4. MATERIAL AND METHODS

4.1 Study site and patients

The study was carried out from January 2012 to March 2016, at the Infectious Diseases Laboratory of YRG Centre for AIDS Research and Education, Chennai, Tamilnadu, which is the largest tertiary referral HIV care centre in southern India providing psycho-social and clinical care to more than 20,000 patients. Participants for this study were enrolled during their visit to YRG CARE Medical Foundation as a part of their routine HIV clinical care and support.

Patient recruitment for the present study was done at YRG CARE hospital between February 2012 - April 2014. Consecutive HIV-1 infected patients fulfilling the study criteria were enrolled. The study protocol was approved by Institutional Review Board of YRG CARE, Chennai and the patients were enrolled with administration of written informed consent. The details of demographic, clinical data and ART regimen were obtained during enrollment in the form of questionnaire. Identification of the patients was coded and these code numbers were used throughout the study period.

4.2 Study Design

This is a longitudinal observational study with 6 month follow- up, totally comprising of 4 visits designated as enrollment (Enrl) visit, 6th month, 12th month and 18th month visit, during the follow- up, among the 30 ART-treated, 21, 19 and 17 participants attended the follow up at 6th month, 12th month, and 18th month visit respectively thus accounting for a total of 87 visits. Among the 57 ART-naive, 40,
31 and 28 attended the follow-up at 6th, 12th, and 18th month respectively and remained drug naïve throughout the follow-up with a total follow-up visits of 156.

4.3 Sample size calculation:

The sample size was calculated with 90% confidence level, with margin of error of 5% and (α 0.05) level of significance with the expected NRTI induced lipodystrophy of 3.2% reported from previous studies (Kumarasamy N. et al., 2008).

A total of 111 study subjects were enrolled under the following groups with stringent inclusion and exclusion criteria.

Low-risk healthy control (LoRHC), n=24.

HIV-1 infected patients who were naïve to antiretroviral drugs, n=57.

HIV-1 infected patients who were on first-line antiretroviral drugs, n=30.

All HIV-infected participants were on vitamin and mineral supplementation.

4.4 Inclusion and Exclusion criteria:

❖ Low risk healthy control

Inclusion Criteria

- Male and female participants > 18 years old.
- Apparently healthy subjects.

Exclusion criteria

- Participants on treatment for chronic illnesses.
- Habitual smokers and alcoholics
HIV infected ART-naïve

Inclusion Criteria

- Male and female participants more than 18 years old.
- HIV-1 infected ART-naïve with an expected CD4 T-cell count of >500 cells/µL.

Exclusion Criteria

- Participants with life threatening opportunistic infections (OIs) or co-infections. Injection Drug Users and HCV co-infected cases have been excluded from the study as HCV is known to cause mitochondrial dysfunction.
- Patients with neoplasm, pregnancy, allergic drug hypersensitivity, non-HIV related metabolic disorders with known history of mitochondrial disease and those receiving other mitochondrial toxic drugs such as aminoglycosides and statins.
- Habitual smokers and alcoholics.

HIV infected HAART treated

Inclusion Criteria

- Male and female participants more than 18 years old.
- HIV-1 infected treated with first-line HAART (the regimen containing NRTI) for more than 6 months with >95 adherence.

Exclusion Criteria

- Participants with life threatening OIs or co-infections. Injection Drug Users and HCV co-infected cases have been excluded from the study as HCV is known to cause mitochondrial dysfunction.
- Patients with neoplasm, pregnancy, allergic drug hypersensitivity, non-HIV related metabolic disorders with known history of mitochondrial disease, those receiving other mitochondrial toxic drugs such as aminoglycosides and statins.
- Habitual smokers and alcoholics.
4.5 Methods

Blood specimen was obtained in a 12 hour fasting state. For lactate estimation blood was collected without tourniquet in sodium fluoride (gray-top) tubes and plasma was separated within 30 minutes of blood draw. Complete blood count (CBC) was performed in Sysmex XT-1800i (Kobe, Japan), CD4 T-cell count by Pan Leukocyte Gating (PLG) was performed in Beckman Coulter (Miami Florida Inc.USA) Cytomics FC500. All the biochemistry testings were analysed in Olympus AU 400 Clinical Chemistry Autoanalyser (Olympus optical, Japan) (Fig 3).

