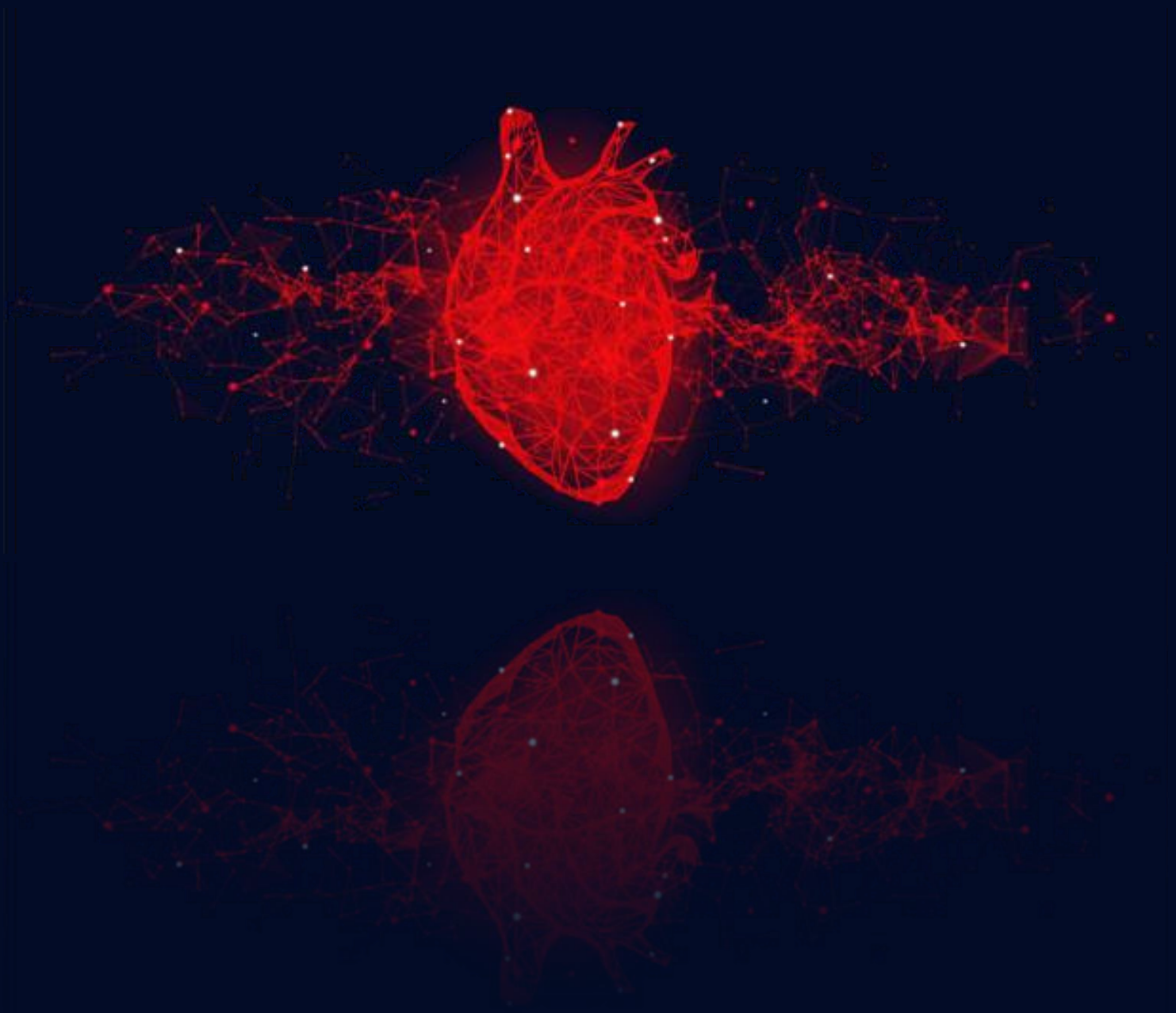
Materials and Methods



Chemicals and reagents

HiEndoXL Endothelial growth medium, EnVzyme Easy, RPMI-1640, phosphate buffer saline (PBS), phorbol 12-myristate 13-acetate (PMA), antibiotic antimycotic solution, bovine serum albumin (BSA), gelatin, Hoechst 33342, and Oil red O (ORO) stain were purchased from Himedia Laboratories, India. Fetal bovine serum (FBS) was procured from Gibco. Oxidized LDL, MitoTracker Red, JC-1 stain, mouse monoclonal β-actin antibody, Alexa Fluor-488 anti-rabbit secondary antibody and Trizol reagent, were purchased from Thermo Fisher Scientific, USA. Protease inhibitor cocktail (PIC), Fluoroshield with DAPI, Direct red 80, hematoxylin, eosin and Carbon monoxide releasing molecule A1 (CORM-A1) were obtained from Sigma Aldrich, USA. RNA-later stabilizing solution was purchased from Ambion Inc. (USA). Rabbit polyclonal β-actin antibody and HRP linked anti-rabbit secondary antibody was purchased from Cell Signaling Technologies, USA. Polyvinylidene fluoride (PVDF) membrane, Precision Plus protein ladder, BioRad Protein Assay Dye reagent concentrate, iScript cDNA synthesis kit, Clarity Western ECL blotting substrate and SYBR green master mix were purchased from Bio-Rad Laboratories, USA. mouse monoclonal CD68 antibody, 3,3'-diaminobenzidine (DAB) and HRP-linked anti-mouse secondary antibody were purchased from Dako, Agilent, USA. MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), dimethyl sulfoxide (DMSO), ethanol, chloroform, Methanol, and isopropanol were purchased from Sisco Research Laboratory Pvt. Ltd. Mumbai, India. Antibodies Clock (PA1-520), β-actin (PA1-183), secondary anti-rabbit (32460) and ActinRed 555 Ready Probes reagent (R37112) were procured from Invitrogen (USA) and SIRT1 (SC-74465) and secondary anti-mouse m-IgGk (SC-516102) were purchased from Santa Cruz (Dallas, USA). miRNeasy kit (217004) and miRScript II RT kit (218161) were procured from Qiagen. Animal diet was procured from VRK

solutions. All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany).

