtDNA depleted but it was also depleted in those with normal ALT values.

As per criteria 2 we found in enrollment AST/ALT ($\rho = 0.038$) and ALT ($\rho = 0.051$) to vary significantly between increased and decreased mtDNA content at a
cut-off of 127.5. Although some of the previous studies have reported on increased lactate levels in those with decreased mtDNA content, not much has yet reported on any other routine laboratory marker to be significantly affected in those with PBMC mtDNA depletion. In contrast to this, in our study we have found AST/ALT ratio to associate significantly with mtDNA content in enrollment visit. The above finding shows that, PBMC mtDNA content has reflected liver injury as ALT is a specific marker of liver which was also evidently seen with a significant correlation with AST/ALT ratio. Moreover the significant increase in ALT during oxidative stress and again in those with oxidative stress and adverse events all documented in enrollment visit implies the greater association between ALT and systemic oxidant stress. But PBMC mtDNA failed to reflect increase in ALT in conditions of adverse events.

On analyzing the correlation between the existence of clinical/laboratory adverse event and mtDNA depletion during the entire follow up we saw that out of n= 41 adverse events 83% (34/41) had mtDNA depletion. mtDNA content of those with adverse events was 98.2(73-123) which is 35% lesser than the LoRHC. On the contrary among the n=38 visits of NRTI treated who had no clinical/ laboratory abnormality, had an overall mtDNA content of 94.5(70-130) which is 30.2% less than the healthy controls. Among these 74% (28/38) had mtDNA depletion. Thus although mtDNA depletion in the symptomatic conditions associated with mitochondrial toxicity signifies a good reflection of mtDNA depletion, the existence of similar trend in the asymptomatic conditions proves that mtDNA changes in PBMCs is not pertinent to the real time existence of adversity.
Thus, the utility of mtDNA of PBMC as a marker of NRTI induced adverse events is at stake due to reasons such as

- PBMC mtDNA depletion being noticed in those without any adverse events because of the existence of compensatory mechanisms to compensate for the reduced mtDNA content.

- PBMC mtDNA being elevated in those treated with NRTI for more than 5.8 years.

- PBMC mtDNA depletion being observed in ART-naïve subjects.

- PBMC mtDNA is found to vary with individuals.

Maagaard A. et al., (2006), has concluded that neither mtDNA in PBMCs nor lactate/pyruvate (L/P) ratio is a good marker of NRTI-associated mitochondrial toxicity as there was no significant difference in mtDNA between ART-treated and untreated which is in line with the results of the current study. Later, the same group of researchers has found 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. 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 [Maagaard A. et al., 2008]. Still it is debatable that if the toxicity is tissue specific, how it will be seen in the CD4 T-cells alone, thus warrants further study exploring on the PBMC subsets.

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 [Maagaard A. et al., 2006], 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 [Cossarizza A. et al., 2003] and cross-validation of mtDNA quantification methods from different laboratories from patient samples will become increasingly important to compare results from different studies [Hammond EL. et al., 2003]. However in the current study, isolated PBMCs were washed additionally to remove platelet contamination as the standard Ficoll gradient separation method does not remove platelets completely, thus with additional washing PBMCs were >95% pure. Removing platelets is crucial in relative quantification as platelets contain mitochondria and are devoid of nucleus hence if not removed can increase the mtDNA content. Patients on mitochondrial toxic drugs and alcoholics/ smokers were excluded and were exposed to NRTI chronically for a median of 46, 23-72 months.

Thus, in spite of the fact that many of the previous studies have totally ignored mtDNA as marker of NRTI induced adverse events, we have documented a substantial source of support discerning its larger association in the case of NRTI induced adverse events of liver and to reflect the metabolic syndrome of blood. Thus further investigations on tissue specificity of mitochondrial dysfunction have to be explored in a larger cohort.

Even though we have documented a significant mtDNA depletion in PBMCs, the diversity of PBMC subsets might be the interfering factor in announcing mtDNA as a biomarker, thus further exploring the mtDNA content of the PBMC subsets
would unravel the mitochondrial dynamics there by would aid in selecting the mtDNA content of the respective PBMC subset to act as a biomarker of NRTI induced adverse events.

In contrast to the above observations, among the ART-naïve those with increased mtDNA content had significantly \( p = 0.0004 \) higher CD4 T-cell count than those with decreased mtDNA moreover there was a significant positive correlation between mtDNA content and CD4 T-cell count, CD4 % and negative correlation with viral load. Thus PBMC mtDNA may be also used as a marker of disease progression.

### 6.6 Impact of thymidine analogues on mtDNA content of PBMC:

The significant ALT elevation in the ART-treated was found to be induced by AZT whereas the association with RBC parameters was triggered by both the thymidine analogues. Thus both the thymidine analogues have unanimously caused mtDNA depletion by inhibiting the enzyme pol \( \gamma \) leading to further release of oxidants that can cause further damage to mtDNA, its further depletion, thus the overall mitochondrial dysfunction causes an increased state of oxidative stress that results in the accumulation of lethal levels of ROS. This causes stress erythropoiesis upon bone marrow suppression by NRTI, which is evidenced as a significant increase in RBC in mtDNA depletion. However, mtDNA has been reported to be an intracellular target involved in the pathogenesis of AZT-associated bone marrow progenitor cell toxicity [Lewis LD. et al., 2004]. Thus HIV infection, bone marrow suppression by ART, mitochondrial dysfunction, oxidant stress and
stress erythropoiesis are highly complex parameters and their underlying mechanisms has yet to be unraveled.

It has been demonstrated in an *in vitro* cell-free chemical system that AZT and its azido-containing derivatives (AZT-MP, AZT-TP, and glucoronidated-AZT) have direct pro-oxidant activities compared to the non-azido containing derivatives [Komarov AM. *et al*., 2004]. In addition, AZT and AZT-MP induce lipid peroxidation mostly in the secondary targets.

Thus, AZT may have pro-oxidant properties inherent in its chemical structure. Lipophilicity of AZT is attributed to the azido group that helps the drug to target the membranes, including those of the mitochondria. This characteristic may partly explain the ability of AZT to cause lipid peroxidation in membrane preparations and to interfere with cardiolipin molecules in the internal mitochondrial membrane [Cazzalini O. *et al*., 2001]. Taken together, the association between mtDNA and plasma F2-IsoP among those on AZT proves the pro-oxidative and the lipid peroxidative properties of the drug.

### 6.7 Differential distribution of mtDNA content in the PBMC subsets:

PBMCs include lymphocytes (T-cells, B-cells, and NK cells), monocytes, and dendritic cells. In humans, the frequencies of these populations vary across individuals. In general, lymphocytes are typically in the range of 70 – 90% of PBMCs, monocytes range from 10 – 30% of PBMCs, while dendritic cells are rare, being only 1 – 2% of PBMCs [Autissier P. *et al*., 2010].
The frequencies of cell types within the lymphocyte population include 70 – 85% CD3+ T-cells (45 – 70% of PBMC), 5 – 20% B-cells (up to 15% of PBMC), and 5 – 20% NK cells (up to 15% of PBMC). The CD3+ compartment is composed of CD4 (25 – 60% of PBMC) and CD8 T-cells (5 – 30% of PBMC), in a roughly 2:1 ratio. Both CD4 and CD8 T-cells can be further subsetted into naïve, and the antigen-experienced central memory, effector memory, and effector subtypes that exist in resting or activated states. CD4 T-cells are known as helper T cells and can be further classified into various functional subtypes based on the expression profiles of specific cytokines, surface markers, or transcription factors. These include regulatory T-cells, TH1, TH2, and TH17 cells as well as other described subpopulations such as TH9, follicular helper, and TR1 types.

Thus the population of cells of PBMCs is highly diverse and is even complicated in HIV infection. In the current study we have dissected the PBMC population into CD4+ T cells and non CD4+ T cells as a preliminary analysis to observe if there is any variation in mtDNA content at the subset level. Though the sample size was small, significant heterogeneity in the distribution of mtDNA among the CD4+ T cells and non- CD4+ T cells of the PBMCs was documented. We did not estimate mtDNA content of CD4+ T cells and non- CD4+ T cells of LoRHC hence we have compared it between ART- naïve and the treated alone.

Among the ART- naïve, keeping in mind the distribution of the PBMC subsets which is much impacted by the chronic immune activation and unlike CD4 T cell counts, absolute CD8 counts have been shown to rise early after HIV infection and maintain a relatively steady level until very late stage. Studies have shown increase
in total CD8 counts in most HIV-infected individuals is primarily due to an expansion of memory cells. Thus, memory CD8 T cells comprise over 80% of the T cells in PBMC from individuals with < 200 CD4/µL [Roederer M. et al., 1995], this study was conducted in HIV infected with a CD4 T cell count of <500 cells/µL whereas in the current study the CD4 T cell count is quite high ie. >500 cells/µL, thus, the expansion of CD8 T cells may not be significant which is why a significant association between PBMC mtDNA content and CD4 T cell count was established in the ART-naïve.

mtDNA content of CD4 T cells was higher than the non-CD4\(^+\) T cells in both the ART-naïve and the treated. In ART-treated, mtDNA content is decreased in both the CD4\(^+\) T cells and the non CD4\(^+\) cells compared to the naïve which highlights the role of NRTI in causing uniform mtDNA depletion in the subsets of PBMC. As the sample size was small and the subjects were selected at random we were able to comment generically on the difference in the distribution of mtDNA content among the PBMC subsets. Further structured studies targeting the long term NRTI treated patients and their PBMC subsets would provide more details in this regard.