Derived variables:

Lipase/Amylase, AST/ALT, LDL-C, VLDL were calculated. The atherogenic ratios [Bhardwaj S. et al., 2013] were calculated from the lipid profile parameters as follows: Atherogenic Index of Plasma (AIP): \( \log \frac{\text{TG}}{\text{HDL-C}} \), Castelli’s Risk Index (CRI-I): \( \frac{\text{TC}}{\text{HDL-C}} \), Castelli’s Risk Index (CRI-II): \( \frac{\text{LDL-C}}{\text{HDL-C}} \), Atherogenic Coefficient (AC) = \( \frac{\text{TC} - \text{HDL-C}}{\text{HDL-C}} \), HDL-C/LDL-C. Absolute Neutrophil Count (ANC): WBC x Neutrophil % x 10 cells/mm\(^3\).

Definitions:

Mild anemia: Hb between 10-12 g/dL
Moderate anemia: Hb between 8-10 g/dL
Severe anemia: Hb less than 8 g/dL.
Thrombocytopenia: Platelets less than \( 150 \times 10^9 /L \).
Leucopenia: WBC less than \( 4 \times 10^9 /L \).
Neutropenia: Absolute Neutrophil Count (ANC) < 2000 cells/µL.

* Dikshit B. et al., 2009
Figure 3: Blood collection and processing

20 mL of peripheral whole blood

3.5 ml Gel tube
- Lipid profile
- AST and ALT
- Amylase and Lipase.

2 ml EDTA tube
- Complete blood count
- Absolute CD4 Count

2 ml NaF tube
- Lactate

12.5 ml EDTA tube
- Plasma viral load
- PBMC isolation

- Plasma stored at -70°C
- PBMCs (5 million cells/vid) stored in liquid nitrogen for research specific assays
4.5.1 HIV-1 Plasma Viral Load Quantification:

Plasma HIV-1 RNA viral load was determined by absolute quantitation with Abbott Real Time PCR, *m2000rt* instrument with the assay range of 40 to 10,000,000 copies/mL.

4.5.2 Isolation of PBMC:

PBMCs are isolated from fresh blood by Ficoll overlay method (Cross-network PBMC Processing SOP -HANC, v4.0, Oct 2011). Live cells were stored in liquid nitrogen for molecular and apoptosis analysis, maintained in RPMI 1640 culture medium (Hyclone, S.Logan, USA), supplemented with 10% fetal bovine serum (FBS) (Hyclone) along with penicillin and streptomycin (Sigma-Aldrich, St. Louis, USA).

4.5.3 DNA Extraction:

PBMC DNA extraction was carried out using Qiagen DNA blood mini kit. Live cells were thawed and checked for platelet contamination, PBMC preparations with platelet contamination of >1 % was washed twice with PBS to remove the platelets (Banas B. *et al.*, 2004; Timmermans EC. *et al.*, 2006). DNA extraction was carried out as per the manufacturer’s instructions. Finally, DNA was eluted in 100µL of elution buffer.

4.5.4 mtDNA Quantification:

Frozen PBMC was thawed and total DNA was extracted using QIAamp DNA Blood Mini Kit following the manufacturer’s instructions (Qiagen®, Hilden Germany). Extracted DNA was quantified using Qubit dsDNA BR
assay kit in Qubit fluorometer (Life Technologies, Carlsbad, USA) for template optimization to a concentration of 5ng/µL.

mtDNA content was quantified by real time PCR based relative quantification method performed in ABI 7500 Fast DX(Applied Biosystems, Foster City, USA) real time PCR instrument.

The assay was duplexed with TaqMan® Gene Expression Assay, part No. 4331182(Life Technologies, Carlsbad, USA) ND2 primer probe (Hs02596874_g1) with FAM™ reporter dye at the 5' end of the TaqMan® MGB probe, for the target and copy number reference assay, part No. 4403326(Life Technologies, Carlsbad, USA) for the endogenous control, RNaseP, which has VIC® dye–labeled TAMRA™ probe.

Briefly, the reaction mixture consists of 10µL, TaqMan Fast Universal Master mix, 1µL each of ND2 and RNaseP primer probe and 4µL of nuclease free water. Finally for 16µL of the master mix, 4 µL of 20 ng DNA target was added. A DNA reference sample was always included in each polymerase chain reaction (PCR) performed throughout the study to assess the inter experiment variation.

All samples were tested in duplicates and the mean Ct value was taken. Relative quantification of mtDNA vs nuclear DNA(nDNA) was expressed in terms of mtDNA content that was calculated using the formula 2-EXP(Ct mtDNA-Ct nDNA) (Ribera E et al., 2008; Curran A et al., 2012), where Ct mtDNA is the cycle threshold of ND2 gene of mtDNA and Ct nDNA, is that of RNase P.