In-vivo Experimentations

Animal maintenance and ethical statement

Animals were maintained as per CPCSEA standard guidelines (23 ± 2°C, LD 12:12, laboratory chow and water ad libitum) for a week-long acclimatization before initiating the experiment. Experimental protocols were approved by Institutional Animal Ethical Committee (IAEC) (Approval no. MSU-Z/IAEC03/01-2019; MSU-Z/IAEC030/03-2019) and experiments were conducted in CPCSEA approved animal house facility of Department of Zoology, The Maharaja Sayajirao University of Baroda, Vadodara, India (827/GO/Re/S/04/CPC-SEA). All animal experimentations were done abiding to the ARRIVE guidelines.

Experimentally induced Chronodisruption in C57BL/6J mice

Male C57BL6/J mice were procured from ACTREC, Mumbai and were housed (3-5/cage) in CPCSEA approved animal house, fed laboratory chow diet and water ad libitum for a week-long acclimatization. Animals were maintained in 12:12 LD cycle, and room temperature at 23-25°C with 50-70% humidity through the phase. At initiation of the experiment mice were randomly divided into 3 groups respectively

I. Time point Group (TP)

The group had 25 animals (n=25), herein animals were maintained at LD 12:12 throughout the experiment and were sacrificed at 5 different time points (ZT 0, 6, 12, 18, 24 (n=5/time point).

II. Control

There were 6 animals (n=6) in the group and were maintained at LD 12:12.

III. Chronodisruption group (CD)

This group had n=6 animals. Herein, animals were subjected to photoperiodic manipulation for instilling chronodisruption. The regimen was structured with phase advance and phase delay in photoperiodic schedule for 18 weeks. Briefly, mice were subjected to 7:00 to 19:00 h light/19:00 to 7:00 h dark period. The photoperiodic regimen was altered by giving 11:00 to 23:00 h dark/23:00 to 11:00 h light period, resulting in a phase advance of 8 hours (lights off at ZT4) and back to (7:00 to 19:00 hours light/19:00 to 7:00 hours dark period) schedule, resulting in a phase delay of 8 hours on 2nd and 5th day, respectively (Fig. M1). Experiment was terminated at end of 18th week in morning 7:00 h (ZT0) (Joshi, 2021). Animals of group I were sacrificed at different time points (ZT 0, 6, 12, 18, 24) and animals of group II and III were sacrificed at ZT0.

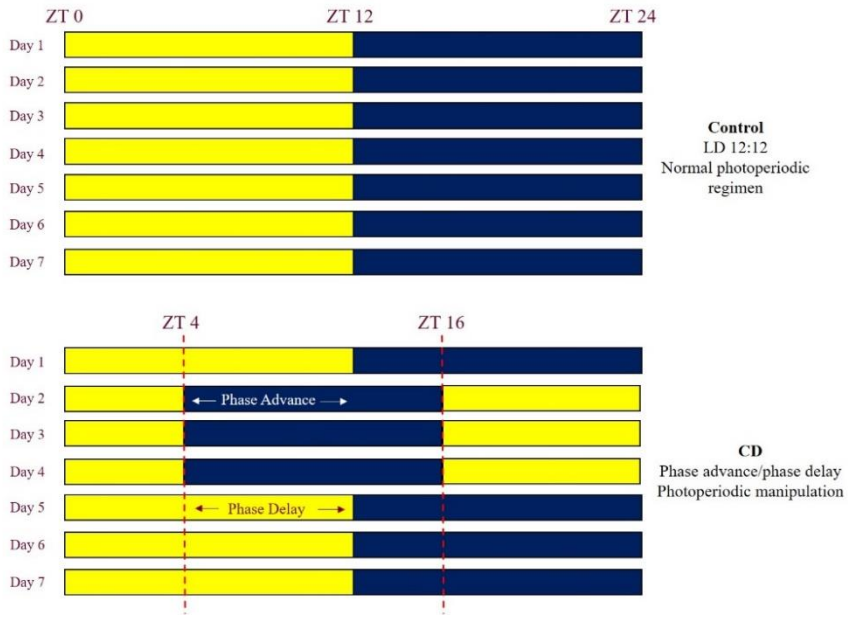


Fig. M1: Schematic representation of phase advance and phase delay photoperiodic regimen followed for instilling systemic chronodisruption in male C57BL/6J mice.

Experimentally induced atherosclerosis in Sprague Dawley rats

Male Sprague Dawley (SD) rats of 6-8 weeks were procured from Sun Pharmaceuticals Pvt. Ltd., Vadodara, India and maintained as per CPCSEA standard guidelines (23 ± 2ºC, LD 12:12, laboratory chow diet and water *ad libitum*) followed by a week acclimatization. Rats weighing 300 ± 20g were randomly divided into 3 groups (n=6 each) respectively.

I. **Control** (n=6) Animals were fed on laboratory chow diet and water ad libitum.

II. **Ath** (n=6) Animals were given single dose of Vitamin D3 (600,000 IU/Kg, *i.p.*) at initiation of the experiment and fed on atherogenic diet (ath diet) and water ad libitum (Jadeja *et al.*, 2011). Vitamin D3 is essential for development of the disease in non-genetic rat model as it facilitates LDL-R expression (Livero and Gasparotto Junior, 2019). Moreover, the active metabolite of vitamin D, 1α,25-dihydroxyvitamin D, binds to the vitamin D receptor that regulates numerous genes involved in fundamental processes of potential relevance to cardiovascular diseases (Norman and Powell, 2014). Vitamin D3 causes hypothyroidism accelerating the pro-atherogenic manifestation when coupled with ath diet (KUNITOMO *et al.*, 1981).

III. **Ath + CORM-A1** (n=6) Animals of this group were administered with CORM-A1 (2mg/kg; *i.p.*) from 2nd week till the experimental termination.

Standard Diet	
Corn	48%
Soybean Meal	20%
Wheat Barn	10%
Flour	10%
Fish Meal	8%
Farina	2%
Vegetable Oil	1%

Atherogenic Diet	
Powdered laboratory chow	81.3%
Lard	10%
Sucrose	5%
6-propyl 2-thiouracil	0.2%
Cholic acid	0.5%
Cholesterol	3%

Blood and Tissue collection

For experimental termination, mice were fasted overnight, and the procedure was initiated at ZT0 (i.e., 0700h). Blood collection was done via retro-orbital sinus puncture under mild isoflurane anaesthesia. Blood was allowed to clot at room temperature (RT) for 30 min and serum was isolated by centrifuging that blood at 3000 rpm at 4°C for 10 min and was preserved at -20°C for further use. Further, mice were euthanized, and thoracic aorta was isolated and cleaned using saline. The tissue was then fixed in 4% PFA for histological analysis, dehydrated in a graded series of ethanol and embedded in paraffin using standard protocol. For immunoblotting, collected tissue were snap frozen in liquid nitrogen whereas for gene expression studies tissues were collected in RNA later and then snap frozen in liquid nitrogen. The frozen tissues were stored at -80°C until further use.