In both the HIV infected groups mtDNA was significantly lower among the non CD4\(^+\) cells than the CD4\(^+\) T cells. Previous studies have reported mtDNA decline in CD8\(^+\) T cells than the CD4\(^+\) T cells among the naïve [Maagaard A. et al., 2008; Casula M. et al., 2007]. Casula M. et al., 2005 has documented a significant increase in CD8\(^+\) T cells in chronic HIV infection of 5 years post sero conversion, moreover HIV-1-induced immune activation involves both CD4 and CD8 T cells, and only peripheral blood total CD4 T cell numbers decline gradually whereas total
CD8 T cell numbers typically remain elevated until the late stage of HIV-1 infection [Hazenberg MD. et al., 2003], thus during chronic immune activation, the population of activated CD4+T cells expands to a lesser extent than the population of CD8+ T cells which could have an impact on the difference in the mtDNA content of CD4+T cells/ non CD4+T cells.

6.8 Mitochondrial parameters before and after ART initiation:

In order to understand the changes in the mitochondrial parameters before and after the initiation of TDF based I line regimen, we longitudinally followed n=10 HIV infected patients before and after ART initiation and found that CD4 T cell counts increased progressively with significance and plasma F2- IsoP and TLA (%) decreased progressively whereas mtDNA content and TLA_\psi^\text{low} (%) were affected initially at the 6\textsuperscript{th} month visit and later improved. Improvement in mitochondrial parameters at 12 months of ART initiation has been reported elsewhere as a common finding irrespective of inclusion of a thymidine analogue. This indicates that ART suppresses the viral load and eventually ameliorates the mitochondrial health of the PBMCs. This finding is in accordance with studies of Casula M et al., 2005 who had studied initiating with d4T sparing and d4T containing PI based regimen and Miura T. et al., 2003 who had reported on initiating AZT/ d4T containing regimen. The explanation for the above finding may be structured as follows:

The suppression of viral load is expected to decrease the level of inflammation and thereby oxidative stress as evidenced by the decrease in plasma F2- IsoP after ART initiation also supported by the finding that expression levels of pro-
inflammatory cytokines (IFN-γ, IL-1β, IL-6, and macrophage inflammatory protein-1α) were reduced after ART [Bucy RP. et al., 1999] which might help in the regeneration of antioxidants especially from the organs such as liver that eventually reduces oxidant stress which recues mitochondria from mtDNA depletion and mitochondrial membrane potential damage thereby reducing lymphocyte apoptosis.

The process of immune reconstitution needs a special mention in this context. During immune reconstitution there is an initial phase of 8-12 weeks during which there is a rapid increase in CD4 and CD8 T cell numbers which are redistributed from the local sites [Pakker NG. et al., 1998]. In line with this the finding that there is no turnover of lymphocytes and a mere redistribution of the sequestered lymphocytes from lymph nodes to the blood, implies that as these are activated lymphocytes mtDNA could have been depleted concurrently with mitochondrial membrane potential damage that was witnessed at 6 months of ART initiation. It might be speculated that this population of lymphocytes might have an inherently reduced mtDNA contributing to the reduction of mtDNA of PBMCs. Hence the physiology at the mitochondrial level has to be studied further on these long hidden special populations of cells that have led to mtDNA depletion at 6 months of therapy. Eventually in the second phase of immune reconstitution there is a slow increase in the number of naive CD4 and CD8 T cells [Douek DC. et al., 1998] and moreover the nonlethal concentrations of reactive oxygen species (reflected by reduction in plasma F2-IsoP after 6 months of ART initiation) have been found to induce nuclear respiratory factor 1 and 2, associated with the transcriptional control of nuclear-encoded mitochondrial proteins involved in the maintenance and
replication of mtDNA, and electron transport chain proteins [Kelly DP. & Scarpulla RC., 2004] would have caused the increase in mtDNA content of the PBMC.

6.9 Plasma F2 Isoprostane- an indicator of oxidative stress:

Systemic oxidative stress as estimated by measuring the plasma F2- IsoP in the present study, when compared between the HIV infected and the LoRHC was significantly elevated among the HIV infected which signifies pro-oxidative role of HIV and ART. Oxidative stress has been implicated in the pathogenesis of HIV disease, and it is considered to play an important role in the progression from the asymptomatic stage to the development of AIDS (Sharma B., 2014). ROS activate the NF-κ B transcription factor, that induces gene expression and HIV replication in human T cells [Pyo CW. et al., 2008]. NF-κ B also acts as a transcription factor for many inflammatory cytokines, such as TNF-α, which further activates HIV replication [Herbein G. et al., 2008]. Abnormal immune function has been found to be stimulated through impairment of T cells and apoptosis of CD4 T cells causing T cell decline in HIV infection [Aukrust P. et al., 2005].

Many studies have documented increased oxidative stress in HIV-infected and in patients with AIDS condition compared to HIV-uninfected controls [Suresh DR. et al., 2009] and in patients receiving ART [Manda KR. et al., 2011].

The significant elevation of plasma F2-ISOp in the ART- naïve than the ART-treated in the present study is also in line with some of the previous studies [Awodele O. et al., 2012; Redhage LA. et al., 2009]. Contrary to this, there are also many studies which have documented increased oxidative stress in ART-treated
[Mandas A. et al., 2009, Hulgan T. et al., 2003] than the naive. On reasoning out the decreased oxidative stress in subjects treated with ART compared to the ART-naïve we found from the literature that patients treated with NNRTI are expected to have reduced oxidative stress. In line with this a previous study has shown significantly high plasma F2-IsoP values in ART-treated containing a regimen without an NNRTI, followed by ART-naïve, and finally by those on ART that included an NNRTI [Redhage et al., 2009]. Similar to the above finding, as all of our study subjects were on NNRTI based first line regimen, NNRTI being either NVP/EFV during the entire study could be seen as a plausible explanation for the difference in the level of oxidative stress in ART-treated and the naïve.

There is a newer revelation that caspase-3-mediated apoptosis accounts for the death of only a small fraction of productively infected cells and the remaining >95% of quiescent CD4 T-cells of the lymphoid tissue die by caspase-1-mediated pyroptosis, an intense inflammatory form of programmed cell death which involves the release of cytoplasmic contents and pro-inflammatory cytokines IL-1β [Doitsh G. et al., 2014]. It is well known that excessive ROS are produced during inflammation (McGregor GP. & Biesalski HK., 2006). Inflammatory cells generate a number of reactive species at the site of inflammation leading to exaggerated oxidative stress [Collins T. et al., 1999]. Reactive oxygen/nitrogen species can initiate intracellular signaling cascade which enhances pro-inflammatory gene expression [Floh´e L. et al., 1997]. Thus, inflammation and oxidative stress are closely related pathophysiological events. As oxidative stress is an expected pathological phenomenon involved in chronic inflammatory disease [Mayne, 2003] and pyroptosis being an intense inflammatory response which accounts for a
majority of the lymphoid CD4 T-cell death could very well reflect the magnitude of systemic oxidative stress among the naive and the fact that ART-treated patients were on NNRTI based regimen gives a clear explanation for the increased oxidative stress in the ART-naïve group of the current study.

Thus, although we have studied the systemic oxidative stress by measuring the plasma F2-IsoP and all the other parameters were estimated from the PBMCs we could confirm an association between the mitochondrial parameters of the PBMC and the systemic oxidative stress by the concomitant increase in the level of plasma F2-IsoP and significant decrease in mtDNA content of the PBMC in the 6th month visit of the study of both the ART-naïve and the treated. The above finding highlights the considerable contribution of mitochondrial dysfunction in causing systemic oxidative stress that has reflected in the mtDNA content of the PBMC, moreover as mentioned previously, activated cells of the immune system such as phagocytes may generate excessive amounts of ROS both locally and in systemic circulation [del Valle LG. et al., 2013], which might lead to oxidative modifications and damage to proteins, nucleic acids, carbohydrates and lipids [Block G. et al., 2002; Kohen R & Nyska A., 2002].

ART does not completely eradicate HIV, hence there is still at least low level on-going immune activation and inflammation [Kuller LH. et al., 2008], which further amplifies the oxidative stress induced by ART. Studies have shown that patients who rigorously follow ART have significantly higher oxidative status than those who do not strictly follow the therapy (poor HAART adherence) [Mandas A. et al., 2009] indicating the stronger role of ART in causing oxidative stress.
Comparing the impact of the NRTI individually upon oxidative stress, we found plasma F2-IsoP level to vary between the two NRTI alternatively during the follow-up without statistical significance. Although many studies have documented mitochondrial toxicity caused by NRTI, in vitro studies on human cells demonstrated that d4T causes marked mtDNA depletion, with simultaneous ROS production [Velsor LW et al., 2004].

