Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Cell Lines: The cell lines THP-1 and 3T3-L1 were sourced from NCCS (Pune, India). THP-1 is a well-established human leukaemia monocytic cell line that has become a fundamental tool in investigating the functions of monocytes and macrophages. This cell line is a great model for understanding the mechanisms underlying macrophage development and investigating the regulation of genes specific to macrophages (Chanput et al., 2014).

The 3T3-L1 is a well-established preadipocyte cell line derived from embryonic mice that is capable of differentiating into adipocytes. The cell line was obtained from the 3T3 fibroblast cell line that was originally created from disaggregated Swiss mouse embryo cells. The 3T3-L1 cell line is frequently used as a surrogate model for adipogenesis in primary human preadipocytes, due to the resemblances in the adipogenic pathway shared by these two cell types (Cave & Crowther, 2019).

THP-1 cells were cultivated in Roswell Park Memorial Institute (RPMI) medium or RPMI 1640 and 3T3-L1 in Dulbecco's modified Eagle's media (DMEM), sourced from Gibco, Thermo Fisher Scientific, USA. Both culture media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% penicillin, streptomycin and neomycin (PSN) antibiotic mixture, all from Gibco, Thermo Fisher Scientific, USA. Both cells were grown at 37°C with 5% CO2.

3.1.2 Chemicals and Reagents:

Phorbol 12-myristate-13-acetate (PMA), Lipopolysaccharide (LPS), Sodium palmitate, Insulin (Human, Bovine), 2-Deoxy-Glucose (2DG), Etomoxir, GW9662, Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) and Oil-red-O stain were acquired from Sigma Aldrich, USA. IFNγ was obtained from Gibco, and IL4 was purchased from Pepro Tech. For RNA isolation, the Krishgen FastPrep-R Mini kit was utilized. Essential laboratory supplies, including the MicroAmp Fast Optical 96-Well Reaction Plate, MicroAmp Optical Adhesive Film, Sybr green, Immun-Blot® PVDF Membrane, and Clarity Western ECL Substrate, were sourced from Bio-Rad Laboratories, USA. DMSO, BSA and glucose were procured from Sisco Research Laboratories (SRL), India. Roche Protease inhibitor cocktail tablets were acquired from sigma.

3.1.3 Antibodies:

The antibodies utilized for Western blot and FACS, along with their respective sources, are provided in the tables 3.1 and 3.2.

Table 3.1 Antibodies used in western blot

Sr. No.	Antibody	Source	Dilution
1	Anti-LC3	Cell signalling	1:2000
2	Anti-p62	Cell Signalling	1:2000
3	Anti-pAkt	Cell signalling	1:2000
4	Anti-total Akt	Cell signalling	1:2000
5	Actin	Cell signalling	1:5000
6	Secondary antibodies HRP-conjugated anti-rabbit	Cell signalling	1:5000-10000

Table 3.2 Antibodies used in FACS

Sr. No.	Antibody	Flourescence Tag	Source
1	CD274	FITC	BD Biosciences
2	CD209	PE	BD Biosciences
3	CD36	PerCP-Cy5.5	BD Biosciences
4	ABCA1	PE-Cy5.5	Novus Biologicals
5	CD64	APC	BD Biosciences

3.2 Methodology:

3.2.1 Dissolving Sodium palmitate:

To prepare a 100 mM stock solution, 27.8 mg of Na-palmitate was dissolved in 1 ml of 50% ethanol using a heating block at 60 °C for 10 minutes. Following this, 200 µl of the 100 mM Na-palmitate solution was swiftly added to 2.3 ml of serum-free RPMI containing 5% non-esterified fatty acid BSA at 37 °C, to get 8 mM palmitate solution. Subsequently, the 2.5 ml of BSA-conjugated palmitate solution was dissolved in 47.5 ml of complete RPMI and filter-sterilized to get final concentration of 0.4 mM of palmitate.