Serum lipid analysis

Isolated serum was subjected to analysis of Triglycerides (TG), Total Cholesterol (TC), Very low-density lipoprotein (VLDL), Low Density Lipoprotein-cholesterol (LDL-c), and High-Density Lipoprotein-cholesterol (HDL-c). using commercially available kits (Reckon Diagnostic kits, Vadodara, Gujarat, India). LDL/HDL and Chol/HDL ratios were calculated from the data. Further, Atherogenic Index of Plasma (AIP) and Cardiac Risk ratio (CRR) were calculated as following (Akici *et al.*, 2020).

$$AIP = \log \frac{TG}{HDL - c}$$

$$CRR = \frac{TC}{HDL - c}$$

Histomorphological evaluation

Histomorphological analysis were done for thoracic aortae. 5 µm thick serial sections were cut from paraffin embedded wax blocks using microtome and stained with Haematoxylin & Eosin (H&E) as described (Kusters and Lutgens, 2015). Sections were observed and photographed with Nikon eclipse Ti2-E, Tokyo, Japan. The morphometric measurements for Intima-media thickness (IMT) were measured using FiJi software (ImageJ, NIH, Bethesda, USA) by an investigator blinded to the experimental grouping.

Elastin autofluorescence

Elastin lamellae were observed for their autofluorescence, and the images were captured using FLoid Cell Imaging Station (Thermo Fisher Scientific, USA) as per (Sawada *et al.*, 2019). Elastin fragmentations, defined as the discontinuity of an elastin fibers with boundaries at both sides clearly visible, were counted manually for each section.

Collagen staining

5 µm think sections of thoracic aorta were de-paraffinized with xylene and rehydrated by immersion in a graded series of ethanol. These sections were then stained with 0.1% Direct Red 80 in saturated aqueous solution of picric acid for 1.5 h at RT and observed under Leica DMRB microscope. Images were captured using Canon power shot S70 digital camera and collagen content was measured relative to vascular area using FiJi, ImageJ. The same sections were also observed for fluorescence of collagen and elastin using FLoid Cell Imaging Station. Captured images were utilized for simultaneous quantification of elastin and collagen as described (Borges *et al.*, 2005; Vogel *et al.*, 2015). Obtained values were used to calculate collagen-to-elastin ratio indicative of arterial stiffness.

Immunohistochemistry

5 µm think sections of thoracic aorta were de-paraffinized with xylene and rehydrated by immersion in a graded series of ethanol and washed with 1X PBS. Antigen retrieval was done by heating the sections in sodium citrate buffer at 95°C for 20 min followed

by masking of endogenous peroxidase with 3% hydrogen peroxide for 20 min in dark. The sections were then blocked in 1% FBS for 30 min at RT and incubated overnight with primary antibodies of SIRT-1 (Santacruz, Mumbai), HSP60 (Cell Signalling Technology, USA) and ICAM-1 (Invitrogen, USA) at 1:200; $CD68^+$ at 1:100 (Dako, Agilent, USA); and CLOCK at 1:250 (Invitrogen, USA) at 4°C in humidified chamber. Sections were incubated with HRP conjugated anti-mouse/anti-rabbit secondary antibody for 1h at RT. Sections were then washed thoroughly with PBS and further DAB substrate was added followed by counter-staining with haematoxylin. Sections were observed under Nikon eclipse Ti2-E (Tokyo, Japan). Quantification of positively stained regions was carried out using Fiji software (ImageJ, NIH, Bethesda, USA) (Crowe and Yue, 2019).

Cell Culture

Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Himedia Laboratories (Mumbai, India). Cells were cultured using HiEndoXL Endothelial Growth medium supplemented with HiEndoXL Endothelial Growth Supplement and 1X antibiotic antimycotic solution. Cells were maintained at 37°C and 5 % CO2. Sub-culturing was done using EnVzyme Easy at a confluency of 70-80%. Exponentially growing cells of passages 2-7 were used for all experiments and cells in passage 1-3 were frozen in DMEM containing 30% FBS and 7% DMSO for storage in liquid nitrogen until further use. Experiments were conducted at about 70% confluency and treatment was given in an incomplete media.

Human monocyte (THP-1) cells were procured from National Centre for Cell Science (NCCS), Pune, Maharashtra, India and maintained in RPMI-1640 medium

supplemented with L-glutamine (2 mmol/l), 10% FBS and 1X antibiotic antimycotic solution. Cells were maintained in a humidified atmosphere with 5% CO2 at 37°C. Culture medium was replaced every 2-3 days until cells reached a maximum density of 1.5-1.8 x 10^6 cells/mL. At this density, cells were collected and centrifuged at 1000 rpm for 2 min. Residual medium was removed and cells were resuspended in fresh culture medium. Cells were seeded in tissue culture flask at a density of 2-3 x 10^5 cells/ml and residual cell suspension was used for experimentation. Exponentially growing cells of passages 22-35 were used for all experiments. Cells were cryopreserved in RPMI-1640 containing 30% FBS and 5% DMSO and stored in liquid nitrogen until further use.

THP-1 differentiation

THP-1 cells were differentiated to Monocyte derived macrophages (MDMs) for all the experiments using PMA. The dose range of PMA was determined as per % adhesion. 1.5 x 10^6 cells/mL were seeded in serum-free RPMI-1640 with varying concentrations (0-100 nM) of PMA for 24 h. After incubation, non-adherent cells were collected, centrifuged and pellet was resuspended in 200 µL PBS. Number of non-adherent cells were counted and % adherence was calculated relative to the number of cells seeded initially. Monocytes were differentiated using 30nM, 50nM and 100nM PMA. For all further experiments, THP-1 monocytes were differentiated with 100 nM PMA for 24 and the differentiated MDMs were washed with 1x PBS before all the experimental treatments (Fig. M2) (Park *et al.*, 2007; Genin *et al.*, 2015).