Also studies have further confirmed such results in cells of adipocytic origin and in hepatic cells. Moreover, compensatory mechanisms such as upregulation of Lon protease, a mitochondrial protein that is able to degrade oxidized substrate proteins are triggered by d4T induced increase in ROS [Pinti M. et al., 2010] which could be the reason for the betterments seen in some conditions such as the increased oxidative stress at a higher CD4 T cell count.

Preliminary studies [Hulgan T. et al., 2003], have identified an association between plasma F2-IsoP and NNRTI use but we could not find any association between them, either with NVP/EFV or with d4T/ AZT by Mann Whitney U test. Thus inspite of the ability of NNRTI to decrease the level of oxidative stress, there was a significantly increased level of oxidative stress in the ART-treated compared to the LoRHC which substantiates the role of NRTI in this pathophysiology. More over the lack of correlation with both the NRTIs and oxidative stress indicate the potential role of both the thymidine analogues in causing oxidative stress. There is a growing body of literature to stress on the association of AZT and oxidative stress, Sun R. et al., 2014 have shown that AZT treatment led to reductions in thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) levels through oxidative stress.
mechanisms and mitochondrially derived oxidative stress inhibits cardiac DNA methylation, alters cardiac gene expression, alters steady-state abundance of S-adenosylmethionine, and promotes characteristic pathophysiological changes of cardiomyopathy [Koczor CA. et al., 2015]. In another study AZT caused greater endothelial dysfunction in human aortic endothelial cell line than d4T because of its pro-oxidative effects. Thus as previous cell line and animal studies have reported a greater impact of AZT on oxidative stress, we in the current study expected a significant level of higher plasma F2-IsoP in those on AZT but we could not appreciate a significant impact of AZT on oxidant stress. However, the association between mtDNA and plasma F2-IsoP has been brought out well in patients on AZT thus implicating its role in mitochondrial dysfunction involving mtDNA depletion and production of ROS.

Oxidative stress has been implicated in cellular senescence and aging, and in the development of several chronic diseases such as non-alcoholic liver disease, cancer, neurodegenerative disorders, or cardiovascular disease [Biswas SK., 2016]. In regard to NRTI associated toxicity conditions, oxidative stress biomarkers were found to be increased in patients with lipodystrophy and symptomatic hyperlactatemia in two cross sectional studies [McComsey GA. et al., 2003; Vassimon HS. et al., 2010], and to be associated with traditional and non-traditional cardiovascular risk factors [Masia M. et al., 2007], but did not predict peripheral neuropathy development in a longitudinal study of patients starting ART [Hulgan T et al., 2006].
In concordance to the above findings in the present study, a significant association between plasma F2-IsoP and HDL-C among the ART-treated in a combination of d4T + NVP and in those on AZT irrespective of the NNRTI was observed which was also reflected in the atherogenic indices. Moreover AZT in a combination with EFV showed a positive correlation between plasma F2-IsoP and TGL, thus we could see an atherogenic dyslipidemic associations which underscores the probable cardiovascular risks in the patients on NRTI highlighting the role of thymidine analogues in causing these cardiovascular changes.

Lipid metabolism is influenced by oxidant stress, and elevated plasma F2-IsoP as a marker of oxidant stress has been linked to coronary artery disease [Shishehbor MH. et al., 2006]. A favourable lipid profile has been attributed to NNRTI and when compared to EFV, NVP has been reported to cause increases in HDL-C levels [Van Leth F. et al., 2004], with decreases in triglycerides [Ward DJ. et al., 2006], or both [Manfredi R. et al., 2005]. Enhanced synthesis or impaired clearance of HDL-C particles and/or apoA1 by NVP [Van Leth F. et al., 2004] and/or stimulation of lipogenic pathways in adipose tissue by EFV [El Hadri K. et al., 2004]. In line with this we have identified a significant negative correlation between plasma F2-IsoP and HDL-C among those on EFV which might be attributed to the increased stimulation of lipogenic pathways by EFV that has caused a decrease in HDL-C in circulation.

ALT, the indirect biochemical biomarker of mitochondrial dysfunction [Haas RH. et al., 2007] was found to be associated positively with F2-IsoP which was negatively correlated with AST/ALT ratio, both of which is found to link ART,
oxidative stress and mitochondrial dysfunction of the hepatic cells as ALT is the specific marker of liver cell damage, however such a association was not observed in ART-naïve individuals. Moreover PBMC mtDNA content although correlated with ALT generally, and during oxidant stress without significance, did not correlate in conditions of drug adversity. Thus, the correlation between ALT and plasma F2-IsoP, ALT and mtDNA and finally the correlation between mtDNA and plasma F2-IsoP among those with mtDNA depletion, all documented in the enrollment visit potentially links liver damage induced by oxidant stress that might have caused mitochondrial dysfunction of the liver but failed to reflect in the mtDNA content of the PBMC in conditions of drug toxicity.

Among the NNRTI, EFV is considered a safer drug for the liver than the NVP, and the frequency of severe increased liver enzymes in patients on NVP ranges from 4-18% but in those on EFV, it ranges from 1-8% [van Leth FR. Et al., 2004]. However, in the present study the correlation between ALT increases was obtained in those with EFV irrespective of the NRTI thus, the liver enzyme elevation would have been triggered by the synergistic effect of the NNRTI and NRTI, more from the NRTI as both d4T and AZT has been associated with ALT increase in the present study.

On the background it is known that damage of certain high-energy tissues, such as muscle and liver, can be because of underlying mitochondrial dysfunction, resulting in elevations in indicators of tissue damage such as creatinine kinase (CK), AST and/or ALT [Rossignol DA. & Frye RE., 2012] which was very well documented in the study indicating mitochondrial dysfunction confirmed by the
association between F2- IsoP and ALT elevation which also supports plasma F2-IsoP as a reliable marker of oxidative stress. Moreover in patients treated with a combination of AZT and EFV we could find an association between oxidative stress and AST which signifies that this combination could even cause damage to other organs such as kidney, brain and muscles in addition to the liver.

The usually associated covariables of oxidative stress even in the general HIV uninfected population such as cigarette smoking and increased BMI, in the present study was not found to be associated significantly with oxidative stress. Plasma F2-IsoP was found to be consistently high among the females in the ART-naïve as reported elsewhere [Redhage L.A. et al., 2009]. As smoking is known to cause mitochondrial dysfunction we have already excluded such individuals. Age is the other demographic factor associated with oxidative stress which in 6th month visit of the ART-naïve showed a significant association.

Increased oxidative stress at a higher CD4+ T cell count among the ART-treated which has been reported elsewhere [Hulgan T. et al.,2003] have also documented a significant negative association between oxidative stress and plasma viral load. Although such an observation seems to be baffling has been confirmed in enrollment visit, although not in terms of reduced viral load but the likely simulation of it that is the higher CD4 T cell count of >700 cells/µL.

It may be contemplated that increased oxidant stress is a component of the dynamic process of immune reconstitution in addition to the impact of ART. Immune reconstitution involves redistribution of lymphocytes from lymphoid tissues to blood, decreased systemic immune activation, changes in lymphocyte turnover
rate, and many other events. Similar finding of higher oxidative stress at a higher CD4 T cell count was also observed in the ART-naïve group which was also witnessed by the increased oxidative stress level in the 12\textsuperscript{th} month visit at a CD4\% of \textgreater{}25 as well as with higher TLC. In both the ART-treated and the naïve the CD4 T cell count was \textgreater{}500 cells/\(\mu\)L. Higher oxidant stress at a higher CD4 T cell count shows that inspite of higher systemic oxidant stress, the compensatory mechanisms at the intracellular level has inhibited CD4 T cell apoptosis which was also confirmed by the significant negative correlation between TLA (\%) and plasma F2-IsoP in the ART-treated. These finding questions the utility of oxidative stress as a marker of disease progression when there are studies which recommend oxidative stress as a predictor of all-cause mortality in HIV-infected patients [Masia M. \textit{et al.}, 2007]. In the above mentioned study as the median CD4 T cell count of the HIV infected was 86 cells/\(\mu\)L would have better reflected oxidative stress. Aukrust P \textit{et al.}, 2005 have documented an inverse correlation between CD4 T cell count and oxidative stress in HIV infected patients with advanced disease.

Thus, those studies that have identified CD4 T cell count as a marker of disease progression are limited by a lower median CD4 T cell count. In the present study, 53\% (16/30) among the ART-treated and 74\% (42/57) among the ART-naïve had a CD4 T cell count of \textgreater{}500 cells/\(\mu\)L, hence the level of oxidative stress and its correlation with CD4 T cell count cannot be compared with these studies involving patients with a lower CD4 T cell counts. There are also other studies that have found oxidative stress markers elevated at an early stage of the disease [Fris Moller N. \textit{et al.}, 2007] and studies involving higher CD4 counts have not documented a significant correlation between oxidative stress and CD4 T cell count [Mandas A. \textit{et al.}]
Taken together, the impact of oxidative stress at a higher CD4 T cell count has not been reported much, hence warrants more such studies to further delineate the level of oxidative stress at a higher CD4 T cell count, as it is more vital with the changing guidelines of early initiation of ART.

