<u>Chapter 2</u>

# 2. Materials and methods

# 2.1. Cell culture

MDA-MB-231, human breast cancer cell line was grown in RPMI 1640 (Roswell Park Memorial Institute) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Tumorigenic human embryonic kidney cell, HEK293, and 'normal' immortalized mouse embryonic fibroblast cells, NIH3T3, were grown in DMEM cell culture medium with 10% FBS and 1% penicillin-streptomycin. The MDA-MB-231 cell line was a kind gift from Prof. Gaurisankar Sa, Bose Institute, Kolkata, India. Cells were incubated at 37°C, with saturated humidity and an atmosphere of 5% CO2. The cell line study was approved by the Institutional Biosafety Committee of Presidency University, Kolkata, India. All cell culture reagents and plastic wares were obtained from ThermoFisher Scientific, USA.

# 2.2. rhTRAIL protein induction and purification

TRAIL extracellular domain (aa 114 – 281) tagged with His-tag was expressed using pQEhTR plasmid. It was obtained from Wafik El-Deiry through Addgene (plasmid #21812)<sup>138</sup>. The resulting 167 amino acid active TRAIL was termed as rhTRAIL (recombination human TRAIL). pET-15b control plasmid was a gift from Dr. Sugopa Sengupta, Presidency University, Kolkata. Induction and purification of rhTRAIL was performed as described by Kim et al.<sup>138,139</sup>. Briefly, pQE-nTR and pET-15b plasmids were transformed in DH5α. Primary culture was prepared by overnight incubation of the positive recombinant bacterial colony in LB medium containing ampicillin (100µg/ml) selection at 37°C. Next day, secondary culture was prepared in LB medium containing ampicillin with 1% primary culture in the presence of 100µM Zinc Sulphate. The culture was grown at 21°C till the optical density (OD) reached 0.3-0.6. Induction of rhTRAIL was carried out by adding 0.5mM Isopropyl-β-D-thiogalactoside (IPTG) to the culture medium at 20°C until the O.D doubled the previous measure. The bacterial cultures were centrifuged at 6000 rpm for 30min at 4°C followed by preparation of cell lysate using lysis buffer (50mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 10 mM  $\beta$ -mercaptoethanol<sup>139</sup>. rhTRAIL was isolated from the soluble fraction using Ni-NTA agarose beads after washing with lysis buffer containing 20 mM imidazole. Isolated protein dialysis was done against phosphate buffered saline with 10 mM  $\beta$ -mercaptoethanol. The purity of the rhTRAIL protein was confirmed by Coomassie blue gel staining and immunoblotting. IC50 of the purified rhTRAIL protein was observed to be 250ng/ml.



**Figure 2.1.** rhTRAIL purification. (a)SDS-PAGE, Coomassie stain of purified rhTRAIL (Lane 1: Ladder; Lane2: Purified TRAIL (~24kDa); Lane 3: Control), (b)Western Blot of purified rhTRAIL with TRAIL specific antibody (Cell Signalling Technologies) (Lane1: Purified TRAIL (~24kDa); Lane 2: Control)

#### 2.3. Microarray of different MDA-MB-231 breast cancer cells

Total RNA from mock treated (control), 50ng TRAIL treated MDA-MB-231, TRAIL resistant and TRAIL sensitive cells were isolated following the manufacturer's instructions (Qiagen RNeasy Mini Kit). This was followed by quantification using NanoDrop spectrophotometer (Thermo Fisher Scientific) and quality assessment using a 2100 Bioanalyzer (Agilent Technologies). An input RNA (200ng) with RNI (RNA Integrity Number) value of greater than 9 was used for the experiment. One color human gene expression experiment was performed by using Agilent Human gene expression array (slide version 3, design ID: 072363) for human differential expression analysis. Each condition was analyzed in duplicate. Experiment was preformed following the Agilent low input gene expression protocol (version 6.9). Agilent Feature Extraction software was used

to obtain raw data from the scanned data Images. Data normalization, grouping, QC and statistical analysis was performed using the GeneSpring software (Agilent Technologies). Differentially expressed genes (DEGs) between different cells were obtained using one-way ANOVA. Benjamini-Hochberg corrected p-value cut-off was less than 0.05. Gene array analysis was performed with the help of employees at the LCGC Life Sciences LLP, Agilent Technologies.