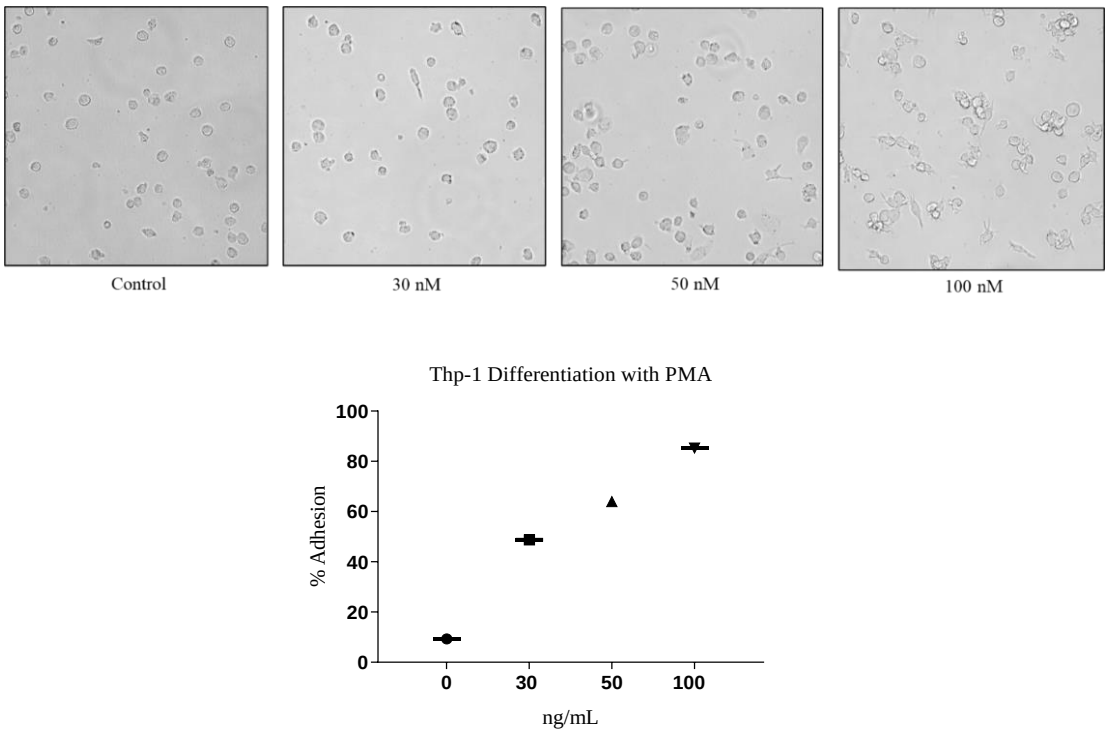


Fig. M2: Microscopic imaged of THP-1 cells differentiated to MDMs using different concentrations of PMA (30, 50 & 100nM). Percentage adhesion of the cells for each dose is represented graphically.

miR34a-5p inhibition

miR-34a-5p was inhibited in HUVEC/MDMs by transfecting the cells with 100 nM of miScript hsa-miR-34a-5p mirVana^{TM} inhibitor or a scramble miR (Ambion, Waltham, MA, USA) used as negative control. Transfection of the cells was achieved by using Lipofectamine 2000 (ThermoFisher, Waltham, MA, USA) in an incomplete media, according to the manufacturer's instruction. Transfection was done at about 75-80% confluency in an incomplete media. Cells were treated with 80 µg/mL ox-LDL for further experiments. Efficacy of the same was determined by assessing miR34a-5p levels.

Cell viability assay

Cell viability was assessed with MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded in 96 well plate in their respective media and treatment were given as per the experimental design for 24h. At the end of the treatment cells were dosed with 0.5 mg/mL of MTT made in an incomplete media and were kept in dark for 4h at 37°C. Resultant formazan crystals were dissolved in DMSO, and absorbance was recorded at 570nm using Synergy HTX Multimode Microplate Reader (BioTek Instruments Inc., USA). The results were represented as percentage cell viability with respect to control.

Luciferase Reporter Gene Assay

Luciferase reporter gene assay was performed using Secrete-Pair TM dual luminescence assay kit (Gene Copoeia, Rockville, MD, USA). HUVEC were transfected either with 100 nM miScript hsa-miR-34a-5p mirVana mimic or negative control (Ambion, Waltham, MA, USA) and with either a plasmid containing a miTarget™ 3′ UTR CLOCK luciferase reporter (pEZX-MT05-CLOCK) or mutated miTarget™ 3′ UTR (pEZX-MT05-Mutant) (Genecopoeia, Rockville, MD, USA) for 48h. Gluc/SEAP (Gaussia Luciferase (GLuc) and Secreted Alkaline Phosphatase (SEAP)) assay was performed with 100 µL of luminescent and 10 µL of the collected supernatant from each group with n=3 technical replicates and read on luminometer (Molecular Devices Gemini XS Fluorescent Microplate Reader; Marshal Scientific, NH, USA). The luciferase units were measured as relative luciferase units (RLU) and normalized to total protein (Thounaojam *et al.*, 2019).

Immunofluorescence staining

HUVEC were cultured on gelatin coated coverslips. Circadian clock of the cells was synchronized by the serum shock treatment done with subjecting the cells to 50% FBS for 2h. Further, cells were dosed with 20 μM LPS for 24h in HiEndoXL media. Briefly, cells were washed with 1X PBS and fixed with 4% PFA for 15 min. Permeabilization was done with 0.1% Triton X-100 in TBS followed by blocking with 3% BSA-PBS for 30 min. Cells were probed with CLOCK primary antibody (1:150; Invitrogen, USA) overnight at 4^{o}C in humidified chamber. Later, cells were washed thrice with 1X PBS and incubated with Alexa Fluor-488 anti-rabbit secondary antibody (Invitrogen, Thermo Scientific, USA) along with ActinRed 555 Ready probes reagent (Invitrogen, Thermo Scientific, USA) for 1.30 hours. Cells were again washed thrice and mounted using Fluoroshield with DAPI (Sigma-Aldrich, USA). Imaging was done on Nikon eclipse Ti2-E (Tokyo, Japan).

Quantification of fluorescence staining

All the fluorescence staining were analysed using FiJi ImageJ software as described (Jakic *et al.*, 2017). In every image, single cells were selected and its area, integrated density and mean gray value were measured. Also, mean gray values (fluorescence intensities) of four different background areas were measured in every image for normalization of background autofluorescence. The corrected total cell fluorescence (CTCF) for each cell was calculated as: CTCF= Integrated density – (area of cell x mean of background fluorescence. The intensities were calculated for more than 80 cells from a total of 5-10 different images for each experimental sample and the values were used for statistical analysis.