Many lines of research findings suggest that HIV-infected patients are under chronic oxidative stress [Wanchu A. et al., 2009], which might be due to increased activation of polymorphonuclear leukocytes or from pro-inflammatory cytokines produced by activated macrophages during the infection, or decreased intake of antioxidants [Morris D. et al., 2011]. Furthermore, the viral protein Tat, increases the intracellular levels of ROS, that is thought to induce apoptosis via FAS/CD95 interactions, thereby contributing to depletion of CD4+ T-cells [Gil L. et al., 2003].

The cells of the immune system are highly sensitive to oxidative stress, since their plasma membranes contain high levels of polyunsaturated acyl lipids, which are vulnerable to peroxidation. Consequently, excessive ROS can damage biomolecules such as DNA, carbohydrates, proteins and uric acids [Devasagayam TP. et al., 2004]. More importantly, this oxidative damage is particularly marked in the phospholipids of the cell membrane [Roberts RA. et al., 2010], therefore making lipid peroxidation a convenient marker of oxidative stress in living systems.

Significant positive correlation between oxidative stress and TLΔΨm^low (%) was observed in those on AZT which may be attributed to the higher impact of AZT in causing mitochondrial membrane potential damage implemented through oxidative damage which was also supported by a previous finding that in endothelial cells, AZT significantly oxidized glutathione redox potential, decreased mitochondrial
membrane potential, increased total cellular and mitochondrial-specific superoxide, increased lactate release, and finally caused cell death and that AZT caused greater endothelial dysfunction than d4T because of its pro-oxidative effects and both AZT and d4T caused mtDNA depletion. Cell type-specific differences in respiratory requirements, nucleotide transport, pol γ activity, mitochondrial turnover, or other factors could explain the difference in mitochondrial dysfunction among the two NRTIs [Kline ER. et al., 2009].

Yet another vulnerable victim of oxidant stress is the RBC which are highly susceptible to oxidative damage due to exposure to high concentrations of oxygen radicals, the lack of nucleus and mitochondria, inability to synthesise new protein and degradation of detoxifying enzymes thus making red blood cells (RBCs) uniquely susceptible to oxidative stress [Bryszewska M. et al., 1995]. In circulation, endogenous and exogenous ROS can cause damage to RBC. Inflammation, reperfusion processes and leukocyte activation can increase the production of ROS from mitochondria that could cause oxidative stress in the RBCs [Lawler JM. et al., 1998].

Thus, analyzing the impact of oxidative stress on the hematological parameters we found hemoglobin and RBC to be significantly reduced among those with higher oxidative stress (> than the median F2 IsoP of the LoRHC) in the ART- naïve which could be attributed to the oxidative damage of the erythrocytes or hemoglobin because studies have revealed that the mechanism of haemolysis as a consequence of the system used to induce the stress [Edward CJ. & Fuller J., 1996]. It might also be explained that as mitochondria is involved in heme biosynthesis, as a result of
mitochondrial dysfunction in HIV infection the heme production might have been affected in the erythroblast cells which could be the reason for the significant reduction in MCHC noticed in those with increased oxidative stress. However, although the RBC parameters during oxidative stress among the naïve were in the normal range, we could identify the subtle changes contributing to the pathophysiology involving RBCs that might transform into a symptomatic condition with further decline in RBCs that occurs with disease progression. Although there are various causes of anemia such as blood loss, decreased RBC production, deficiencies in iron, folic acid, or vitamin B$_{12}$ a CD4 T cell count of <200 cells/µL [Volberding PA. et al., 2004] being a risk factor, in the current study the CD4 T cell count of the ART-naïve patients was 624(481-738) cells/µL and as per standard of care and treatment patients were on vitamin and mineral supplementation. Thus the underlying mechanisms for the reduction in Hb levels may be attributed to oxidative stress and mitochondrial dysfunction. Further studies to corroborate the finding might unravel the involvement of mitochondria in causing changes in vital parameters such as Hb/RBC.

One of the first cellular responses to hypoxia is increased production of ROS by mitochondria, with evolution, organisms have developed an efficient and rapid way to respond to erythroid challenges by using ROS to increase the RBC numbers, defined stress erythropoiesis, which induces hematopoietic/stem progenitor cells to produce RBCs to compensate losses [Suda T. et al; 2011] especially in conditions of bone marrow suppression due to chemotherapy. Much has been discovered in recent years on the mechanisms that regulate stress erythropoiesis. ROS stimulates kidney cells to produce greater levels of erythropoietin, the hormone that specifically
stimulates RBC production [Haase VH. et al., 2013]. In addition, ROS stimulates hematopoietic cells in the marrow to produce factors that induce hematopoietic stem cells to generate ‘stress-specific erythroid progenitors’ with higher ability to produce RBCs [Paulson RF. et al., 2011]. However, the evidence of stress erythropoiesis in the current study can be also confirmed by the elevated MCV which is a marker of stress erythropoiesis; although it is elevated in both with and without oxidant stress, significant elevation in PCV without significant difference in the RBC count in oxidant stress could be because of macrocytosis. During the maturation of such erythroid cells the accumulation of Hb causes increase in ROS which if not checked could activate p53-dependent pathway of cell death. Thus these cells inorder to neutralize the effects of ROS, increases the expression of antioxidant enzymes such as catalase which is controlled by the transcription factor forkhead-box protein O3 (FoxO3) activated by AKT (Protein kinase B) [Zhang P. et al., 2013]. The subsequently reduced ROS could alleviate mitochondrial dysfunction of the erythroblast cells during stress erythropoiesis for the significantly increased Hb levels despite the increased level of systemic oxidative stress.

Mitochondria are the major source of oxidative stress because the unavoidable electron leakage during electron transfer leads to the constant generation of superoxide anion which, despite the presence of an efficient mitochondrial/cellular antioxidant system, is responsible for 90% of the endogenous ROS [Andreyev AY. et al., 2005]. It is suggested that dysfunctional mitochondria are less efficient producers of ATP but more efficient producers of ROS, which could represent a major source of oxidative imbalance. As oxidative stress has been implicated in various forms of mitochondrial dysfunction, in the present study, when we intended
to find a correlation between the mitochondrial dysfunction parameters and F2-IsoP, we could find a significant negative correlation between oxidative stress and mtDNA content in those on AZT in the enrollment visit, a significant positive correlation with TLΔΨm^low (%) in those on EFV and significant negative correlation with TLA (%) among the ART-treated. Although there were no significant correlations between plasma F2-IsoP and other mitochondrial parameters, in the 6th month visit there was a significant decrease and increase in mtDNA content and plasma F2-IsoP respectively in the ART-naïve group that signifies their association. Plasma F2-IsoP gives the picture of the systemic oxidative stress and PBMC mitochondrial dysfunction gives an estimate of the mononuclear cells of the blood, however we have documented the interlinking pathophysiological mechanisms operated in oxidant stress and mitochondrial dysfunction and their probable outcomes in the HIV infected ART-naïve and the treated patients.

6.10 Total lymphocyte apoptosis (%) and ΔΨm^low (%):

In this study, TLA% and TLΔΨm^low (%) of HIV-infected, ART-naïve and treated patients were significantly high in comparison with LoRHC. This is in line with other previously published results showing the effect of HIV infection and ART on mitochondrial function of blood lymphocytes [Karamchand L. et al., 2007; Sternfeld T. et al., 2007] and the subsequent induction of apoptosis. The mechanisms of apoptosis are highly complex, involving an energy dependent molecular mechanism. There are two main apoptotic pathways: the extrinsic pathway in response to death signals and the intrinsic pathway in response to internal cellular stress signals, this pathway is mitochondrial mediated [Elmore S.,
and that if the extrinsic pathway fails it can even stimulate the intrinsic pathway through p15 truncated Bid (tBid) [Li H. et al., 1998]. Increase in mitochondria membrane permeability is triggered by pro-apoptotic signals during the intrinsic pathway of apoptosis [Boya P. et al., 2001]. This change in the mitochondria membranes results in the opening of the mitochondrial permeability transition pore, loss of the mitochondrial transmembrane potential ($\Delta \psi_m$) and release of pro-apoptotic proteins from the intermembrane space into the cytosol [Saelens X. et al., 2004] that leads to apoptosis in a caspase dependent manner.

Infection by HIV is characterized by gradual CD4 T lymphocyte decline, which leads to immunodeficiency. However, the particular mechanism by which HIV causes T lymphocyte depletion was made clear to an extent only recently, that is, productive infection of CD4$^+$ T cells induces apoptosis, mediated by caspase-3 activation [Cooper A. et al., 2013], and abortive infection induces pyroptosis, mediated by caspase-1 activation [Doitsh G. et al., 2014]. T cell apoptosis had been proposed as the mechanism responsible for T cell depletion in patients infected with HIV [Laurent-crawford AG. et al., 1991]. Later it was established that inappropriate apoptosis induced by HIV is central to the pathogenesis of AIDS [Wan ZT. & Chen., 2010]. Bystander T helper apoptosis is primarily mediated by extrinsic pathway of apoptosis involving Fas ligand and/or tumor necrosis factor (TNF), whereas infected T helper cells are spared from autonomous Fas- or TNF-related apoptosis by the inhibition of apoptosis signal-regulating kinase-1 by Nef protein [Geleziunas R. et al., 2001].
HIV infection may induce apoptosis of both infected cells and uninfected cells through various mechanisms, which include: (i) direct role of HIV-specific proteins, (ii) direct infection of T lymphocytes, (iii) activation-induced cell death (AICD), (iv) autologous cell-mediated killing of uninfected T cells and (v) dysregulation of cytokine/chemokine production [Saelens X. et al., 2004].