# 2.4. DAVID analysis of the candidate differentially expressed genes (DEGs)

Database for Annotation, Visualization, and Integrated Discovery (DAVID) database is an online tool for functional enrichment and pathway enrichment analysis. The algorithm is used to condense the group of candidate genes into biological terms (Gene ontology/GO term) consisting of functionally related genes<sup>140</sup>. Next, the candidate genes were categorized into GO Biological Processes (BP), GO Cellular Components (CC), and GO Molecular Function (MF). This enabled us to efficiently interpret the list of candidate genes that we obtained through the high-throughput microarray analysis. KEGG (Kyoto Encyclopedia of Genes and Genomes) is a database that organizes experimental data into molecular networks or pathways<sup>141–144</sup>. We used KEGG pathways through DAVID in order to categorize the candidate genes based on signaling pathway networks. For both, GO term and KEGG pathway fold enrichment was calculated<sup>145</sup>. Benjamini-Hochberg corrected p-value was considered significant at and below 0.05.

# **2.5. PPI (protein-protein interaction) network analysis**

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) is a biological database of known and predicted protein-protein interactions. These interactions can either be physical (direct) or functional (indirect) association between the proteins<sup>146</sup>. We used the STRING (version 11.0) for construction of PPI network for the candidate genes. The active interactions were set to include 'Text mining', 'Experiments', 'Databases', 'Co-expression', 'Neighborhood', 'Gene fusion' and 'Co-occurrence' with a minimum interaction score of 0.4. PPI network obtained from STRING was imported into Cytoscape software (version 3.8.0). The modules were identified through Cytoscape plugin MCODE.

Modules are clusters of highly interconnected regions in a network<sup>147</sup>. This was followed by identification of hub gene using the Cytoscape plugin CytoHubba.

# 2.6. GeneMANIA

Functional network predictive analysis for TRAIL and complement related genes were performed using the GeneMANIA (https://www.genemania.org/), which is a user-friendly platform for predicting similar gene function using available genomics and proteomics data. TRAIL, CFB, C2, C1S, C1R, C4B, CFP, CFH, CFI and CD59 genes were given as query genes for generating functional networks between then in the human genome.

# 2.7. Survival and correlation analysis

To understand the prognostic value of the candidate genes, Kaplan–Meier plotter (kmplot.com/analysis), an online database was used [29, 30]. The primary source for the database includes GEO (Gene Expression Omnibus), EGA (European Genome-phenome Archive), and TCGA (The Cancer Genome Atlas) datasets. The software was set to provide analysis based on recurrence free survival (RFS) with respect to median of candidate gene expression in triple negative breast cancer (TNBC) patient cohort. This was performed by restricting the analysis to estrogen receptor (ER) negative, progesterone receptor (PR) negative and Her2 negative category of breast cancer patients. Using the above parameters, the analysis was run on 255 TNBC patients from E-MTAB-365, GSE19615, GSE21653, GSE2603, GSE31519, GSE45255 datasets. Pearson's pairwise correlation analysis between the candidate hub gene and TRAIL was performed via Kaplan–Meier plotter (kmplot.com/analysis) on the same group of 255 TNBC patient samples.

# 2.8. Trypan Blue assay

For trypan blue assay  $5*10^4$  cells were grown in 24-well tissue culture plate for 24 h at 37°C and 5% CO<sub>2</sub>. Treatment reagents such Theophylline (Sisco Research Laboratories, India) dissolved in sodium hydroxide was added to the wells at a final concentration of 10

mM or TRAIL (in different concentreation) were used as per the experiments. In some experiments Sodium hydroxide was used as a negative control (The above concentration of theophylline and NaOH control was used for all the other experiments). After 24 hrs of treatment, cells were trypsinized and counted on a hemocytometer at 20x magnification after staining with trypan blue in 1:1 ratio (Sigma-Aldrich, now part of Merck, Germany). The experiment was carried out in triplicate and repeated three times. Representative experiment is shown in the figure.

# 2.9. Cell viability assay

A cell proliferation assay using thiazolyl blue tetrazolium bromide (MTT) reagent (SRL Pvt. Ltd., India) was performed. Briefly,  $1*10^4$  cells seeded in 96-well cell culture plates were treated with treatment and control. After 24 hrs of treatment, from each well media was replaced with 100 µl fresh media containing 10 µl of 5 mg/ml MTT reagent. After 4 hrs of incubation, 85 µl of media was removed carefully from each well followed by addition of 50 µl DMSO. The solution was then mixed to solubilise the formazan crystals. Next, the absorbance was measured at 590 nm on a SynergyH1 microplate reader with Gen5 software (Biotek, USA). The experiment was carried out in triplicate and repeated three times. Representative experiment is shown in the figure.