3.2.2 Differentiation of THP-1: For differentiation, 500,000 cells per well were seeded in a six well plate and stimulated with 5 ng/ml PMA for 24 hours to generate primed macrophages (M0). Subsequently, these M0 cells were stimulated with LPS (50 ng/ml) and IFNγ (20 ng/ml) for M1 differentiation. While IL4 (20 ng/ml) was used for M2 stimulation. Palmitate (0.4 mM) in combination with Glucose (30 mM) and Insulin (10 nM) was given to induce the MMe phenotype. These stimulations were given for 24 hours. (modified from (Kratz et al., 2014)

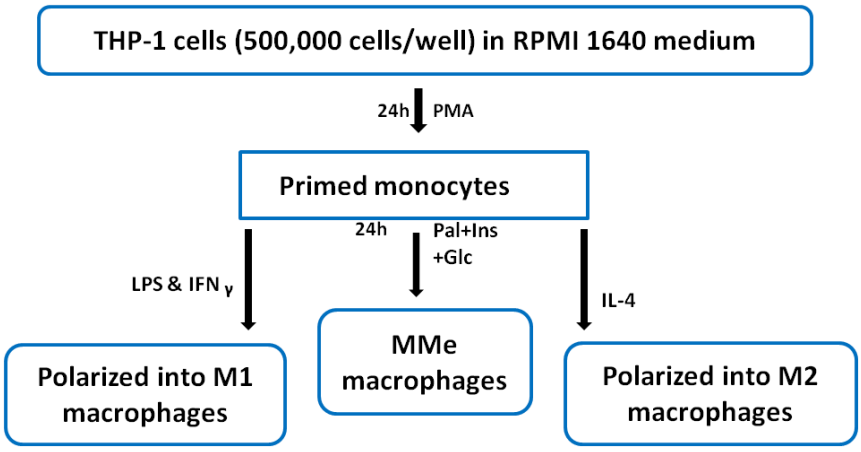

Figure 3.1 Protocol for THP-1 differentiation into M1, M2 and MMe

Treatments for metabolic inhibition: 2-Deoxy-D-Glucose (2-DG) was used for glycolytic inhibition, Etomoxir (Cpt1α inhibitor), was used to block fatty acid oxidation and GW9662 was used for inhibiting PPARγ. All treatments were given along with differentiation stimuli (24 hrs). 2-DG was dissolved in RPMI complete media and filter sterilised. The final

concentration of 25 mM was used for THP-1, M1 and M2. MMe was treated with 30 mM of 2-DG which is equimolar to the glucose concentration in the media. Final concentration used for Etomoxir was 100 µM. GW9662 was used at a final concentration of 10 µM.

3.2.3 Differentiation of 3T3-L1:

Approximately, 40,000 cells were seeded per well in six well plate. When the cells reached approximately 80% confluency (usually after 48 hrs), they were subjected to induction using differentiation media (MDI), which included 0.5 M IBMX (3-Isobutyl-1-methylxanthine), 1 µM dexamethasone, and 2 µg/ml insulin for a duration of 48 hours. Subsequently, the media was aspirated, and the cells were maintained in DMEM with 2 µg/ml insulin until adipocyte differentiation was achieved nearly on day 10.

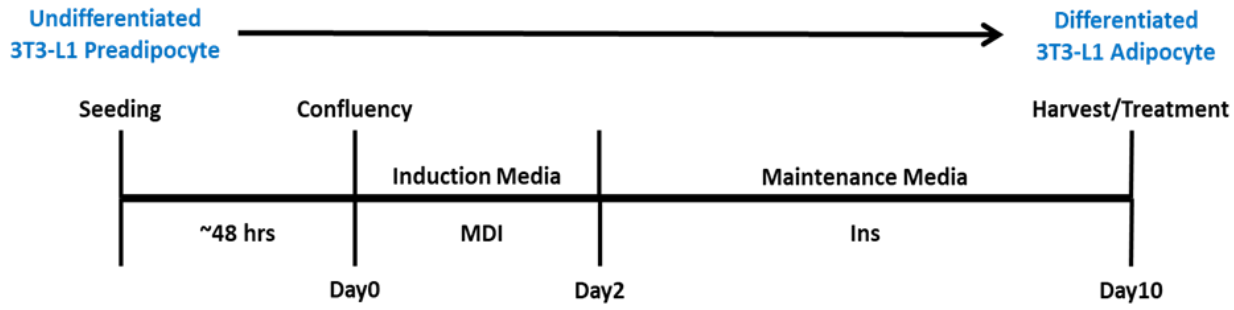

Figure 3.2 Protocol for 3T3-L1 differentiation

3.2.4 Macrophage Conditioned Media (CM) and their treatment to 3T3-L1 derived adipocytes:

For conditioned media collection, THP-1 and differentiated M1, M2, and MMe cells were thoroughly washed with PBS, and allowed to grow in 1 ml of serum-free medium. After 24 hours, conditioned media (CM) was collected and cleared by centrifugation.