Percentage change of mtDNA content among the HIV infected was calculated from the median mtDNA content of LoRHC which was arbitrarily assigned to 100% [Garrabou G. et al., 2009].
4.5.5 **mtDNA- ND1 sequencing:**

ND1 gene of mtDNA codes for one of the seven subunits of respiratory complex 1 and is involved in the first step of the electron transport chain of the mitochondrial energy-generating pathway, OXPHOS. *ND1* gene is sequenced as per Yusnita Y. *et al.*, 2010 with few modifications using ABI 3100 Avant 4 capillary genetic analyser (Applied Biosystems Inc., Foster City, USA).

ND1 gene spans from nucleotide position 3307 to 4262 encompassing 956 base pairs which is amplified as 2 fragments of 555bp and 626 bp with 2 sets of overlapping primers 5’-CAG AGC CCG GTA ATC GCA TAA- 3’ with 5’- GGA GAG GTT AAA GGA GCC ACT-3’ and 5’-ATG AAG TCA CCC TAG CCA TCA-3’ with 5’-AGG GAT GGG TTC GAT TCT CAT-3’, respectively. The reaction mixture was subjected to pre-denaturation at 96° C for 10 mins; 95° C for 30 secs, 64°C for 1 min, 72°C for 2 mins, 15 cycles; 95° C for 30 secs, 58° C for 1 min, 72° C for 2 mins 30 secs, 15 cycles; and a final extension for 10 mins at 72°C. PCR products were subsequently purified using a spin column to remove unincorporated primers and excessive dNTPs (QIAGEN QIAquick Purification Kit) (Fig 4).

The purified PCR products were subjected to sequencing PCR bi-directionally using Big Dye Terminator, version 3.1 cycle sequencing chemistries (Applied Biosystems) as per standard protocol. The plate was
Fig 4. *ND1* Qualitative non-nested PCR showing the 555 bp and 626 bp fragments of *ND1* gene.
then purified with 75% isopropanol and finally 10 µL of formamide was added and linked to ABI 3100 Avant genetic analyzer.

The sequences generated were aligned with the published human mitochondrial DNA revised Cambridge Reference Sequence (rCRS) using SeqScape version 2.5 software (Applied Biosystems). The FASTA sequences were then compared to the MITOMAP: a human mitochondrial genome database to identify the mitochondrial genome sequence variants (Fig 5A & B).

4.5.6 Detection of total lymphocyte apoptosis (%) (TLA%)

Annexin V-FITC/ Propidium Iodide (PI) staining (Beckman Coulter Inc, Brea, USA):

Annexin V-FITC was used for detecting the total lymphocyte apoptosis (%) (TLA%). Annexin V-FITC binds to phosphatidylserine which is exposed on the cell membrane of early apoptosis and PI intercalates with DNA of the necrotic cells. Thus, cells undergoing apoptosis and necrosis are differentiated. Positive Control cells were obtained from HIV negative controls and were induced with Staurosporine (50µM) which is an apoptosis inducing agent. Uninduced healthy cells from HIV uninfected controls were used as negative control. In addition control cells from HIV uninfected controls incubated without annexin V-FITC was used as auto-fluorescence negative control cells.

PBMCs were thawed with 10mL Complete RPMI medium (cRPMI) in 37°C water bath according to standard protocol.
To $1 \times 10^6$ cells resuspended in 100 $\mu$L of 1x binding buffer and kept in a ice bucket, 1 $\mu$L of annexin V- FITC and 5 $\mu$L of PI were added and incubated for 15 minutes in dark and finally 400$\mu$L of ice cold binding buffer was added.

Cell preparations were analysed by flow cytometry (Cytomics FC 500, Beckman Coulter, CA, USA) within 30 minutes at an emission spectra of 520 nm for annexin V- FITC in FL 1 and at 560- 680 nm for PI in FL 4.

Optimisation was done with unstained cells, individually stained cells and with double stained cells.

Lymphocytes were gated on forward scatter and side scatter parameters to exclude debris. A total of 50,000 events were acquired for each assayed sample.

C4 quadrant gives the TLA% which is the percentage of apoptotic lymphocytes (Fig 6A & B).

4.5.7 Detection of total lymphocyte $\Delta \psi_m^{\text{low}}$ (%) (TL $\Delta \psi_m^{\text{low}}$ (%))

JC-1 MitoScreen Assay (BD Biosciences, San Jose, USA):

Total Lymphocyte with depolarized mitochondrial transmembrane potential ($\Delta \psi_m^{\text{low}}$) was determined flow cytometrically with the JC-1 dye which is 5,5',6,6'-tetrachloro-1,1',3,3'- tetraethylbenzimidazolcarbocyanine iodide, a lipophilic fluorochrome that is used to evaluate the status of the $\Delta \psi_m$.