# 2.10. Acridine Orange (AO) and Ethidium Bromide (EtBr) Staining

For AO/EtBr staining,  $5*10^4$  cells were seeded in 24-well culture plates the night before treatment. Treatment and control were added to separate wells followed by incubation at  $37^{\circ}$ C. After 24 hrs cells were harvested and resuspended in 100 µl of PBS. Cells were stained with 8 µl of AO/EtBr mix containing 100 µg/ml acridine orange and 100 µg/ml ethidium bromide. 10 µl of stained cell suspension was aliquoted on a clean microscope slide and covered with a coverslip. Cells were observed and counted using Axio A1 (Zeiss, Germany) inverted microscope at 10x magnification with excitation filter 502/605 nm. Pictures were taken with a Tucsen digital camera. Experiments were done in triplicate and repeated three times, counting a minimum of 50 total cells each. Representative experiment is shown in the figure.

#### 2.11. Comet Assay

For both alkaline as well as neutral Comet assay (single cell gel electrophoresis),  $1*10^5$ cells were seeded in 6-well culture plate and incubated for growth for 24 hrs at 37°C and 5% CO<sub>2</sub>. Treatment and control were added to the wells and incubated for 6 hrs at 37°C and 5% CO<sub>2</sub>. Next, the cells were harvested via trypsinization and counted. Coverslips were coated with a layer of 1% low melting agarose. Subsequently, 30,000 cells were resuspended in 300 µl of PBS and transferred to 900 µl of low melting agarose that had been kept at 38°C. The cell-agarose suspension was transferred on the agarose coated surface of the pre-treated slides(slide surface was made rough by use of diamond pensil). Slides were allowed to dry for 30 min and then transferred to either alkaline lysis (sodium choride 1.2M; Na<sub>2</sub>EDTA 100mM; Sodium lauryl sulphate 0.1%; Na<sub>0</sub>H 0.26M) or neutral lysis buffer (NaCl 2.5M; disodium EDTA 100mM; Sodium lauryl sulphate 0.1%; Tris base 10mM; TritonX-100 1%) for overnight incubation.. alkaline wash buffer (NaOH 0.03M; disodium EDTA 2mM) or neutral wash buffer (disodium 10mM; HEPES-KOH 10mM; KCl 100mM) for alkaline and neutral comet assay, respectively. This was followed by electrophoresis for 10 min at 35 V. Slides were stained with propidium iodide (10 µg/ml) and observed using an excitation of 535 nm at 10x magnification in confocal microscope (Leica Microsystems, Germany). Comet tail moments were measured for a minimum of 50 cells using CometScore 2.0 software. The experiment was carried out in triplicate and repeated three times. Representative experiment is shown in the figure.

#### 2.12. RNA Isolation

After different treatments cells were trypsinized and washed with PBS, then the cellswere taken for RNA isolation. Cells were homogenised with TRIzol reagent (1ml/50-100mg tissue or cells) (Ambion, Life technologies) and incubated for 10min at room temperature (RT). Added 0.2ml of chloroform/ml of TRI reagent used. After that coverd the samples tightly and shook them vigorously for 15sec and allowed to stand for 10min at RT. Centrifuged the resultant mixture at 13000g for 10min at 2-8°c. Then the aqueous phase was transferred to a fresh tube and 0.5ml of isopropanol/ml of TRI reagent was used. Sample were allowed to stand for 10min at RT. Again, centrifuge at 13000g for 10min at 4°c. RNA pelleted down on the side and bottom of the tube. Next, supernatant is removed and RNA pellet were washed by adding 1ml of 75% ethanol/ml of TRI reagent added.

Then samples were vortexed and centrifuged at 7500g for 5min at 4°c. Discarded the supernatant and dryed the RNA pellet for 5-10min by air drying or 55°c heating block (Didn't let the RNA pellet dry completely as this will greatly reduce its stability). Finally, added an appropriate volume of DEPEC treated water/5% SDS to RNA pellet. Mixed by pipetting. Heated the RNA solution at 60°c for 10min. Followed by placing the eppendrof in ice.