Owing to the fact that the proportion of cell loss is greater than the number of infected cells, there are more of chances for the killing of the bystander uninfected T cells. Furthermore, apoptosis occurs predominantly in bystander cells but not in productively infected cells of HIV-infected lymph nodes [Ferri KF. et al., 2000]. So, it is logical to assume that HIV infection induces bystander lymphocyte apoptosis, which in turn leads to dysfunction of the immune system.

The Bcl-2 family of proteins regulate mitochondrial membrane permeability, thus Bcl-2 family proteins regulate the release of proapoptosis proteins. Binding of gp120 to CD4 may lead to regulation of the Bcl-2 protein family by inducing the down regulation of Bcl-2, an anti-apoptosis protein, and upregulation of Bax, a pro-apoptosis protein, leading to mitochondrial pathway of apoptosis [Somma F et al., 2000]. In addition binding of gp120 to CXCR4 induces mitochondrial transmembrane depolarization, cytochrome-C release and activation of the caspases-9, which are the hallmarks of intrinsic apoptosis [Roggero R. et al., 2001].

Env-induce mitochondrial intrinsic apoptosis pathway and the process do not involve activation of the stress- and apoptosis-related mitogen-activated protein kinases (MAPKs) p38 and JNK [Biard-Piechaczek M. et al., 2000].
It is the mitochondrial mediated apoptosis that occur in syncytium apoptosis [Roumier T. et al., 2003]. Tat proteins target the mitochondria and tigger mitochondrial membrane potential damage with subsequent cell death.

Other HIV-1 proteins, Vpr, Env, and PR, may also affect mitochondrial function; Vpr via direct transition pore binding [Jacotot E. et al., 2000], PR by cleaving procaspase-8 and/or Bcl-2 [Nie Z. et al., 2008] and Env by Bax activation [Perfettini J. et al., 2009]. Thus the HIV viral proteins target the mitochondria and trigger cell death. This hints at the possibility that several apoptogenic HIV-1 proteins influence at the mitochondrial level, contributing to HIV-related cell damage in lymphocytes [Cummins NW. & Badley AD., 2010].

Apoptosis has been shown to be triggered by ROS overproduction, m lowering, or network disruption. Oxidative stress mediated mechanisms reduce the lymphocyte count in HIV infection [Pasupathi P. et al., 2009]. Though that NRTIs suppresses viral load and increases CD4 T cell counts, it does not produce an enhanced immunological benefit than the PIs which has anti-apoptotic activity [Sloand EM. et al., 1999; Badley AD. et al., 1999] that leads to a dramatic increase in CD4+ T cells, thus mitochondrial dysfunction could possibly be the underlying reason for this lacunae because NRTI are well established to cause mitochondrial dysfunction that could trigger apoptosis. Moreover the ability of NRTI to inhibit pol $\gamma$, and its ability to generate oxidants may collectively damage the mitochondrial membrane potential which could trigger the mitochondrial mediated apoptosis. Thus many lines of research have identified the progressive decrease in CD4 T cell count in HIV infection is due to the intrinsic pathway of apoptosis possibly by the
induction of mitochondrial membrane depolarization. Though it is expected that upon ART initiation, with the suppression of viral load, there is a low level of immune activation, with successful immune reconstitution, lymphocyte apoptosis is reduced. However, as per the mitochondrial dysfunction hypothesis, NRTI could inhibit pol \( \gamma \) enzyme with the eventual energy deprivation, stimulation of oxidant stress causing mitochondrial dysfunction with the possible induction of apoptosis.

In the current study, although CD4 T cell count of the ART-naïve and the treated showed a progressive decrease and increase respectively, TLA (%) of the treated also was significantly higher than the LoRHC inspite of virologic suppression, thus proving the existence of mitochondrial dysfunction mediated cellular loss caused by NRTI.

6.11 Correlation between TLA (%) and TLA\( \Psi \)^{low} (%):

The concept of an apoptotic cascade, with reduction of \( m \) as a pivotal step prior to apoptotic cell death [Castedo M. et al., 1996] has been confirmed once again in the present study in the ART-naïve and also in the ART-treated in enrollment and 6\(^{th}\) month visit. However, this correlation was not very evident in individual visits but only in the cross-sectional analysis in the ART-naive. Opening of the mitochondrial permeability transition pore in mitochondrial dysfunction stimulated by HIV or NRTI induces depolarization of transmembrane potential eventually causing release of apoptogenic factors and loss of oxidative phosphorylation resulting in apoptosis. However the absence of such a correlation has also been reported by Karamchand et al., 2007. In the 12\(^{th}\) month visit, TLA\( \Psi \)^{low} (%) was found to be higher than the TLA (%), which is also confirmed by the increase in
CD4 T cell count \((p= 0.055)\) compared to the enrollment visit. However owing to the longer duration of exposure, the mitochondrial parameters though were improving in the 12\(^{th}\) month visit was still affected or probably there is the existence of compensatory mechanisms to overcome the depolarized transmembrane potential thereby reducing TLA (\%) atleas\(t\) partly at a higher CD4 T cell count. The above theory was also supported by the finding that there was a higher level of \(\text{TL} \Delta \Psi^\text{m}_{\text{low}}\) (\%) and plasma F2- IsoP at a higher CD4 T cell count.

mtDNA content another marker of mitochondrial health which correlated well with CD4 T cell count, also correlated with \(\text{TL} \Delta \Psi^\text{m}_{\text{low}}\) (\%) at a higher viral load in the ART- naï\(\ve\). Moreover under oxidant stress, TLA (\%) correlated significantly with mtDNA content and with CD4 T cell count and faintly with \(\text{TL} \Delta \Psi^\text{m}_{\text{low}}\) (\%) in the 6\(^{th}\) month visit. This implies the greater role of oxidant stress in linking lymphocyte apoptosis and mitochondrial functioning. Overproduction of ROS may therefore directly decrease m and lead to a lowered ATP supply, and may also cause mitochondrial network fragmentation and subsequent mitochondrial autophagy (mitophagy), cell apoptosis or cell senescence. Of note, in the 6\(^{th}\) month visit of the ART-naï\(\ve\) there was no significant difference in plasma F2-IsoP at any of the CD4 T cell count cut-offs.

However, the observation that higher oxidative stress at a higher CD4 T cell count shows that there are mechanisms that could mitigate the effect of oxidative stress thus helping the lymphocytes to overcome the eventual consequence of ROS which is cell death. When the lymphocytes succumbs to the detrimental effects of ROS then the lymphocyte undergoes apoptosis and such a scenario is found to exist.
at very low level of mtDNA content which seems to be the ultimate stage for lymphocyte cell death. These regulatory mechanisms could be the antioxidant system of the cell and the mitochondrial mechanism such as mitophagy, mitochondrial fission or fusion that could increase the mtDNA content to a level to save the cell from apoptosis.

In addition, a decreased m was previously reported in PBMCs from ART-naive with a median CD4 T cell count of 429 cells/µL [Sternfeld T. et al., 2009]. This decrease could be linked to HIV-1-encoded Vpr and other viral proteins through their binding to mitochondrial permeability transition pore components.

Moreover, in the ART-treated a significant positive correlation between TLA (%) and TLΔΨm low (%) in enrollment confirms that mitochondrial membrane depolarization has eventually induced lymphocyte apoptosis as studies [de Oliveira Pinto LM. et al., 2002] have documented a significant level of persistent lymphocyte apoptosis in about 70% of chronically treated (up to 55 months) patients. Moreover induction of apoptosis was also a pathophysiological mechanism illustrated in mitochondrial dysfunction hypothesis which was also supported by studies documenting persistence of lymphocyte apoptosis during treatment with d4T to be ascribed to mitochondrial toxicity through NRTI induced mtDNA depletion which is predominant during chronic ART treatment [Miro O. et al., 2003]. The lack of correlation in 12th month may be attributed to the indigenous compensatory mechanisms such as mitophagy which can be also evidenced by the increasing mtDNA content seen in 12th month.
Though high levels of ROS may harm cells by inducing DNA damage and promoting apoptosis, moderate levels of ROS are essential for hematopoiesis during embryonic development, and they are also required in adult hematopoietic homeostasis [Harris JM. et al., 2013]. In view of this in the current study among the ART-naïve we noticed a significant positive correlation between TLA(%) and CD4 % in 12th month among those with normal level of F2 IsoP conversely in those with oxidative stress there was a significant negative correlation with CD4%. Thus we report oxidative stress induced apoptosis as an internal stimuli that stimulates apoptosis via mitochondrial membrane depolarization accounting for the negative correlation and the optimal level of ROS in inducing normal hematopoiesis as evidenced by the significant positive correlation. Further to this finding we could also see by and large a significant reduction in other cells of the blood such as the platelets, lymphocytes and a reduction in RBCs reflected as lowered MCV in ART-naïve patients with oxidative stress which signifies that oxidative stress has induced apoptosis of CD4 T cells through mitochondrial dysfunction and oxidative damage induced hemolysis of RBCs.