Monocyte endothelial adhesion assay

Cell adhesion assay was done as described by Yanaka et al. (2011) with minor modifications (Yanaka *et al.*, 2011). HUVECs were seeded in gelatin coated 12 well plate. At about 70% confluency cells were treated with OxLDL (80µg/ml) and/or CORM-A1 (40µM) for 24 h. THP-1 monocytes were labelled with 1 µg/ml Hoechst 33342 in incomplete medium for 15 min in dark. These were washed with warm PBS twice and the Hoechst labelled THP-1 monocytes (0.5 x 10^5 cells/well) were laid over HUVEC and incubated for 30 min. The unbound monocytes were removed by aspiration of residual medium followed by washing the wells with warm PBS thrice. Monocytes bound on HUVEC were observed and photographed under FLoid Cell imaging station (Thermo Fisher Scientific, USA). Number of HUVEC and bound THP-1 monocytes were counted and % monocyte adhesion was calculated relative to endothelial cells (Yanaka *et al.*, 2011).

Oxidative stress Determination

Cellular oxidative stress was determined using 2',7' –dichlorofluorescin diacetate (DCFDA) that quantifies cellular ROS. DCFDA assay was performed on the cells treated with ox-LDL and/or CORM-A1 for 24h. DCFDA is deacetylated by esterases to a nonfluorescent compound, Cellular ROS oxidises it to 2', 7'-dichlorofluorescein (DCF) which is highly fluorescent and used for detection. Cells were incubated with 10 µM DCFDA for 20 min. After 20 min cells were washed with 1X PBS for about 3 times or till the background noise was minimum. Images were taken on FLoid Cell Imaging

Station using green filters. Intensity of green fluorescence witnessed is directly proportional to intracellular cellular ROS.

Mitochondrial membrane potential

The mitochondrial integrity was monitored by detecting the change in mitochondrial membrane potential (MMP; ΔΨm) using lipophilic fluorescent probe JC-1. JC-1 is a membrane permeable dye that exhibits potential-dependent accumulation in mitochondria leading to formation of aggregates. The formation of J-aggregates in a healthy mitochondrion result in shift of its fluorescence maxima from green (~529 nm) to red (~590 nm) making it an indicator of mitochondrial health (Smiley *et al.*, 1991). Briefly, Cells were cultured in 6 well plate at about 70% confluency, cells were dosed with ox-LDL and/or CORM-A1 for 24h. Cells were washed with 1X PBS and incubated with 5 µM JC-1 dye prepared in an incomplete media for 20 min at 37˚C. Cells were then with warm 1X PBS (37°C) to remove excess dye and fluorescent images of JC-1 monomer (green fluorescence) and JC-1 aggregates (red fluorescence) were captured using FLoid Cell Imaging station. Total integrated intensity of green- and red-fluorescence in the individual cells was measured using FiJi Image J software (NIH, Bethesda, USA) and red/green fluorescence ratio was calculated. The ratio of red fluorescence to green fluorescence was considered as a change in the Mitochondrial Membrane Potential (ΔΨm) (Smiley *et al.*, 1991).

MitoTracker Red Staining

Cellular mitochondrial quantity was assessed by MitoTracker Red staining. Briefly, HUVECs/MDMs were treated with ox-LDL and/or CORM-A1 for 24h. cells were washed and dosed with 50nM Mitotracker Red co-stained with 1µg/mL Hoechst 33342 prepared in an incomplete media for about 15-20 min. Cells were washed thrice with 1X PBS to remove excess of stain. Imaging was done using Floid Imaging station (Thermo Scientific, USA).

Mitochondrial DNA Copy number

Mitochondrial DNA (mtDNA) was used to determine mitochondrial density using q-PCR. Total DNA was isolated from thoracic aortae or HUVEC/MDMs cells using GeneJET genomic DNA purification kit (Thermo Scientific, USA) as per manufacturer's instructions. Isolated DNA was further used as template for quantification of ND1 and Nuclear 18s rRNA / β-globin using qPCR. Primers for the same were designed using NCBI BLAST and procured from IDT. Further, mitochondrial DNA copy number was calculated from the ratio of ND1 (mitochondrial encoded gene) to Nuclear18s rRNA/β-globin (nuclear encoded gene) (Liu *et al.*, 2003; Naha *et al.*, 2020).

DNA Methylation Assay

Genomic DNA was isolated from HUVEC and MDMs using GeneJet Genomic DNA (gDNA) purification kit (Thermo Scientific, USA) as per manufacturer's protocol. To access the genomic methylation pattern, gDNA was deaminated using EpiJet Bisulfite

conversion kit (Thermo Scientific, USA) according to manufacturer's instruction. The deaminated gDNA was further used as template for running methylation specific PCR (MSP Assay). CpG islands were determined in the promoter region of miR34a, several hundred base pairs upstream of precursor transcription start site and 5 sets of primers were used for methylated and unmethylated DNA region each (Fig. M3; Table M4). Real-time PCR was performed with following conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 60°C for 30 seconds (Meng *et al.*, 2012). A reaction tube w/o template was used as negative control and all the samples were run with n=3 technical replicates.