# 2.13. Real-Time PCR

Different treated cells were harvested and RNA was isolated using TRIzol reagent mentioned above. RNA quality was determined via A260/A280 absorbance ratio and by performing electrophoresis. 1µg of total RNA was taken from different samples and treated with DNase for conversion to cDNA using reverse transcriptase as per the manufacturer's instructions (Biorad, USA). The Real-Time PCR reactions were performed for different primers listed in Annexure Table1 using SsoFast Evagreen supermix (Biorad) on PCR system 9700 (Biorad).  $\beta$ -actin was used as endogenous control. Threshold cycle (CT) number was measured using CFX Manager (Version 3.1.1517.0823) software (Biorad). Relative gene expression was calculated using the comparative CT method.

# 2.14. Genomic DNA isolation

The cells were seeded in T75 flask at a density of  $1.2*10^6$  cells/flask and grown for 24hrs. After trypsinization, cells were washed with PBS and taken out in fresh centrifuge tube for lysis and extraction. Genomic DNA lysis buffer (0.4M NaCl, 0.01M Tris and 0.002M EDTA) was added and vortexed vigorously. Thereafter, 10% SDS was added and gently vortexed until its mixed properly. Samples were incubated at  $55^{\circ}c$  for 15mins, then saturated phenol was added and centrifuged at 6000 rpm for 5 minutes. Supernatant was collected, then again, the mixture of chloroform: isoamyl alcohol was added and mixed gently. Samples were centrifuge at 6000 rpm for 5 minutes added and mixed gently. Samples were centrifuge at 6000 rpm for 5 minutes added and mixed gently. Samples were centrifuge at 6000 rpm for 5 minutes and supernatant was collected. Genomic DNA present in the supernatant was precipitated using 95% chilled ethanol. Pellets were washed thrice with 70% chilled ethanol to remove all the traces of phenol. Pellets were vacuum dried and re-suspender in sterile water.

### 2.15. qPCR for Copy Number Variation

Firstly, genomic DNA was isolated of both resistant and sensitive cells with the help of above mentioned protocal. To determine the efficiency of each primers used for CNV, we have done quantitative PCR using graded dilution of genomic DNA. The dilutions are  $100ng/\mu$ l,  $10 ng/\mu$ l,  $1 ng/\mu$ l,  $0.1 ng/\mu$ l. After that using the Ct value directly, we have calculated R2 and slope of the standard curve. From the slope we have determined the primer efficiency using this formula Efficiency (%) =  $(10^{(-1)}) + 100^{(-1)}) + 100^{(-1)}$ . For further qPCR reactions 100ng/reaction concentration of DNA was used for each of the genes listed in Annexure Table 1. Syber green qPCR was performed with the genomic DNA and relative CDH1 and DACH1 gene copy number was calculated using the comparative CT method.

### 2.16. Competent cell preparation

For primary culture, inoculated the DH5 $\alpha$  stock in 10ml LB[5ml]. Grown overnight at 37°c. Reinoculated 50ml LB (secondary culture) with 1ml (2%) of the primary culture. Kept another culture as negative control (LB without bacteria). Cultured them at 37°c shaker for 2 1/2 hrs until the O.D reaches 04-0.6(600nm). Next, left the cultures in ice for 2 1/2 hrs. Then centrifuged at 3000rpm for 5min at 4°c. Resuspended the pellets in 2ml of 100nM CaCl2[chilled]. Again, left on ice for 3hrs and then added glycerol (or can be even kept overnight in CaCl at -20°c). Aliquoted 200µl of cells in each tube.

#### 2.17. Plasmid DNA Isolation

At first primary culture was done with the desired strain. Next, 5ml overnight culture was centrifuged at 8000rpm for 5 min. Then soup was discarded. Solution I(Cold) [25mM Tris-Cl, pH-8; 10mM EDTA, pH-8; 10µg/ml lysozyme] 250µl added and kept on ice for 5 min. Next, solution II (freshy made) [1ml 10%SDS; 1ml 0.2N NaoH; 9ml distil water] 250µl added and incubated for 2-3 min. Solution III [5M sodium or potassium acetate, pH-5.2] 350µl wasadded and kept for -10 min centrifuged at (mixed well by inverting all the samples). Centrifuged at 12000rpm for 10min. Then collected the soup in fresh eppendrof. Added equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Next, added

RNase (10mg/ml){approx. 2-3µl in 200 soup}.Incubated at 37°c for 1 hr. Added equal volume of chloroform. Again, centrifuged at 10000rpm for 10min at 4°c. Collected the aqueous layer and added 1/10th volume of 3M sodium acetate and equal volume of Isopropenol. Incubated for 30min at -20°c. Centrifuged at 13000 rpm for 25 min at 4°c. Washed the pellet in 1ml of 70% ethanol. Finally, centrifuged at 13000rpm for 12 min. Air dried the plasmid pellets. Lastly, added 50µl distil water.