6.12 TLΔΨmlow (%), TLA (%) and CD4 cell count:

Apoptosis of infected and uninfected cells is regarded as the mechanism mainly responsible for CD4 T cell loss during HIV infection. In the present study, CD4T cell counts significantly negatively correlated with TLΔΨmlow (%) and with TLA (%) in the ART-naïve but in the treated although CD4 correlated negatively with TLA (%) but positively with TL mlow (%). Though many studies have documented a positive correlation between TLA (%) and TLΔΨmlow (%), these
studies are limited to the CD4 T cell count of <500 cells/µL where as in our study CD4 T cell count was >500 cells/µL in both the groups. Increased oxidative stress at a higher CD4 T cell count could explain the significant positive correlation between CD4 T cell count and $\Delta \Psi_m^{\text{low}}$ (%) as ROS is known to influence m. It cannot be denied that there is no compensatory mechanism for these apoptotic cell death induced by mitochondria because Perrin S. et al., 2012 has reported that there is significant alterations in the lymphocyte mitochondrial parameters namely ROS production and mitochondrial membrane depolarisation but without irreversible damage that may lead to mitophagy and/or apoptosis in ART-naive patients with controlled CD4T cell count similar to the CD4 cell count of the present study.

But looking at the consistency of the correlation during the follow up we saw that among the naïve we could demonstrate the correlation between CD4 and TLA% only in enrollment and 6th month and not in 12th month, as a probe on this when we analysed the correlation between CD4 T cell count and TLC we found a significant correlation only in enrollment and 6th month visit and not in 12th month visit which shows that there is a marked expansion in the count of non CD4 cells of lymphocytes probably the CD 8 T cells. Similarly in the ART-treated, correlation between apoptosis and CD4 T cell count was not seen significantly in any of the visits in spite of the significant correlation between CD4 T cells and TLC in all the visits, this again signifies the generic inhibitory effect of the thymidine analogues irrespective of the subsets of the PBMCs as seen with mtDNA content of the ART-treated.
6.13 TLΔΨm\textsuperscript{low} (%), TLA (%) and viral load:

HIV proteins might be directly responsible for induction of PBMC apoptosis. HIV proteins have been related to influence mitochondrial membrane potential, release of cytochrome C and subsequent induction of apoptosis. Thus a significant association between viral load and TLA (%) and TLΔΨm\textsuperscript{low} (%) was expected among the ART-naïve. In this regard, we found a positive correlation between HIV viral load and percentage of apoptotic cells, and not with TLΔΨm\textsuperscript{low} (%). Although a significant correlation between viral load and TLΔΨm\textsuperscript{low} (%) has been reported in previous studies we could not demonstrated such a correlation in any of the visits. However previous studies which has shown a significant correlation between viral load and TLΔΨm\textsuperscript{low} (%) differ from the current study by factors such as CD4 T cell count of <500 cells/µL. However there is one study by Perrin et al., 2012, in which the ART-naïve had a CD4 T cell count of 548.4 ± 220 cells/µL and a viral load of 3.8 ± 0.8 log copies/mL which is almost similar to the viral load of the present study of 4 log copies/mL(3.0-4.7), but CD4 count was 624 cells/µL (481-738) we could only find a trend of positive correlation between viral load and TLΔΨm\textsuperscript{low} (%). Reduction of m does not lead to cell apoptosis automatically.

Induction and inhibition of apoptosis is complex and HIV replication itself is only one possible trigger besides the general immune activation during HIV infection. The depletion of the CD4 T-cell compartment is explained mainly by apoptosis of uninfected cells caused by indirect mechanisms and syncytia formation. HIV infection can even result in inhibition of apoptosis by modulating the mitochondrial pathway of apoptosis independently from active viral replication. It
was shown recently that reduction of viral load in ART-treated patients was not correlated to the detected increase of m after starting therapy, which again indicates determinants of m loss and PBMC apoptosis other than viral replication \textit{per se} (Sternfeld T \textit{et al.}, 2007).

Moreover among the ART-treated there was a characteristic finding documented in the study that in those treated there was a significant negative correlation between TLA (\%) and VLDL. As it has been mentioned already that in the condition of mitochondrial dysfunction, there can be decreased hepatic production of VLDL due to the decreased TGL production which was also documented in conditions of oxidant stress and mtDNA depletion.

Irrespective of the co-administered NNRTI, AZT was found to significantly associate with the blood cells such as WBC, TLC, CD4 T cell count and CD 4 \%, all showing a negative correlation with lymphocyte apoptosis. And in a combination with EFV, AZT had an impact on the positive and negative correlation between lymphocyte apoptosis and neutrophil count and platelet count respectively. The reason for the above finding could be attributed to the increased utility of neutrophils to clear up the cells undergoing apoptosis which is not much replaced subsequently due to the myelosuppressive activity of AZT. In line with these associations in the ART- naive group we found a significant negative association between increased \% of lymphocyte apoptosis and platelet count which was pronounced among those with increased oxidative stress, in enrollment. Although ROS has a significant role in thrombocytopoiesis in healthy controls, lethal levels of ROS causing oxidative stress has been reported to cause oxidative damage as the underlying mechanism in
thrombocytopenia. In addition, it is a well established phenomenon that HIV can directly infect the megakaryocytes [Chelucci C. et al., 1998] that would possibly account for the decline in platelet count, in order to affirm this finding we also grouped the naïve based on their plasma viral load of 4 log copies/mL and found that there was a marked decrease in platelet count at a higher viral load [287 x10⁹/L, 225-345 vs 251 x10⁹/L, 212-289, p= 0.077].

HIV results in impaired survival of bone marrow megakaryocytes and their precursors. HIV also decreases the number and activity of human progenitor cells and decreases megakaryocyte maturation and ploidy. HIV surface glycoprotein gp120 leads to increased megakaryocyte apoptosis in vitro due to increased TGFβ and down-regulation of the proliferation-inducing ligand tumor necrosis factor ligand superfamily member 13 (TNFSF13). Further, gp120 interacts with CD4, which is expressed by immature megakaryocytes, which also express CCR5, and leads to their infection [Louache F. et al., 1991]. Furthermore, HIV infection of megakaryocytes can lead to reduced TPO receptor (c-Mpl) expression. Thus as there was significant difference in the CD4 T cell loss at every visit with increasing viral load, with the increasing lymphocyte apoptosis there is a significant depletion of platelets of which being catalysed by elevated oxidative stress and viral replication.

6th month visit of the naïve cohort was very unique because all the clinical and mitochondrial parameters were very badly affected documenting a median CD4 T cell count of 493 cells/µL in a subgroup with a median viral load of 4.7 copies/mL. Correlation analysis in this critical cohort showed that during increased oxidant stress there was a significant negative association between lymphocyte
apoptosis and mtDNA content, thus the cohort in this particular visit has precisely portrayed the potential link between the increased viral load and thereby its proteins that has induced the inflammatory reactions causing mitochondrial release of oxidants that would trigger mtDNA depletion and concomitant lymphocyte apoptosis causing CD4 T cell decline thus causing a viscious cycle that ultimately sets up the stage leading to further disease progression.

Among the ART-treated a significant positive correlation was documented between TLA (%) and TLΔΨm^low (%) in enrollment and a negative correlation with oxidative stress at 12th month which can be directly explained by the observation that increased oxidative stress occurs at a higher CD4 T cell count. However to confirm this finding we estimated the level of TLA (%) at every visit among those with increased oxidative stress of >118pg/mL and found that in 6th month visit and 12th month, TLA (%) was lesser in those with oxidative stress but there was no marked difference in TLΔΨm^low (%) in enrollment but was again lower in oxidative stress of 12th month. In general it is canonical that excess ROS production and mitochondrial dysfunction induce mitochondria-mediated apoptosis, to prevent the accumulation of harmful oxidants [Puddu P. et al., 2005]. However there are previous studies that indicated NRTI treatment increases ROS production and mitochondrial dysfunction without inducing apoptosis [Jiang B. et al., 2007], thus our findings from this study signifies the existence of compensatory mechanisms which allows mitochondria to combat NRTI-induced oxidant injury. This compensation may be derived from upregulated antioxidant response pathways or alternatively the recently discovered phenomenon of mitophagy. Mitophagy, which has been documented in numerous cell types, is the selective autophagic degradation
of damaged mitochondria or mitochondria producing excess ROS to prevent the accumulation of mtDNA mutations and additional cellular damage. Moreover it is conditional that for mitophagy to occur mitochondrial membrane depolarization is vital, in line with this it was observed that TLΔΨm\text{low} (\%) was high in the ART-treated at higher CD4 T cell count. It may be contemplated that such TLΔΨm\text{low} (\%) cells remove their damaged mitochondria by mitophagy thereby decreasing CD4 T cell loss. Thus, the transcriptomic changes in both the nucleus and the mitochondria observed by Desai VG. \textit{et al.}, 2008 may reflect cellular efforts to mitigate damage. Eventually, after ROS accumulate to levels that irreparably damage the mitochondria, these cells likely undergo mitophagy, thus preventing cell death [Goldman SJ. \textit{et al.}, 2010].

