1. INTRODUCTION

Increasing experimental evidence supports the existence of a link between inflammation and mitochondrial dysfunction. Mitochondrial defects, systemic inflammation and oxidative stress are central to many diseases such as diabetes, atherosclerosis, cancer, Parkinson’s disease, Alzheimer’s disease, other neurodegenerative diseases, heart and lung disturbances, obesity, and autoimmune diseases [Ouyang J. et al., 2013; Pagano G. et al., 2013; Osellame LD. et al., 2012; Enache I. et al., 2013]. Chronic inflammation is the hallmark of Human Immunodeficiency virus (HIV) infection and HIV infected patients suffer chronic oxidative stress [Morris D. et al., 2011].

HIV and many other viruses either induce or inhibit various mitochondrial processes in a highly specific manner so that they can replicate and produce progeny, moreover, mitochondria has a vital role in anti–viral immunity. Thus there is a growing concern to identify the role of mitochondria in the immunopathogenesis of HIV infection.

Mitochondria, in addition to energy production, perform a variety of functions and perturbation of mitochondrial functioning causes devastating outcomes. Mitochondria is a major producer of reactive oxygen species (ROS) and because it lacks histone proteins and many of the DNA repair mechanisms [Liang Q. & Dedon PC., 2001], and being proximal to ROS production it is more vulnerable to the damage caused by ROS. Thus one can visualize a vicious cycle of mitochondrial dysfunction upon constant stimulation in HIV infection. Unfortunately, although Anti-Retroviral Therapy (ART) initially suppresses the virus induced mitochondrial
malfunctioning, Anti-Retrovirals (ARVs) such as Nucleoside analogue Reverse Transcriptase Inhibitor (NRTI) have the potential to inhibit mitochondrial DNA (mtDNA) polymerase gamma (pol γ) [Martin JL. et al., 1994; Lewis W. et al., 2003], the sole enzyme responsible for mtDNA replication that results in mtDNA depletion and thereby causes ART induced mitochondrial dysfunction. Thus HIV infected patients are under prolonged mitochondrial dysfunction caused both by HIV before ART and by NRTI after initiation of treatment, moreover impaired mitochondrial activities has been attributed to aging. In addition, lifestyle changes such as cigarette smoking and alcohol are the known agents that can harm the mitochondrial health. There are a number of other drugs that are known to affect mitochondria such as antibiotics and coadministration of other classes of drugs is inevitable in the settings of HIV/AIDS (Acquired Immunodeficiency Syndrome). Taken together studying mitochondrial impairments is vital in the settings of HIV infection and its treatment in order to understand the pathophysiology involved in mitochondrial dysfunction during HIV infection and eventually upon ART that would help in devising newer treatment strategies targeting mitochondria. Moreover understanding mitochondrial dysfunction will also help in identifying novel biomarker to determine NRTI induced adverse events in HIV infected patients.

In 2015, an estimated 67.6 [46.4–106.0] thousand people died of AIDS-related causes nationally. This decline is consistent with the rapid expansion of access to ART in the country. It is estimated that the scale-up of free ART since 2004 has saved cumulatively around 4.5 lakhs lives in India until 2014[India HIV estimations 2015, NACO Report]. Thus with increasing coverage of treatment there are more reports on the emergence of toxicity.
ARVs are associated with a broad range of toxicity, ranging from low-grade intolerance, which may be self-limiting to life-threatening conditions. As a general principle, mild toxicities do not require the discontinuation of ART or drug substitution, symptomatic treatment may be given. Moderate or severe toxicities may require substitution of the drug with another of the same ARV class, but with a different toxicity profile. Severe life-threatening toxicity requires discontinuation of ARVs until the patient is stabilized and the toxicity is resolved.

In India, currently more than 26 drugs have been licensed for treatment of HIV-1 infection, each belonging to one of five classes: NRTIs; protease inhibitors (PIs); non-nucleoside reverse transcriptase inhibitors (NNRTIs); fusion inhibitors (FIs), chemokine receptor blockers and HIV-1 integrase inhibitors. Not surprisingly, it soon became evident that these highly effective drug combinations were also associated with a variety of potentially serious complications that have paved the way for the black box warnings from Food and Drug Administration (FDA), USA. The serious complications range from, the worsening of existing or the de novo development of medical conditions such as diabetes but also the insurgence of adverse effects such as peripheral neuropathy, hepatic steatosis sometimes accompanied by lactic acidosis, (cardio) myopathy, drug-induced hypersensitivity, and also the emergence of the lipodystrophy syndrome [Carr A. et al., 1998]. The lifelong commitment to antiviral therapy means that HIV-infected patients and clinicians have to come to terms with the possible development of long-term toxicities. Highly Active Antiretroviral Therapy (HAART) drug combination is continuously evolving, and therapy guidelines have to take into account the balance between the long-lasting suppression of the virus and the limitation of potential side
effects. Treatment monitoring still remains indispensable especially in the setting of HIV/AIDS due to the factors such as inter-individual variability, long-term administration and multiple drug regimens. NRTI occupies a vital position in ARV regimen of both first line and second line HAART, but owing to their long-term toxicity profile; there are attempts such as NRTI sparing regimens to simplify therapy, reduce toxicity and to overcome resistance but to date none of such studies have resulted in better efficacy and safety than the standardized first line treatment [Raffi F. 2013] which underscores the importance of NRTI in the treatment of HIV for years to come.