### 2.18. Transfection

We performed transient transfection using pcDNA3.1-E-cadherin-GFP which as a gift from Jennifer Stow (Addgene plasmid #28009). Vector control pcDNA3-EGFP was a kind gift from Dr. Abhik Saha, Presidency University, Kolkata. For transient transfection cells were seeded in 6 well plates at a density of  $0.8 \times 10^5$  cells/well. At 24hrs, when the cells have reached a confluency of 70% old media was removed and a mixture of 4µg of DNA and 10µl of Lipofectamine<sup>TM</sup> 2000 (Invitrogen) in serum free media was added to each of the wells containing serum free media. The cells were incubated for 6hrs at 37°C, with saturated humidity and an atmosphere of 5% CO2. Then the media was removed and fresh media with serum was added. After 48hrs of transfection, cells were used for downstream experiments.

# 2.19. The Cancer Genome Atlas (TCGA) analysis

For generation of data regarding various molecular formation of 30 different types of human tumors, Cancer Genome Atlas (TCGA) was used as it is an open publicly funded program. TCGA data helps in better understanding of cancer [21]. For our study, we have obtained the transcript (mRNA Z-score), copy number variation (CNV) and survival data for the 953 TCGA breast cancer samples from the platform of cBioPortal (http://www.cbioportal.org). We have also obtained transcript data (mRNA Z-score) for 19 breast cancer cell lines from Cancer Cell Line Encyclopedia (Novartis/Broad, Nature 2012), which was downloaded on 09.08.2020 through cBioPortal v3.4.4 [22, 23]. For transcript expression, mRNA z-score for each gene e.g. CDH1, TRAIL, DR4, DR5, DCR1 and DCR2 was obtained. Z-score indicates the number of standard deviations from the mean expression for the respective gene in the reference population. For tumor samples,

the reference population is the sub-set of tumor samples out of the 953 breast cancer samples that are diploid for the respective gene<sup>148</sup>. The clinical data for human samples were acquired from TCGA database (https://www.cancer.gov/tcga). An Institutional Review Board was not required as TCGA is publicly accessible data where all the patient information have been de-identified<sup>149</sup>.

# 2.20. TMRE-Mitochondrial Membrane Potential Assay

1\*10<sup>4</sup> cells were seeded in 96-well culture plate for each treatment and control, then incubated for growth for 24 hrs at 37°C and 5% CO<sub>2</sub>. After 6 hrs incubation at 37°C, TMRE (tetramethylrhodamine, ethyl ester) was added to label the active mitochondria in all treated and control samples. As per the kit protocol (Cell Signalling, USA), samples after 30mins of TMRE incubation were analysed on plate reader at excitation about 550nm and emission about 580nm.

# 2.21. ROS DCFDA Assay

 $1*10^4$  cells were seeded in 96-well culture plate for each treatment and control, then incubated for growth for 24 hrs at 37°C and 5% CO<sub>2</sub>. Next, cells were pre-stained with 2',7' –dichlorofluorescin diacetate (DCFDA) as per the manufacturer's instruction (Abcam, USA). Then different treatment and control were added separately in the respective wells and incubated for 4 hrs. The wells were read end point in a fluorescent plate reader with excitation at 485 nm and emission at 535 nm (SynergyH1, Biotek, USA). The experiment was carried out in triplicate and repeated three times. Representative experiment results were shown.