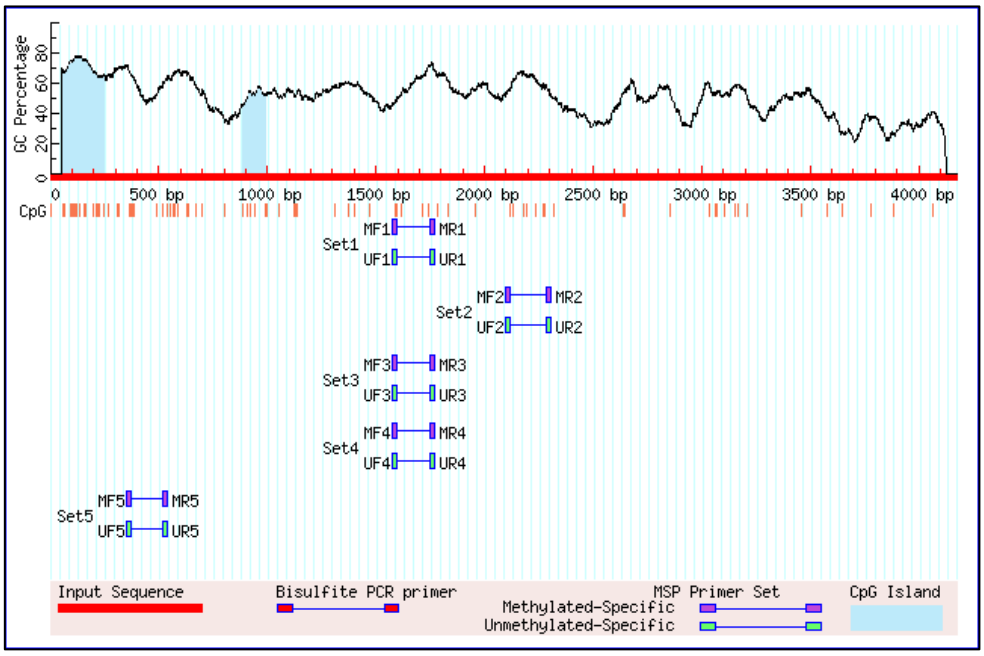


Fig. M3: Pictorial representation of regions selected for assessing methylation pattern in the promoter of miR34a-5p using MSP Assay (Meng *et al.*, 2012).

RNA isolation and cDNA synthesis

Total RNA was isolated from thoracic aorta, HUVEC and MDM samples using Trizol reagent (Invitrogen, Thermo Scientific, USA). Lysis/homogenization was followed by phase separation using chloroform. RNA was precipitated from aqueous phase using pre-chilled isopropanol, centrifuged to pellet that was subsequently washed once with pre-chilled 75% ethanol. The entire process was done on ice. The RNA pellet allowed to dry and dissolved in diethyl pyrocarbonate (DEPC)-treated water. Quantification and purity check (260/280 and 260/230 ratios) were done on nanodrop. 1 µg of RNA was used to synthesize cDNA by reverse transcription using iScript cDNA synthesis kit (Bio-Rad Laboratories, USA) according to manufacturer's protocol and cDNA was used as template for quantitative RT-PCR.

miRNA isolation and cDNA synthesis

miRNA isolation was done using miRNeasy Kit (Qiagen, Germany). Tissues were homogenized, and cells were directly lysed into QIAzol lysis reagent. The lysate was further processed as per manufacturers instruction. Yield quantification and purity check (260/280 and 260/230 ratios) were done using nanodrop.

miRNA was further converted to cDNA using miScript II RT kit. Template quantity was taken and processed as per manufacturers instruction. The product was further used as a template for qPCR reactions.

Quantitative RT-PCR

Quantitative RT-PCR was performed using SYBR Green supermix (BioRad, USA) as per the manufacturer's protocol and the reaction was run on the QuantStudio-3 Real-Time PCR System (Applied Biosystem, Thermo Fisher Scientific, USA). mRNA expression of the genes of interest were analysed using $2^{-\Delta\Delta CT}$ method using 18s and 5S as endogenous control for RNA and miRNA respectively. Primers used for this study are listed in the tables M1-M4.

Western blotting

For extracting total protein lysate, tissue was homogenized in RIPA buffer (50 mM tris (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% triton X-100) containing PIC and 1 mM PMSF followed by incubation on ice-bath for 2 h. For HUVEC and THP-1 samples, cell pellets were washed twice with ice-cold PBS and lysed in RIPA buffer and incubated for 30 min on ice. The tissue and cell lysates were centrifuged at 10,000 rpm at 4˚C for 20 min and resultant supernatant was subjected to protein estimation using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, USA). Equal quantity of protein from each sample was denatured in 6X loading dye at 95°C-100°C for 5 min. 6X Loading Dye comprised of 12% SDS, 30% β-Mercaptoethanol, 60% Glycerol, 0.012% Bromophenol Blue and 0.375 M Tris HCl (PH=6.8). 20 µg of each protein samples was separated by SDS-PAGE and subsequently transferred onto PVDF membrane using standard protocol of Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, USA). Complete transfer of proteins was checked with 0.05% Ponceau S staining. Membrane was destained in distilled

water followed by blocking with 5% milk in Tris buffered saline for 1 h at RT. The membrane was incubated overnight in primary antibody prepared in 3% BSA. After washing thrice with TBS containing 0.1% Tween 20 (TBST), the membrane was probed with HRP-linked anti-rabbit/ anti-mouse secondary antibody (1:2000) for 1 h at RT. Expression of the immune-reactive proteins was detected using ClarityTM Western ECL Substrate (Bio-Rad Laboratories, USA) according to the instruction manual and chemiluminescence was captured on X-ray sheets. The membrane was stripped using stripping buffer and the same were blocked and re-probed overnight with primary β-actin antibody.

Total ATP content

ATP content was assessed in a freshly prepared lysate using ATP Determination Kit (A22066, Molecular Probes). Equal volume of lysate was used for each reaction. The reaction mixture constituted of 50 mM D-luciferin, 1.25 mg/ml firefly luciferase and 1 mM dithiothreitol in 1 reaction buffer and tissue/cell homogenate. After 15-min incubation luminescence was measured using Synergy HTX Multimode Readers (Bio-Tek instruments, Inc., Winooski, VT). The lysate was used for protein quantification using Bradford Assay. Results were expressed as Mean intensity/µg of protein and compared to that of the control group.

Mitochondrial respiration (Seahorse XF Analyzer)

The Seahorse XF Analyzer (Seahorse Biosciences, North Billerica, MA) was used according to the manufacturer's protocol to measure OCR and ECAR of the cells.

HUVEC/MDMs cells were seeded in Seahorse Flux Analyzer mini plates (10000 cells/well) and incubated overnight at 37˚C. For one group, cells were transfected with miR34a-5p antagomir. Overall cells were treated for other groups with ox-LDL and ox-LDL + CORM-A1. Thereafter, the culture medium was changed to XFp base medium minimal (Seahorse Biosciences) and placed in a non-CO2 incubator for at 37˚C. Mitochondrial function was assessed using the Seahorse XFp Analyzer by monitoring changes in OCR and ECAR. Three OCR measurements were obtained under basal conditions and upon sequential injection of 2 µM oligomycin, 2 µM fluoro-carbonyl-cyanide phenylhydrazone (FCCP), and 0.5 µM rotenone plus 0.5 µM antimycin A. OCR values were calculated from 3-min measurement cycles. The OCR measurements were adjusted to cell numbers. Glycolysis was assessed by analysing ECAR in cells cultured in glucose-free medium after sequential addition of 10 mM glucose, 2 µM oligomycin, and 100 mM 2-deoxyglucose. The final data was obtained using the Seahorse XFp software and calculated as per the instruction of manufacturer.