Establishing mitochondrial toxicity is not a FDA requirement for drug approval [Sims K. 2010], which is a main reason for the toxicity issues that we face today. Adding to the situation, treating for co-morbidities complicates the condition hence monitoring for the emergence of toxicity becomes very essential. Moreover, as the currently available toxicity monitoring is not well delineated in the circumstances of mitochondrial toxicity, a search for new, early, easily accessible biomarker is underway. Even evaluating new treatment approaches for primary mitochondrial disorders has been hampered due to lack of biomarkers to monitor disease status or response to treatment [Enns GM. 2014]. In addition the limitations that are associated with traditional methods for assessing mitochondrial dysfunction have discouraged routine evaluation of mitochondrial dysfunction thus there is a surge for identifying a novel new biomarker of mitochondrial dysfunction that could avoid late-stage attrition during drug development.
1.1 Mitochondrial dysfunction mechanisms:

The only enzyme that is responsible for mtDNA replication, DNA pol \( \gamma \), is inhibited to a varying extent by NRTIs used in HAART. Through this mechanism, NRTIs can easily induce depletion of mtDNA, resulting also in depletion of mtDNA-encoded mitochondrial enzymes and this will finally lead to mitochondrial dysfunction. In fact, nearly all side-effects that have been attributed to the use of NRTIs, such as polyneuropathy, myopathy, cardiomyopathy, pancreatitis, hepatotoxicity, bone-marrow suppression, and lactic acidosis, greatly resemble the spectrum of clinical manifestations seen in inherited mitochondrial diseases. The pol \( \gamma \) hypothesis suggests that inhibition of pol \( \gamma \) causes mtDNA depletion which inturn causes an altered expression of mitochondrial proteins, which could eventually lead to oxidative phosphorylation (OXPHOS) defects with the generation of ROS which causes oxidative damage of proteins, mtDNA and lipids and thus causes mitochondrial dysfunction [Lewis W. & Dalakas MC., 1995]. Various studies have reported on the possible role of NRTIs in the development of HAART related lipodystrophy, suggesting an alternative to PI contribution to the syndrome [Gervasoni C. et al., 1999]. The mitochondrial toxicity hypothesis postulated by Brinkman K. et al., 1999, offered a possible link between the mitochondrial DNA disruption caused by NRTIs and the development of HAART related lipodystrophy, as a possible manifestation of the resulting mitochondrial toxicity.

Steady-state and pre-steady-state analysis of DNA pol \( \gamma \) inhibition \textit{in vitro} has shown a hierarchy of mitochondrial toxicity for the NRTIs as zalcitabine (ddC) = didanosine (ddI) = fialuridine (FIAU) = stavudine (d4T) >> lamivudine (3TC) >
zidovudine (AZT) > abacavir (ABC) [Lim SE. & Copeland WC., 2001; Johnson AA. et al., 2001].

Mutations can result from spontaneous errors of replication or from unrepaired chemical damage to DNA, such as oxidation or exposure to ultraviolet irradiation. DNA pol- is the sole DNA polymerase found in mitochondria and is responsible for the replication and repair of mtDNA. So, when DNA pol- is inhibited, mtDNA copy number decreases. The decrease in mtDNA inhibits the synthesis of adequate proteins that are essential for OXPHOS. Disruption of OXPHOS leads to energy loss that is, a decrease in the production of adenosine triphosphate (ATP) and an increase in electron leakage from the electron-transport chain, which increases the production of ROS [Lewis W. et al., 2001].

Immune activation and inflammation are the characteristic features of HIV infection, both of these conditions causes oxidative stress, which is also seen in the ART- treated patients due to the ARVs, thus the HIV infected are under prolonged oxidative stress. Moreover, the cells of the immune system are remarkably sensitive to oxidative stress, since their plasma membranes contain high levels of polyunsaturated acyl lipids, which are vulnerable to peroxidation. More importantly, this oxidative damage is particularly marked in the phospholipids, which constitute the cell membrane, therefore making lipid peroxidation a convenient marker of oxidative stress in living systems.