On the 10th day of adipogenic differentiation of 3T3-L1 cells, media was removed and replaced with conditioned media (CM) obtained from THP-1, M1, M2 or MMe.

Adipocytes were incubated in this media for 24 hours. This was followed by RNA isolation and expression analysis.

For pAkt analysis, after 24 hours of incubation with CM, cells were washed with PBS and were subsequently subjected to a 2-hour period of serum and glucose starvation. Following starvation cells were treated with insulin (10 nmol/ml) for 30 minutes. Cells were then harvested in RIPA buffer for further evaluation.

3.2.5 RNA isolation, cDNA conversion:

Total RNA extraction was carried out using the Krishgen RNA Fast PrepR-mini RNA isolation kit. Subsequently, 2 µg of RNA was reverse transcribed to synthesize cDNA using Applied Biosystems cDNA synthesis kit.

Table 3.3 2X RT reaction Mix

Component	Volume per 20 µl reaction (µl)
10X RT buffer	2
25X dNTP Mix (100mM)	0.8
10X RT random primers	2
MultiScribe™ Reverse Transcriptase	1
RNase inhibitor	1
Nuclease-free H_2O	3.2
Total per reaction	10

For RT reaction 10 µl RNA is mixed with 10 µl 2X RT reaction mix, and loaded on the thermocycler using following the conditions mentioned below in table 3.4.

Table 3.4 RT-PCR conditions for cDNA synthesis

Settings	Step1	Step2	Step3	Step4
Temp. (°C)	25	35	85	4
Time (minutes)	10	120	5	∞

3.2.6 qRT-PCR:

To assess gene expression, a quantitative PCR (qPCR) reaction was performed using iTaq SYBR green from Bio-Rad, following the manufacturer's instructions. The calculation of fold change was executed using the 2^-ddCt method (Livak & Schmittgen, 2001), with β-actin as an endogenous control.

Table 3.5 RT-qPCR reaction mixture

Component	Volume per 10 µl reaction (µl)
iTaqTM Universal SYBR® Green Supermix.	5
Forward Primer	1
Reverse Primer	1
DNA Template	2
Distilled Water	1

qPCR was performed with a final concentration of 40 ng/µl cDNA.

Table 3.6 RT-qPCR reaction conditions

Step	Temperature(°C)	Duration
Initial Denaturation	95	2 mins
Denaturation x40	95	10 sec
Annealing & Extension	60	35 sec
Melt Curve stage	95→60	60 mins

The details of the primers utilized in the study are provided in table 3.7 and 3.8.

Table 3.7 List of primers sequence used in RT-qPCR (THP-1, human)

Genes	Sequence (5'-3')
β-Actin	Forward- GACGACATGGAGAAAATCTG Reverse- ATGATCTGGGTCATCTTCTC
IFNγ	Forward- GGTAACTGACTTGAATGTCC Reverse- TTTTCGCTTCCCTGTTTTAG
IL6	Forward- GCAGAAAAGGCAAAGAATC Reverse- CTACATTTGCCGAAGAGC
IL1β	Forward- CTAAACAGATGAAGTGCTCC Reverse- GGTCATTCTCCTGGAAGG
TNFα	Forward- AGGCAGTCAGATCATCTTC

	Reverse- TTATCTCTCAGCTCCACG
STAT1	Forward- ACCCAATCCAGATGTCTATG Reverse- GAGCCTGATTAAATCTCTGG
CD319	Forward- CATTGAAGAGAAGAAGAGAGTG Reverse- CTATTAGTGTGAGGGATTGTG
CD38	Forward- CAGACCTGACAAGTTTCTTC Reverse- GATGACATTAAACCACAAGGAG
CD209	Forward-CGTAATCAAAAGTGCTGAGG Reverse- TAGATCTGAAAGTCCCATCC
CD36	Forward- AGCTTTCCAATGATTAGACG Reverse- GTTTCTACAAGCTCTGGTTC
PLIN2	Forward- GTTCACCTGATTGAATTTGC Reverse- GAGGTAGAGCTTATCCTGAG
FABP4	Forward- CAAGAGCACCATAACCTTAG Reverse- CTCATTTTCTCTTTATGGTGG
CD63	Forward- GTTTGCCATCTTTCTGTCTC Reverse- TTCGGGTAATTCTCCATCTG
PKM2	Forward- ATGTTGATATGGTGTTTGCG Reverse- ATTTCATCAAACCTCCGAAC
PFKFB3	Forward- TGGAAGTTAAAATCTCCAGC Reverse- CATAGCAACTGATCCTCTTC
PDK1	Forward- ATGATGTCATTCCCACAATG Reverse- AAGAGTGCTGATTGAGTAAC
Glut1	Forward- ACCTCAAATTTCATTGTGGG