Insilico studies for miRNAs

miRNA targets for CLOCK gene in human and mice were investigated using computational prediction algorithms (miRDB V 6.0 and TargetScan V 7.0) software (M Witkos *et al.*, 2011; Wong and Wang, 2015). Potential miRNAs were further screened for functionality test (miRDB/ PubMed/ preliminary data from lab). Based on the results, miR34a-5p was identified as a potential candidate and subjected to gene target prediction using miRDB and TargetScan Version 7.0. PubMed evaluation for 899 genes targets of miR34a-5p was conducted and the percentage genes relevant to cardiovascular diseases were identified.

Molecular Docking studies

For P53 protein target (available protein structure for p53 protein target in Protein Data Bank with PDB ID: 1TUP) a grid around the two amino acid residues R248 and R273 were generated followed by protein preparation which included removal of water molecules and unwanted ions/chains, Cleaning the protein structure and Energy minimization for further docking studies. For the protein target NF-κB, the mutated amino acid residue Cys 61 (as deciphered from literature review), was found to bind with DNA. In NF-κB, (available protein structure for NFkB in Protein Data Bank with PDB ID: 1SVC) as confirmed from pairwise sequence alignment with its canonical sequence, The position of Ala61 in the structure residue index is Ala62. Using Schrodinger tool, alanine at 62nd position was then mutated to Cys. Both the protein targets were prepared in the similar way after downloading and removal of mutations from the structure. The ligand CO was prepared using LigPrep.

Glide version 11.4 was used for molecular docking. By optimizing geometries through the OPLS_2005 force field and producing potential ionization at pH 7.0±2.0, ligand structures were created using the LigPrep tool (Schrödinger, LLC). The protein preparation wizard (Schrödinger, LLC) was used to generate high-resolution protein structures downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB). Charges and bond ordering were ascribed to the protein structure after hydrogen atoms were added, heteromolecules and water molecules were removed. The resultant protein structure was then optimized and minimized using the OPLS_2005 force field to eliminate steric conflicts, while the rest of the parameters were left at their default values. The grid was created at the active site

$$G\ Score = a*vdW + b*Coul + Lipo + Hbond + Metal + BuryP + RotB + Site$$

of the protein molecule using the receptor grid generation module. Molecular docking of CO with both the targets was carried out using the Glide extra precision method (XP). The Glide score (Gscore) was calculated using the following formula in kcal/mol.

Where Van der Waals energy is represented by vdW, Coul is the symbol for Coulomb energy. Lipo is a symbol of lipophilic interaction. Metal binding is represented by Metal, and hydrogen bonding is represented by H-bond. BuryP represents the penalty for buried polar groups, while RotB represents the penalty for freezing rotatable bonds. This glide score (Kcal/mol) was used to further determine the final ligand-protein binding evaluation.

Free energy Calculations

Free energy calculations (MM-GBSA) of ligand CO with the targets NFkB and P53 were performed using Prime suite of Schrodinger using VSGB 2.1 solvation model. Enumeration of Protein -ligand binding energy using OPLS3 force field was done, and the following equation was utilised by the tool in order to calculate the binding free energies of Protein-ligand complexes.

$$\Delta G(\text{bind}) = \Delta G(\text{solv}) + \Delta E(\text{MM}) + \Delta G(\text{SA})$$

where:

- ΔG *(*solv) is the difference in GBSA solvation energy of the protein-ligand complex and the sum of the solvation energies for unliganded target and ligand.

- Δ*E (*MM) is a difference in the minimized energies between protein-ligand complex and the sum of the energies of the unliganded target and ligand.
- Δ*G*SA is a difference in surface area energies of the complex and the sum of the surface area energies for the unliganded target and ligand.

Prime MM-GBSA suite calculates the energy of optimized free receptors, free ligand, and a complex of the ligand with a receptor. It also calculates the ligand strain energy by placing ligand in a solution which was auto- generated by VSGB 2.1 suite. The prime energy visualizer presented the visualization of energy.

Statistical Analysis

All the statistical analysis were carried out using GraphPad Prism 8.0.1. Differences between two groups were compared by unpaired two-tailed Student's t-test and for more than two group with one variable, one-way ANOVA followed by Tukey's test was carried out. Results were expressed as mean ± S.E.M. For all analysis, differences were considered statistically significant at *P <0.001

Table M1: List of mice primers for qPCR (FP: Forward Primer; RP: Reverse Prime).

Target	Primer	Sequence (5' → 3')
miR34-5p	FP	GCAGTGGCAGTGTCTTAG
	RP	GGTCCAGTTTTTTTTTTTTTTACAAC
5S	FP	TCTCGTCTGATCTCGGAAGC
	RP	AGCCTACAGCACCCGGTATT
18s rRNA	FP	GCAATTATTCCCCATGAACG
	RP	GGCCTCACTAAACCATCCAA
CLOCK	FP	CACTCTCACAGCCCCACTGTA
	RP	CCCCACAAGCTACAGGAGCAG
BMAL1	FP	ACATAGGACACCTCGCAGAA
	RP	AACCATCGACTTCGTAGCGT
PER1	FP	CATGACTGCACTTCGGGAGC
	RP	CTTGACACAGGCCAGAGCGTA
PER2	FP	GGCTTCACCATGCCTGTTGT
	RP	GGAGTTATTTCGGAGGCAAGTGT
CRY1	FP	GGTTGCCTGTTTCCTGACTCGT
	RP	GACAGCCACATCCAACTTCCAG
CRY2	FP	TCGGCTCAACATTGAACGAA
	RP	TCGGCTCAACATTGAACGAA
SIRT1	FP	GATACCTTGGAGCAGGTTGC
	RP	CTCCACGAACAGCTTCACAA

Table M2: List of human primers for qPCR. (FP: Forward Primer; RP: Reverse Prime).