Possible explanations for the relationship between lower BMI and higher TLA (\%) documented in 6\textsuperscript{th} month visit and 12\textsuperscript{th} month visit and with TLΔΨm\text{low} (\%) in enrollment may include the effects of adipokines such as leptin, differences in thymic size, differences in lymphocyte population dynamics in the gastrointestinal tract and other mucosal sites, and differences in T-lymphocyte apoptosis. On the other hand, several studies have demonstrated that increasing BMI positively correlated with higher CD4 T-lymphocyte counts in HIV-seronegative women [Womack J. \textit{et al.}, 2007] and children [Zaldivar F. \textit{et al.}, 2006]. Therefore, persons with higher BMI may naturally have higher CD4 T-lymphocyte counts, and the greater CD4 T-lymphocyte recovery on ART in HIV-infected patients with higher BMI could be explained simply by a “return to health” phenomenon. This association of lower BMI with high apoptosis was more pronounced in patients treated with d4T, which is known to cause lipodystrophy, although overweight is a
well known risk factor for lipodystrophy, in the context of lymphocyte recovery it may have a role to play in causing adipose cell apoptosis there by reducing the levels of leptins. Adipocytes produce cytokines and adipokines, including leptin and adiponectin. Serum leptin levels are higher in obese patients and are positively correlated with percentage of adipose tissue [Considine RV. et al., 1996].

The leptin-deficient state in mice is associated with reduced thymic development and peripheral lymphocyte counts and function [Lord GM. et al., 1998]. In humans, there are reduced lymphocyte numbers and function; leptin replacement therapy reverses these defects [Oral EA. et al., 2006].

Although TLA (%) correlated significantly with CD4 T cell count by Mann Whitney U test, by spearman correlation test also correlated negatively with mtDNA in 6th month visit which further substantiates that the mtDNA depletion induced mitochondrial dysfunction has triggered the intrinsic pathway of apoptosis leading to CD4 T cell depletion. The pattern of dyslipidemia is different in the HIV infected ART-naïve especially in more advanced disease in whom low total, LDL-C and HDL-C and TGL than HIV-negative controls [Anastos K. et al., 2007].

The role of mitochondria in lipid homeostasis has been strongly emphasized in recent studies focusing on mitochondrial respiratory deficiency. Conditions of chronic mitochondrial dysfunction may cause lipid metabolism disorders [Kakuda TN., 2000]. Thus although the molecular mechanisms underlying the changes in lipid profile in HIV infection is not clear, in this study we have seen a significant association between the mitochondrial parameter, $\text{TLM}^{\text{low}}$ (%) and the elevated TGL, VLDL.
Systemic inflammation in HIV infected may contribute to hypertriglyceridemia. TG concentration was found to correlate with serum interferon-alpha (IFN-α) [Grunfeld C. et al., 1992], which is overproduced in HIV infection, moreover the activity of lipoprotein lipase (LPL) and hepatic lipase, which are both involved in TG clearance from the circulation, are decreased compared to controls. Sofar it is known that the activity of cholesterol ester transfer protein (CETP), which transfers cholesterol esters from HDL-C to apolipoprotein- B containing proteins, CETP is elevated in HIV infection, and its activity correlates inversely with serum HDL concentrations [Rose H. et al., 2008]. Thus, decreasing the level of HDL-C in the HIV infected. Cholesterol and fatty acid biosynthesis depends on the export of acetyl–coA from the mitochondria, sterol biosynthesis also takes place in the inner mitochondrial membrane. Mitochondrial Triglyceride (TG) transfer protein (MTP) is a key enzyme for apoB-containing lipoprotein assembly and secretion. This is mostly attributed to its capacity to transfer lipid components (TGs, cholesterol esters and phospholipids) to the endoplasmic reticulum lumen, where these lipoproteins are assembled. Inhibition of MTP may cause the disturbances in the lipid profile [Kostapanos MS. et al., 2013]

6.14 Viral load and mtDNA content, TLA (%), TLAψm<sub>low</sub> (%):

During uncontrolled viral replication, there is a marked decline in the CD4 T cell count as a result of increase in lymphocyte apoptosis. In patients with HIV infection, neutropenia can result from the disease or related malignancies, drug therapies, or opportunistic infections. HIV can cause neutropenia by directly or indirectly impairing hematopoiesis. Similarly, microorganisms that cause
opportunistic infections, such as cytomegalovirus and Mycobacterium avium complex, can infiltrate the bone marrow and cause myelosuppression. The association between \( TLA \) (\%), TL\( \Delta \Psi m^\text{low} \) (\%) with neutrophils might also be attributed to the increased clearance of apoptotic bodies produced in addition to the aforementioned reasons. Neutropaenia, was observed only in 7/57 patients at various visits during the follow up, however ANC was significantly increased in the ART-naïve than the treated. Correlation between mtDNA content and TL\( \Delta \Psi m^\text{low} \) (\%) was observed at a higher viral load implying the intense effect of the viral proteins in causing various mitochondrial defects.

HIV infection leads to a disturbed T-cell homeostasis, featured by a depletion of CD4 T-cells and a persistent elevation of CD8 T-cells over disease progression. The dynamics of CD8 T-cell responses to intracellular infection have been extensively studied in model systems. Infection typically stimulates a rapid burst of proliferation in antigen-specific CD8 T cells with division occurring as quickly as once in 4–6 h. This expansion results in a large population of effector CD8 T cells that aid in clearance of infected cells. Naïve CD8 proliferation is driven by HIV viral RNA [Catalfamo M. et al., 2008], thus although the vivid expansion of CD8 T cells was much pronounced at the advanced disease stage, it may be expected that there is concomitant CD8 T cell expansion at least in those with higher viral load which was reflected on the TLC which increased with TL\( \Delta \Psi m^\text{low} \) (\%).

6.15 Impact of age on mitochondrial parameters and oxidative stress:

In the current study, among the ART-naïve, it was quite surprising to see mtDNA content decrease in <40 years which was also confirmed by the increase in
TLA(%) and $\text{TL} \Delta \Psi_m^{\text{low}}$ (%), however, increased oxidative stress was seen in >40 years of age. Among the ART-treated, $\text{TL} \Delta \Psi_m^{\text{low}}$ (%) was associated with >40 years of age. Possible reasons for the frequent and accelerated disease progression in elderly HIV-infected patients are an age-related decrease in thymic function, replicative senescence of the immune system associated with accelerated telomere shortening, and lower CD4 cell counts at baseline. Other factors proposed to contribute to the greater risk of disease progression in elderly HIV-infected patients are increased expression of the CCR5 HIV-1 co-receptor, reduced production of IL-2 and its receptor that may promote immunosenescence, and reduced CD8 cytotoxic T cell function. Although a recent study has shown that CD4 T cells from elderly individuals show high levels of apoptosis, in line with this, the age group of the elderly individuals in the age related studies were in the range of 50–86 years, where as in the present study in those with the age group of > 40 years, only 3/57(5%) were more than 50 years of age [Heigele A. et al., 2015]. However, the strongest predictors of progression to AIDS in HIV-infected humans of all ages are increased levels of immune activation and apoptosis. Moreover, those with the age group of <40 years had a lower CD4 T cell count hence the mitochondrial parameters must have been well reflected in this age group.

6.16 mtDNA content, $\text{TL} \Delta \Psi_m^{\text{low}}$ (%), TLA(%) and systemic oxidative stress- the interlink:

The correlation between mtDNA content and plasma F2- IsoP/ $\text{TL} \Delta \Psi_m^{\text{low}}$ (%) was found to be pronounced in conditions of mtDNA depletion among the ART which implies the pol $\gamma$ inhibiting role of NRTI leading to mtDNA depletion that
could trigger other downstream pathways such as inhibition of OXPHOS, ATP depletion, increased ROS production that could possibly cause mitochondrial membrane depolarization that can potentially induce mitochondrial mediated apoptosis. However in the present study, in the 6th and 12th month visit we have observed higher TLΔΨm low (%) at a higher CD4 T cell count implying the possible compensating mechanisms such as mitophagy, which is a process by which mitochondria with depolarized mitochondrial membrane are removed by autophagy. Generally many research findings have studied the pro-oxidative effects of AZT as a characteristic feature involved in AZT mediated pathologies of mitochondrial dysfunction thus resulting in a significant correlation between mtDNA content and systemic oxidative stress. Moreover the association between TLΔΨm low (%) and plasma F2-IsoP was pronounced in oxidative stress and mitochondrial membrane depolarization occurs only when there is a marked depletion in mtDNA content. Thus it may be structured that in the ART treated, upon inhibition of pol γ by the NRTI there is increased release of ROS as estimated by plasma F2-IsoP level though the intracellular ROS was not measured, could eventually cause mitochondrial membrane depolarization. However when the TL m low (%) increases the compensatory mechanisms probably intervene to regulate the CD4 T cell number which was also supported by the progressive increase in CD4 T cell count in the ART treated. It is also conceivable that when there is a uniform level of cellular toxicity in all the subsets of the blood caused by the mitochondrial dysfunction of the bone marrow progenitor cells induced by NRTI.