	Reverse- GAAGATGAAGAACAGAACCAG
FAS	Forward- CTGTCCTCCAGGTGAAAG Reverse- TGTACTCCTTCCCTTCTTG
PPARγ	Forward- AAAGAAGCCAACACTAAACC Reverse- TGGTCATTTCGTTAAAGGC
PGC1α	Forward- GCAGACCTAGATTCAAACTC Reverse- CATCCCTCTGTCATCCTC
ACACA1	Forward- CAGTGAAGGCTTATGTTTGG Reverse- CGTCATATGGATGATGGAATC
ACOX1	Forward- AAAGCAGAGGTCCAGG Reverse- CCACAAAATTTGAGTTGCAC
LAMP1	Forward- TAAGAACATGACCTTTGACC Reverse- GTCAGAAGTGTTCTCTTTTCC
LAMP2	Forward- AACAAAGAGCAGACTGTTTC Reverse- CAGCTGTAGAATACTTTCCTTG
ATG5	Forward- AGACCTTCTGCACTGTCCATC Reverse- GCAATCCCATCCAGAGTTGCT
ATG7	Forward- AAATAATGGCGGCAGCTACG Reverse- TCGTTCAGCTTTTGGGTCACC
XBPus	Forward- CAGCACTCAGACTACGTGCA Reverse- ATCCATGGGGAGATGTTCTGG
XBPs	Forward- CTGAGTCCGCAGCAGGTG Reverse- TGGCAGGCTCTGGGGAAG
CHOP	Forward- AGAACCAGGAAACGGAAACAGA

	Reverse- TCTCCTTCATGCGGCTGCTTT
GADD34	Forward- GGAGGAAGAGAATCAAGCCACG Reverse- TGGGGTCGGAGCCTGAAGA
ATF4	Forward- GTTCTCCAGCGACAAGGCTA Reverse- ATCCGTGTTGCTGTTGTTGG
ATF3	Forward- GCACTGCACAGCTCTCTTCT Reverse- AGCATCATTTTGCTCCAGGC

Table 3.8 List of primers sequence used in RT-qPCR (3T3-L1, mouse)

Genes	Sequence (5'-3')
β-Actin	Forward- GCAGGAGTACGATGAGTCCG Reverse- ACGCAGCTCAGTAACAGTCC
AdipoQ	Forward- TGACGACACCAAAAGGGCTC Reverse- ACCTGCACAAGTTCCCTTGG
PPARγ	Forward- GAGGGACGCGGAAGAAGAG Reverse-CACAGGCTCCTGTCAGAGTG
C/EBPα	Forward- CCCTTGCTTTTTGCACCTCC Reverse-GCTTTCTGGTTCTGACTGGGG
FATP1	Forward- GCCAGGGATCTCTCTCTCCA Reverse-GTGCTGGAGCTTGCCTGAT

3.2.6 Cytokine Measurement:

After 24 hours of differentiation stimuli, culture supernatants were collected and centrifuged to remove any debris. TNFα was measured with ELISA using Human TNF-alpha ELISA Kit – Quantikine (R&D Systems, #DTA00D) by following the manufacturer's instructions. Briefly, 50 µl of standards and samples along with 50 µl of assay diluent were added per well and left to incubate at room temperature (RT) for 2 hours. Following the incubation, wells were aspirated and washed with wash buffer 3-4 times. Then 200 µl of substrate solution was added to each well and allowed to incubate in dark at RT for 30 minutes. After incubation, 50 µl of stop solution was added and absorbance was measured at 540 nm after thorough mixing.

3.2.7 FACS:

THP-1 cells were differentiated into M1, M2 and MMe following the protocol mentioned above. After differentiation, media was removed and cells were washed with PBS. The cells were then detached using 5 mM EDTA in PBS on ice, centrifuged, and then resuspended in FACS buffer (2% FBS in PBS). THP-1 cells were spun down, washed with PBS and resuspended in FACS buffer. Subsequently, they were stained with the antibodies CD274 FITC, CD209 PE, CD36 PerCP-Cy5.5 or ABCA1 PE Cy5.5 for 30 minutes in the dark at room temperature. Following the staining procedure, cells were washed with PBS to remove any unbound antibodies, resuspended in FACS buffer and utilized for acquisition using the BD FACSCalibur BD flow cytometer.