JC-1 can exist in two different states, aggregates or monomers, each with different emission spectra. JC-1 forms monomers at low dye concentrations, and
Fig: 6A & B. Flow cytometry scatter plots with Annexin V-FITC/PI of PBMCs stimulated with A) Staurosporine (Positive control) and B) the test cells. C4 quadrant gives the total lymphocyte apoptosis (%).
aggregates at higher concentrations. Both JC-1 aggregates and monomers exhibit fluorescence in the green end of the spectrum which is measured in the green (FL-1) channel on flow cytometers.

When live cells are incubated with JC-1, JC-1 penetrates the plasma membrane of cells as monomers. The Δψm of normal, healthy mitochondria is polarized and JC-1 is rapidly taken up by such mitochondria. This uptake increases the concentration gradient of JC-1 leading to the formation of JC-1 aggregates (known as J-aggregates) within the mitochondria. JC-1 aggregates show a red spectral shift resulting in higher levels of red fluorescence emission which is measured in the Red (FL-2) channel on most flow cytometers.

JC-1 does not accumulate in mitochondria with depolarized Δψm and remains in the cytoplasm as monomers. These monomers do not have the red spectral shift, and therefore have lowered fluorescence in the FL-2 channel. The formation of JC-1 aggregates is reversible. Thus, in mitochondria undergoing a transition from polarized to depolarized Δψm (due to apoptosis or other physiological events), JC-1 leaks out of the mitochondria into the cytoplasm as monomers resulting in a decrease of red fluorescence.

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (50 μM) which is a known mitochondrial membrane potential disruptor was used to generate positive control cells. From the thawed PBMCs, 1x10^6 cells were resuspended and incubated in 500 μL of JC-1 solution at 37°C (5% CO₂) for 15 mins. Stained PBMCs were washed twice in JC-1 MitoScreen wash buffer and resuspended in 500 μL of assay buffer. All flow cytometric data were acquired immediately after staining on
Monomeric form of JC-1 emits at 527 nm and J aggregates emits at 590 nm.

Mitochondria with depolarized $\Psi m$ ($\Delta\Psi m^{\text{low}}$) shows decreased red fluorescence. Lymphocytes were gated on forward scatter and side scatter parameters to exclude debris. A total of 50,000 events were acquired for each assayed sample. Samples were analysed simultaneously for TLA (%) and $\text{TL}\Delta\Psi m^{\text{low}}$ (%) (Fig 7A & B).

**4.5.8 Estimation of oxidative stress:**

F2- IsoP is the marker of oxidative stress estimated using OxiSelectTM 8-iso-Prostaglandin F2 IsoP ELISA kit (CELL BIOLABS, INC, CA, USA). Isoprostanes are prostaglandin like compounds produced non-enzymatically through the oxygen radical induced peroxidation of tissue phospholipids and lipoproteins. It is a competitive Enzyme-linked immunoassay (ELISA) for determining levels of F2-IsoP.

**Method:**

**Sample preparation:**

100µL of 10N NaOH was added to 400µL of plasma sample and incubated at 45°C for 2 hours. 100µL of 10N HCl was added to the hydrolysed sample and centrifuged at 12,000 rpm. The supernatant was used for the assay. Standards of concentrations from 49 pg/mL to 200,000 pg/mL were run with the samples for the standard curve (Fig 8).
Enzyme-linked immunosorbent assay

100µL of 1:1000 diluted anti-8-iso-PGF2 antibody was added to the Goat anti-Rabbit antibody coated plate and incubated for 1 hour at 25°C on an orbital shaker.

After washing, 55µL of conjugate mixed to 55µL of the processed sample was added to the well and incubated at 25°C for 1 hour in an orbital shaker.

Finally 100µl of substrate was added and incubated in dark for 10-30 and the reaction was stopped by adding 100µL of stop solution and read at 450nm as the primary wavelength.

Plasma F2-IsoP concentration in pg/mL was calculated from the standard curve.

4.5.9 Isolation of CD4⁺ T cell and non-CD4⁺ T cells:

EasySep™ Human CD4⁺ T cell enrichment cocktail and EasySep™ D magnetic particles label non-CD4⁺ T cells for magnetic separation. These reagents are designed to enrich CD4⁺ T cells from PBMCs by depletion of non-CD4⁺ T cells. CD4⁺ T cells are isolated by negative selection.