In general, among the ART-naïve, the significant negative association between mtDNA content and CD4 T cell count decline is strengthened by the negative
association between mtDNA and TLA (%) along with the positive association between TLA (%) and $\Delta \Psi_m^{\text{low}}$ (%) potentially linking mtDNA depletion triggered depolarization of mitochondrial membrane potential that ultimately causes CD4 T cell apoptosis.

Thus the current study with Indian HIV patients has documented mitochondrial dysfunction among HIV infected and the NRTI treated. Though the extent of mtDNA depletion, $\Delta \Psi_m^{\text{low}}$ (%) and TLA (%) were similar among the patients who were on ART and drug-naïve, interestingly observed that oxidative stress was higher among drug-naïve patients. Moreover higher level of mitochondrial dysfunction was observed even at a higher CD4 T cell count. Thus further studies on mitochondrial dysfunction would suggest newer treatment strategies in the future.

6.17 mtDNA- ND1 gene variations:

Mitochondria is the host of oxidative phosphorylation and hence a major producer of cellular oxidants. There is an imbalance between the oxidants and the antioxidants causing oxidative stress during HIV infection in both ART-naïve and treated patients which damages mtDNA that leads to mtDNA variations as per the mitochondrial dysfunction hypothesis [Lewis W. et al., 2001]. In mtDNA, so far over 100 pathogenic point mutations, rearrangements and 200 deletions and insertions have been identified. About 35% of these mutations affect polypeptide subunits of the respiratory chain. Traditionally, mutations in mtDNA and dysfunction of the proteins encoded by mtDNA were regarded as classic mechanisms causing mitochondrial disorders [Zhang L. et al., 2011]. Diseases generally included in mitochondrial disorders are the clinical syndromes caused primarily by disruption of energy production through oxidative phosphorylation (OXPHOS). Hence in the present study, ND1 gene coding for one of the 7 subunits of NADH dehydrogenase was sequenced by Sanger’s method to study the impact of NRTI in causing variations in mtDNA, a number of variations were observed both in the ART-treated and in ART-naïve and also in the LoRHC which seemingly questions the possible effect of both the virus and NRTI in causing mutations. Further, all mutations and polymorphisms observed were single base substitution,
deletions and insertions were not identified and all the variations are homoplasmic except for 3 heteroplasmic (coexistence of wild-type and mutant mtDNA) variations found in 2 ART-naïve patients. These variations upon 18 month follow-up still remained heteroplasmic. Though the proportion of individuals with variations was high in LoRHC, the total number of variations was very high in the ART-naïve than the 2 other groups. However the distribution of non synonymous and pathogenic variations associated with mitochondrial disease was almost the same in all the three groups. The higher number of variations (77%) among the ART-naïve could be probably because of the impact of HIV infection mediated by excessive ROS. Although the intracellular ROS levels of PBMC was not estimated in the present study, systemic oxidative stress was significantly high in the ART-naïve than the treated which could be simulated to the higher level of intracellular ROS that might have induced the higher level of mutations. However whether is it acquired or inherited remains a question unless further confirmed by sequencing the siblings of the study subjects.

The polyploid nature of the mitochondrial genome — up to several thousand copies per cell — gives rise to an important feature of mitochondrial genetics, homoplasmy and heteroplasmy. The value of these terms is apparent when we consider mtDNA mutations that lead to disease. Some mutations affect all copies of the mitochondrial genome (homoplasmic mutation), whereas others are only present in some copies of the mitochondrial genome (heteroplasmic mutation). In the presence of heteroplasmy, there is a threshold level of mutation that is of importance for both the clinical expression of the disease and for biochemical defects, as
routinely demonstrated by the cytochemical assessment of cytochrome C oxidase activity in an individual cell.

The concept of homoplasmy is more apparent than real. In most individuals there is no evidence of heteroplasmy, but all available evidence indicates that mtDNA is constantly undergoing mutation, with clonal expansion or loss of either point mutations or deletions.

Because these acquired mutations occur at random, all acquired mutations will be present at a low level and therefore might not be detected in a tissue homogenate or blood sample. Thus the additional low level of heteroplasmic variations that are not detectable by our current method could be identified by using clonal sequencing.

Thus the overall variations observed in the HIV infected has to be confirmed whether they were the inherited or the acquired variations induced by HIV or NRTI. In order to do this either the siblings of the patients has to be screened for similar variations or by clonal sequencing.

Defining pathogenic mutation of mtDNA is still contentious, most workers in human mitochondrial genetics suggest that three out of the four following criteria must be met if a mutation in mtDNA is to be considered pathogenic[Naviaux RK., 2000], i) it is not seen in controls ii) it is seen in unrelated pedigrees with similar disease presentations iii) the nature and location of the mutation suggests a logical mechanism of disease and iv) it is heteroplasmic. However, with the increasing number of reports of homoplasmic mtDNA mutations other parameters to define pathogenicity should be considered.
Among the reported pathogenic mutations, G3316A reported to be associated with Non insulin dependent diabetes mellitus (NIDDM) has been also detected in the healthy controls. T4216C associated with Insulin resistance (IR), A3397G was reported to be associated with Alzheimer’s and Parkinson's Diseases (ADPD) and cardiomyopathy, A3505G and A3480G with prostate tumour. The pathogenic mutations were found to be distributed almost equally among the three groups.

Further impact of the variations on the clinical outcome has to be elucidated in patients with specific toxicity to corroborate on the role of these mutations in the emergence of toxicity and the impact of HIV per se. Previous studies involving genome wide analysis of peripheral blood mtDNA mutations in NRTI-treated individuals showed that the use of these drugs provides conditions permissive for mutagenesis in vivo [Martin AM. et al., 2003]. In contrast studies have shown absence of common pathogenic mtDNA point mutations, deletions, and DNA rearrangements in NRTI exposed patients [McComsey G. et al., 2002]. To this end, though the mutations of this study have not been reported in HIV/AIDS setting because of few of such studies been carried out, it has been well documented in various other studies on mitochondrial disorders and in mitochondrial mediated diseases.

It should be pointed out that although synonymous mutations do not result in amino acid changes, there are differences in the usage frequency of different codons [McComsey G. et al., 2002]. Most remarkably the aminoacid change from CTA to TTA(C3757T and C3637T), the relative codon usage frequency of CTA and TTA are 70% and 17.3% respectively. Thus when TTA is incorporated in place of CTA,
the efficiency of incorporating lysine at this position is markedly reduced thus affecting
the overall translational efficiency. Thus it may be contemplated that many of the
polymorphic variations might have subtle effects in controls that are aggravated in the
presence of HIV and ART to result in clinical disease.

6.18 Study Limitations:

Based on our results, 70% of the HIV infected had mtDNA depletion compared to 41% of
the controls, which had adequate power (84%) which was calculated using openepi power
calculator. Previous reports have identified that the number of normal mtDNA copies must
fall below 20–40% of basal levels to induce mitochondrial dysfunction and severe adverse
events [Igoudji A. et al., 2006, Ducluzeau PH. et al., 2002 & Rossignol R. et al., 2003], in
line with this, ART-treated in the enrollment visit with symptomatic events and the ART-
naïve showed % change of mtDNA content of 24% and 34% respectively, compared to
the controls with the calculated sample size of the study, which is a reasonable
reflection of the underlying mitochondrial dysfunction. However, due to loss to
follow-up, the sample size was eventually reduced which also reduced the comparable
sample size in the subgroup analysis. Thus the small sample size was a limitation.

Though the inclusion of LoRHC was to estimate the level of mtDNA and other
mitochondrial parameters of Indian ethnicity, the current study has limitation in enrolling
the low-risk healthy control limiting to 24 study subjects. Moreover there is a
significant difference in age between the LoRHC and the HIV infected. Thus lack of
age and sex matched controls is another limitation of the study.
Although plasma F2-IsoP is a reliable marker of oxidative stress, knowing the status of the antioxidants levels would give a better picture of the impact of the oxidants.

Along with assessing the systemic oxidant stress, knowing the intracellular level of oxidant stress of the PBMC would suggest the greater impact of oxidant stress on the other mitochondrial parameters of the PBMCs.

Clonal sequencing in addition to population sequencing, to identify minor variations, lack of prospective follow up of the ART-naïve patients and their subsequent ART initiation to identify the time point of the emergence of variations and to identify whether they are the acquired variations and lack of sufficient number of patient population with a specific toxicity condition are the study limitations.

Thus we recommend that future studies should focus on mitochondrial dysfunction involving a larger HIV infected cohort who should be followed prospectively to measure the changes in mitochondrial functions.