The population was first gated with THP-1 cells on FSC vs SSC to exclude the debris. The FSC and SSC were also acquired for all samples. Further, the single fluorophore stained samples were acquired to compensate the overlapping signals. On the compensated setting samples stained with multiple dyes were acquired. At least 10,000 events were acquired within the gate. The acquired data were analysed using Flowing Software.

3.2.8 Oil-red O staining:

100,000 THP-1 cells/well were seeded in a 12 well plate and differentiation protocol was followed as mentioned above. After differentiation, media was removed and cells were

rinsed in PBS followed by fixing with 10% formalin for 60 minutes. After fixation, cells were washed with distilled water and then treated with 60% isopropanol for 5 minutes. Subsequently, isopropanol was discarded and cells were covered with a 0.05% Oil Red O (ORO) solution for 5 minutes. The cells were then thoroughly washed to remove excess stain. Finally, the plate was observed under a microscope (Nikon Ti2E Live Imaging Microscope) to assess the formation of lipid droplets.

3.2.9 Western blotting:

Cell lysis was performed using RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, 5 mM EDTA, 0.4% NP40, 1% Glycerol, and 1x protease inhibitor cocktail). Total protein content in the lysates was quantified using the BCA method. Equal amounts of proteins were then separated on a 10% SDS-PAGE gel, or for LC3 western blotting on a 15% gel. The proteins were subsequently electro-blotted onto a PVDF membrane (Immun-Blot® PVDF Membrane, Bio-Rad, USA) at 100 V for 75 minutes. Following electroblotting, the membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) for 1 hour at room temperature. It was then incubated with the primary antibody overnight at 4 °C. After incubation, the membrane was washed three times with TBS-T (TBS containing 0.1% Tween-20) and subsequently incubated with a secondary antibody for 1 hour at room temperature. Protein bands were detected using Clarity Western ECL Substrates (Bio-Rad, USA) and visualized using the ChemiDoc MP Imaging System.

3.2 .10 Mass Spectrometry:

Protein Precipitation (TCA method): A total of 4 million THP-1 cells were seeded in a 100 mm petri plate and subsequently differentiated into M1, M2 and MMe following the previously stated protocol. Cells were then washed three times with 1X PBS after 24 hours of differentiation. Subsequently, a volume of 1 ml of Radio immunoprecipitation (RIPA) lysis buffer, containing both buffer and a protease inhibitor cocktail, was added onto the cells. Next, the cells were placed on ice and incubated for a duration of 15 minutes, while periodically swirling the dish to ensure the buffer is evenly distributed. Following a duration of 15 minutes, the cells were gently removed using a cell-scraper and collected in a micro-centrifuge tubes. The cells were then sonicated to disrupt the cell membranes and get the

complete cell lysate. Following sonication, the samples were spun at 15,000 g for 20 minutes at 4 ℃ and supernatant was collected in a new tube.

The proteins were precipitated by adding 100% (w/v) trichloroacetic acid (TCA) at a ratio of 1:4 (TCA volume to cell lysate volume) and tubes were inverted repeatedly until white precipitates become apparent, and then, the tubes were left to incubate overnight at -20 ℃. The pellet was thawed on ice and then the tubes were centrifuged at a speed of 15000 rpm for a duration of 10 minutes. The pellet was subjected to three washes with 500 µl of Acetone in order to remove residual TCA and other contaminants.

Protein Digestion and Sample preparation: The precipitated protein samples were dissolved in 6 M urea at a temperature of 35 ℃ while continuously shaking for 1hr and protein estimation was carried out by BCA kit (Sigma). Subsequently, 10µg of protein were reduced using 1,4-Dithiothreitol (DTT) (final conc. 10 mM) and alkylated with iodoacetamide (final conc. 37 mM). The chemical reactions were stopped by adding 30 mM of dithiothreitol (DTT). These linearized proteins were diluted with 50 mM ammonium bicarbonate to reduce urea concentration to 0.6 M and pH to 7.8, parameters optimum of trypsin activity. Samples were then subjected to overnight trypsin digestion at a ratio of 1:5 enzyme to proteins. The reactions were stopped by adding 2 µl of trifluoroacetic acid. The samples were condensed using vacuum centrifugation to a final volume of 100 µL. The peptides were then desalted using custom-made stage tips that were constructed by attaching Empore™ C-18 discs and pre-treated with acetonitrile. Stage tips were conditioned using 100 % acetonitrile (ACN) and pre-equilibrated with 0.1% formic acid in water. Then the acidified samples were loaded and passed through the stage tips three times to ensure peptide retention. Samples were washed with 0.1% formic acid three times, before their extraction using 50% acetonitrile in 0.1% formic acid. For LC-MS analysis, the samples underwent drying in a vacuum centrifuge to remove ACN and were then dissolved in 100 µl 0.1% formic acid.