Target	Primer	Sequence (5' → 3')
miR-34a-5p	FP	CGAGTGGCAGTGTCTTAGCT
	RP	CCAGTTTTTTTTTTTTTTTTACAACC
5S	FP	GGCCATACCACCCTGAACGC
	RP	CAGCACCCGGTATTCCCAGG
18s rRNA	FP	CGTTCAGCCACCCGAGATT
	RP	GACCCGCACTTACTGGGAATT
CLOCK	FP	CGAGCGCTCCCGAATTTTTA
	RP	AGGTATCTAGTGAGACTTGCCA
BMAL1	FP	GGCTCATAGATGCAAAAACTGG
	RP	CTCCAGAACATAATCGAGATGG
PER1	FP	CATGACTGCACTTCGGGAGC
	RP	CTTGACACAGGCCAGAGCGTA
PER2	FP	GACTCCTCGGCTTGAAACGG
	RP	GTGTCACCGCAGTTCAAACG
CRY1	FP	GTGGATCAGCTGGGAAGAAG
	RP	GGCTTGCGCTCCTTTTCCTC
CRY2	FP	GTGCCTCAAATCCTGACCCA
	RP	GCCTCCCACAAGATTGACGA
SIRT1	FP	GATACCTTGGAGCAGGTTGC
	RP	CTCCACGAACAGCTTCACAA

PGC1α	FP	AGTCTTCGGCTGTTTGGTGA
	RP	TGGAAGAACAGATGTGCCCC
NF-κB	FP	GAGGTCTCTGGGGGTACCAT
	RP	AAGGCTGCCTGGATCACTTC
P53	FP	ATTGGCCAGACTGCCTTCC
	RP	TCCGGGGACAGCATCAAATC
ZEB1	FP	GCGGCGCAATAACGTTACAAA
	RP	TTCCTTTCCTGTGTCATCCTCCC
SNAI1	FP	CTCGGACCTTCTCCCGAATG
	RP	TCATCAAAGTCCTGTGGGGC
STAT3	FP	ATCACGCCTTCTACAGACTGC
	RP	CATCCTGGAGATTCTCTACCACT
SOD2	FP	GCACTAGCAGCATGTTGAGC
	RP	CCGTTAGGGCTGAGGTTTGT
TrxR2	FP	GATTAGGAGGGCGCTTCCG
	RP	GTTGGGGGCATCTTGGATCA
18s rRNA	FP	CGTTCAGCCACCCGAGATT
	RP	GACCCGCACTTACTGGGAATT
GAPDH	FP	TGTGAACGGATTTGGCCGTA
	RP	ACTGTGCCGTTGAATTTGCC

Table M3: List of rat primers for qPCR. (FP: Forward Primer; RP: Reverse Prime).

Target	Primer	Sequence (5' → 3')
miR34a-5p	FP	GCAGTGGCAGTGTCTTAG
	RP	GGTCCAGTTTTTTTTTTTTTTACAAC
p53	FP	ATGGGTTCCAGCACTTAGCC
	RP	GAAGATTCCCTGGTAGCGCA
SIRT1	FP	TATGCTCGCCTTGCTGTGGA
	RP	GCTGAGTTGCTGGATTTTGTGT
PGC-1α	FP	TTCAGGAGCTGGATGGCTTG
	RP	GGGCAGCACACTCTATGTCA
ZEB1	FP	TGCCCAAACTGCAAGAAACG
	RP	GGACTGCCTGGTGATGTTGA
SNAI1	FP	AGTTGTCTACCGACCTTGCG
	RP	TGCAGCTCGCTATAGTTGGG
ICAM-1	FP	GCCTGGGGTTGGAGACTAAC
	RP	CTGTCTTCCCCAATGTCGCT
VCAM-1	FP	CCTCTCGGGAAATGCCACC
	RP	GTCAGAACAACGGAATCCCCA
U6	FP	CTCGCTTCGGCAGCACA
	RP	AACGCTTCACGAATTTGCGT
GAPDH	FP	CAACGGGAAACCCATCACCA
	RP	ACGCCAGTAGACTCCACGACAT
TrXR2	FP	AGGGCAGCAGAACTTTGATCT
	RP	GGGTTCCACATAGTCAGCCA
SOD2	FP	CGGGGGCCATATCAATCACA
	RP	TAGCCTCCAGCAACTCTCCT

Table M4: List of primers used for Methylation Specific PCR Assay. (FP: Forward Primer; RP: Reverse Prime).

Target	Primer	Sequence (5' → 3')
M-P1	FP	GGTTGAAAGGTTTTAAGAGTAGAATC
	RP	TTATTTCAAAAAATCGACTTACGTA
M-P2	FP	GGTTGAAAGGTTTTAAGAGTAGAATC
	RP	TTATTTCAAAAAATCGACTTACGTA
M-P3	FP	TGAAAGGTTTTAAGAGTAGAATCGA
	RP	TTATTTCAAAAAATCGACTTACGTA
M-P4	FP	TAGGTTTGTTTTTCGAGTTTTTTTC
	RP	CTCCCACTAATCTAAACATCTCTCG
M-P5	FP	TAGGTTTGTTTTTTGAGTTTTTTTTG
	RP	CCCACTAATCTAAACATCTCTCACT
UM-P1	FP	TGAAAGGTTTTAAGAGTAGAATTGA
	RP	TTATTTCAAAAAATCAACTTACATA
UM-P2	FP	TGAAAGGTTTTAAGAGTAGAATTGA
	RP	TTTATTTCAAAAAATCAACTTACATA
UM-P3	FP	TGAAAGGTTTTAAGAGTAGAATTGA
	RP	TTATTTCAAAAAATCAACTTACATA
UM-P4	FP	AGGTTTGTTTTTTGAGTTTTTTTTG
	RP	TCTCCCACTAATCTAAACATCTCTCA
UM-P5	FP	TAGGTTTGTTTTTCGAGTTTTTTTC
	RP	CTCCCACTAATCTAAACATCTCTCG

Table M5: List of primers for mtDNA Assay. (FP: Forward Primer; RP: Reverse Prime).

Target	Primer	Sequence (5' → 3')
hsa-ND1	FP	ATGGCCAACCTCCTACTCCT
	RP	GCGGTGATGTAGAGGGTGAT
hsa-18s rRNA	FP	ACGGACCAGAGCGAAAGCA
	RP	GACATCTAAGGGCATCACAGAC
rno-ND1	FP	TCCTCCTAATAAGCGGCTCCTTCTC
	RP	GGTCCTGCGGCGTATTCGAC
rno-β globin	FP	CAGTACTTTAAGTTGGAAACG
	RP	ATCAACATAATTGCAGAGC