F2-Isoprostanes (F2-IsoP), are a family of prostaglandin F2-like molecules produced by nonenzymatic free-radical-catalyzed peroxidation of esterified arachidonic acid and then cleaved and released into the circulation by
phospholipases before excretion in the urine as free isoprostanes. Reports have shown that F2-IsoPs are authentic, reliable biomarkers of lipid peroxidation and are useful in vivo indicators of oxidative stress in various clinical conditions, such as acute and chronic inflammation.

Increase in the concentration of ROS damages proteins, lipids and mtDNA, setting off a cascade of further oxidative damage. There is a -180 mV difference in the electrical potential between both sides of the mitochondrial internal membrane, known as mitochondrial membrane potential (ΔΨm). In case of mitochondrial dysfunction there is depolarization of membrane i.e. loss of mitochondrial membrane potential that causes the release of apoptogenic factors from the intermembrane space such as cytochrome C, second mitochondria derived activator of caspases (SMAC) or its homologous in mouse (DIABLO), apoptosis inducing factor (AIF) or endonuclease G, among others. The release of these factors causes apoptosis of these cells.

Since the first reports on mitochondrial damage in myocardial tissue obtained from patients who had died of AIDS [Flomenbaum M. et al., 1989], the idea has emerged that HIV-1 infection by itself might also affect mitochondria. HIV-1 infection itself in untreated individuals has been reported to be associated with the development of pathologies such as cardiomyopathy and distal symmetric polyneuropathy and nephropathy [Moroni M. et al., 2003].

Furthermore, mitochondrial alterations have been demonstrated in T-lymphocytes from untreated HIV-1-infected patients, suggesting a potentially increased susceptibility to apoptosis of these cells, consistent with the immune cell
dynamics during HIV-1 infection [Carbonari M. et al., 1997; Cossarizza A. et al., 1997]. More recently several studies reported on the adverse effects on mitochondria of distinct viral-encoded proteins such as transactivating regulatory protein (tat) and viral protein R (vpr) [Raidel SM. et al., 2002; Muthumani K. et al., 2002]. Increased production during HIV-1 infection of pro-inflammatory cytokines, like Tumour Necrosis Factor- (TNF- ) and interferon- (IFN- ) have also been shown to damage mitochondria [Geng YJ. 1992]. Finally, although the underlying mechanism is still unknown, a number of studies have demonstrated that HIV-1 infection itself, in the absence of ART, is associated with a decline of mtDNA in several tissues including adipocytes and peripheral blood mononuclear cells (PBMCs) [Shikuma CM. et al., 2001; Cote HCF. et al., 2002; McComsey G. et al., 2002; Miro O. et al., 2004; Casula M. et al., 2005].

There is great inter-individual variation in the presentation of toxicities induced by mitochondrial dysfunction and also marked differences in organ susceptibility may occur within the same individual. The mechanisms underlying these variations are not fully understood. One possible explanation is a differential effect of mtDNA variants on susceptibility to toxicity. In line with this, mitochondrial haplogroup T was reported to be associated with increased risk for peripheral neuropathy in individuals treated with d4T and AZT [Hulgan T. et al., 2005], whereas haplogroup J might possibly protect from lipoatrophy [Hulgan T. et al., 2008], thus mitochondrial toxicity varies with ethnicity. In addition individuals with an A1555G mutation in mtDNA are unusually susceptible to aminoglycoside-induced ototoxicity [Estivill X. et al., 1998], and lactic acidosis has been reported in patients carrying mtDNA polymorphisms in the16S rRNA gene.
Thus both HIV and NRTI have been reported to cause mitochondrial dysfunction. To the best of our knowledge, so far no study has been conducted on mitochondrial function/dysfunction among HIV patients in India. Hence, in the current study we intended to analyse mitochondrial dysfunction among HIV infected ART-naïve and the NRTI treated patients in south India. In order to study mitochondrial dysfunction, mtDNA content of PBMC was determined. Mitochondrial membrane depolarization and apoptosis of lymphocyte were measured as total lymphocyte mitochondrial membrane potential low designated as $\Delta\Psi_m^{\text{low}}$ (%) and total lymphocyte apoptosis (%) designated as TLA (%) respectively. In addition systemic oxidative stress was estimated by measuring the plasma F2- IsoP. Mutations in a representative gene of mtDNA, NADH dehydrogenase subunit 1 (ND1) were determined among the study participants.