PBMC suspension was prepared at a concentration of 5X10⁷ cells/mL in a medium containing phosphate buffered saline (PBS) + 2% FBS with 1mM EDTA. Cells must be placed in a 5 mL polystyrene tube to properly fit into the Purple EasySep™ magnet.
Add the EasySep™ Human CD4⁺ T cell enrichment cocktail at 50 µL/mL cells. Mix well and incubate at room temperature for 10 minutes.

Vortex the EasySep™ D magnetic particles for 30 seconds. Ensure that the particles are in a uniform suspension with no visible aggregates.

Add EasySep™ D magnetic particles at 100 µL/mL cells, mix well and incubate at room temperature for 5 minutes.

Bring the cell suspension up to a total volume of 2.5 mL by adding PBS containing 2% FBS and 1mM EDTA. Mix the cells gently and place the tube into the magnet, set aside for 5 minutes.

In one continuous motion invert the magnet and the tube pouring off the negatively selected CD4⁺ T-cells into a new tube. The magnetically labeled cells that remain bound in the original tube held by the magnetic field of the EasySep™ magnet are the non-CD4⁺ T-cells.

Purity of CD4⁺ T-cells was measured by flow cytometry after staining with phycoerythrin to CD4 and the CD4 T cell purity was >95% (Fig 9).

### 4.5.10 Assays performed

Biochemistry/hematology, CD4 T-cell count, plasma viral load, mtDNA relative quantification was done for all the four visits of the HIV infected and in the LoRHC. CD4 T-cell count and plasma viral load were not performed in LoRHC. Quantification of plasma F2-IsoP, estimation of TLA% and TLΔΨ<sup>m</sup> low (%) were performed for the HIV infected during the Enrl, 6<sup>th</sup> month and 12<sup>th</sup> month visit and
for the LoRHC. Isolation of CD4\(^+\) T-cells and the non-CD4\(^+\)T-cells and subsequent estimation of mtDNA content was carried out in n=10, ART-naïve and n=12, ART treated subjects. mtDNA- ND1 gene sequencing was done for the enrollment samples of the three groups.

4.6 Statistical Analysis

Data was analysed as longitudinal which is recorded at every visit and cross-sectional which is recorded combining all the visits (total visits) [Mc Comsey G. et al., 2005]. Results were expressed as median, interquartile range (IQR) unless stated otherwise. Qualitative variables were expressed as percentage (%). All the study specific parameters namely mtDNA content, TLA (%), TL\(\Delta m\)low (%), plasma F2-IsoP and mtDNA-ND1 mutations were compared with the laboratory and clinical data between the three groups to analyze mitochondrial dysfunction caused by HIV and NRTI.

Two group comparisons were done by Mann-Whitney U test. More than two groups comparison was done by Kruskal Wallis test. Longitudinal paired data between two visits was analysed by Wilcoxon matched pairs test. During the follow-up, n=13 in the ART- treated and n=20 in the ART- naïve group attended all the follow-up visits, from this cohort, difference in the mitochondrial parameters was analysed by Friedman test which is a non-parametric repeated measures test.

The study specific parameters were stratified at various cut-offs of covariables and analysed by Mann-Whitney U test in order to study their association both
longitudinally and cross-sectionally. The covariables used were age, gender, BMI, CD4 T cell count, CD4%, viral load, d4T/AZT, duration of infection, duration on ART, adverse events, baseline CD4 T cell count, WBC count and total lymphocyte count. The cut-off values were set either as reported or in order to adjust for the comparable sample sizes in each subgroup. Correlation between variables was analysed by using Spearman rank correlation coefficient test to test for the association within the study specific parameters and also with the biochemical/hematological parameters.

During the follow-up, n=10 ART-naïve patients initiated ART as per the standard of care and treatment. Thus we were able to identify a prospective cohort of HIV infected patients before and after initiating ART in whom the study specific parameters were analysed prospectively.

In an attempt to probe for the association between the study specific parameters and the clinical/ laboratory variables in conditions of mtDNA depletion and oxidative stress, in the present study, a cut-off value of 127 was set for mtDNA content and 118 pg/mL for plasma F2-IsoP which was calculated from the median values of the LoRHC. mtDNA content of <127 was denoted as mtDNA depletion and plasma F2-IsoP >118 pg/mL was regarded as a status of oxidative stress, correlation between variables in these subgroups was also analysed by Spearman rank correlation coefficient test.

All statistical tests were two-tailed and performed at a level of statistical significance of 0.05. GraphPad Prism, Version 5 (GraphPad Software, Inc., La Jolla, USA) was used for statistical analyses.