# 2.22. Lipid peroxidation assay

 $2.5*10^5$  cells were seeded in T25 culture flasks and incubated for growth for 24 hrs at 37°C and 5% CO<sub>2</sub>. Respective treatment and control were added into separate flasks. After 6 hrs of treatment, cells were harvested via trypsinization followed by resuspension in 0.9% NaCl. Then cells were sonicated and 100 µg of protein from each sample was taken for

further analysis. Thiobarbituric acid (TBA) - Trichloroacetic acid (TCA) - HCl (0.375% TBA, 2.5% TCA dissolved in 0.1 M HCl) reagent was mixed separately with each protein sample and incubated at 80°C for 20 min followed by centrifugation. Supernatant was collected and OD was measured at 530 nm using a spectrophotometer (Shimadzu, Japan). The experiment was carried out in duplicate and repeated three times. Representative experiment was shown.

# 2.23. NAC treatment

NAC (N-acetyl-L-cysteine) is an inhibitor of ROS. Cells were pre-treated for 1hr with NAC (5mM) followed by treatment with theophylline for 4hrs. This was followed by measurement of ROS using DCFDA reagent as described above. Consequently, these NAC treated cells were subjected to lipid peroxidation assay, trypan blue assay (figure 5C), cell viability assay and apoptosis assay. All the experiments were carried out in triplicate and repeated two times with representative experiments being shown in the figure. TNFR1 immunoblotting was performed thrice as described above. Representative blot has been shown along with average of three biological replicates.

# 2.24. R7050 Treatment

R7050(TOCRIS, 303997-35-5) is a known compound for TNFR1 inhibition. R7050 was dissolved in DMSO at a final concentration of 200µM and stored in -80°C. MDA-MB-231 cells were pre-treated with R7050 for 1 hr followed by 6 hrs of theophylline treatment. R7050 and theophylline treated cells were used to carry out cell viability assay and apoptosis assay.

# 2.25. Adenosine Treatment

adenosine (Cat. No- 54865, SRL) in a given concentration was dissolved in 1N HCL to obtain a final concentration of 0.1M. For treatment 200µM adenosine was added individually and in combination with 10mM theophylline in MDA-MB-231 cells. Treated cells were subjected to DCFDA assay, trypan blue assay and cell viability assay.

#### 2.26. Cathepsin assay

 $5*10^4$  cells were seeded in 24-well culture plate for growth for 24 hrs at 37°C and 5% CO<sub>2</sub> and then were exposed to theophylline and control NaOH. After 6 hrs incubation, the cells were harvested via trypsinization and protein was isolated using RIPA (Sigma-Aldrich, now part of Merck, Germany) buffer. 5 µg of protein from each sample was plated in 96-well plate with 20 µM Z-LR-AMC fluorogenic peptide substrate (R&D systems, USA) in assay buffer (sodium acetate 0.1M; EDTA 1 mM; DTT 2 mM; BSA 100 µg/ml) and incubated for 30 min. Measurement was carried out using a fluorescence plate reader with excitation at 380 nm and emission at 460 nm (SynergyH1, Biotek, USA). The experiment was carried out in triplicate and repeated three times.

# 2.27. Scratch Assay

Scratch assay is performed in vitro to measure cell migration. It involves 'scratching' the cell monolayer and following the movement of cells by microscope to close the scratch. The TS and TR cells were seeded in 6 well plates at a density of 1x105 cells/well in complete media. After 24hrs, when the cells have reached 90-100% confluency, the media was removed and the wells were scratched with a 200µl pipette tips in absence of any media. The wound was generated and then wells were washed with PBS. After addition of fresh serum free media (to inhibit the proliferation of cells), few photographs of the scratch area were taken for the 0hr reading, then the plate was incubated at 37°C, with saturated humidity and an atmosphere of 5% CO2. At 24hrs of incubation again scratch wound was photographed with Lieca fluorescent microscope for measurement of the gap area.

# 2.28. Western Blotting

After 6 hrs of respective treatments, cells were harvested via trypsinization and protein was isolated using RIPA buffer (Sigma-Aldrich). Cells were incubated on ice for 30 min, in between 3 times sonication was done for 1 min each. The entire solution was centrifuged

at 16000g for 20 min at 4°C. Then the supernatant was collected. The protein extracts were separated by SDS-PAGE at 120V and transferred onto a polyvinylidene difluoride membrane (MerckMillipore, Germany) at 80V for 180min at 4°C. Different specific proteins were detected using antibodies against different genes enlisted in Annexure Table 2.

# 2.29. Data analysis

All comparisons between groups were performed using the unpaired two-tailed t-test using SPSS statistical software (SPSS Inc., Chicago, IL, USA). P value was considered significant at and below 0.05.