LCMS Analysis: The desalted peptides were separated using an Ultimate 3000 RSLC nano-UPLC system (Thermo Fisher Scientific) that was connected to an Orbitrap Elite hybrid mass spectrometer. The automated injections were used to load 500 ng of peptides onto a trap column (Acclaim PepMap™ 100, 3 µm particle size, 75 µm × 2 cm) for a duration of 4 minutes. The flow rate was set at 5 µL/min with 0.1 % formic acid. The peptides were then eluted from the trap column gradually by increasing the concentration of acetonitrile. The

samples were separated on a C-18 analytical column (PepMap™ RSLC, 2 µm particle size, 100 Å pore size, 75 µm × 50 cm) at a flow rate of 250 nL/min. The solvents used for peptide separation were solvent A, which consisted of 0.1% formic acid in water, and solvent B, which consisted of 0.1% formic acid in 100% acetonitrile (ACN). Following a 10-minute equilibration period, a rapid increase in concentration from 1% to 5% B was run for 3 minutes. Subsequently, it was gradually increased in concentration from 5% to 22% B over a period of 135 minutes, which was further extended to reach 28% B in an additional 20 minutes and quickly raised to 90% B for column cleaning.

The temperature of the analytical column was set to 40 °C and it was directly linked to a nanospray electron ionisation source (Thermo Fisher Scientific) that produced a consistent spray at a voltage of 1.8 kV. The capillary temperature was adjusted to 275 °C to provide efficient nebulization and facilitate the movement of peptide ions into the mass spectrometer. The data were procured in the positive mode utilising data-dependent acquisition (DDA). The top twenty most prevalent parent ions within each MS1 spectra, ranging 350 to 2000 m/z, were consecutively separated and subjected to fragmentation using collision-induced dissociation (CID). The MS1 and MS2 spectra were obtained using the Orbitrap at a resolution of 60,000 and the ion trap in rapid scan mode, respectively. The maximum duration for filling MS1 was limited to 100 ms, with a maximum of 1 million ions. While, for MS2 spectra, the filling period was limited to 50 ms, with a target of 5 thousand ions. The peptides were fragmented using a normalised collision energy of 35%, and an activation duration of 10 ms. A dynamic exclusion of 30 seconds was provided, with a single repeat count.

Protein Identification: The raw data for all the samples were collectively analysed using Maxquant (version 2.0.3.0). The Uniport human proteome (UP000005640) was used as a reference to identify the peptides using the internal peptide search engine Andromeda. The peptide and protein identifications were determined using the following parameters: The maximum number of missed cleavages allowed was 2. The mass tolerance for the first and main search was 20 and 4.5 ppm respectively. The mass tolerance for fragment ions was 0.5 Da. The variable modifications used were N-terminal acetylation and methionine oxidation. At least 1 unique peptide was required for identification, and the minimum peptide length for identification was 7 amino acids. The maximum peptide mass allowed was 4600 Da. The peptide spectrum match (PSM) identification and protein inference false discovery rate were

kept at 0.01. Dependent peptide and match between run options were allowed with a match time window of 0.7 min and an alignment window of 20 min. The iBAQ mode was activated to quantify proteins.

3.2.11 Pathway Analysis:

"VENNY 2.1" was used to get the list of unique proteins among each subtype. Further, the unique proteins were used to analyze the pathways enriched. For enrichment analysis, "Enrichr" was used (https://maayanlab.cloud/Enrichr/). "**BioPlanet**" library was used to get the pathways enriched from the input set of genes. The genes involved in those pathways were visualised through bar graph and clustergrammer with rank based on their enrichment score. Clustergrammer were plotted using python. For PPi network string was used. (https://string-db.org)

3.2.12 Statistical Analysis:

All data in this study are presented as mean ± SD for n observations. Group comparisons were conducted using the unpaired Student's t-test to determine differences in means, and GraphPad Prism 8 software was utilized for statistical analyses. Results were considered statistically significant when the p-value was less than 0.05. For normalized graphs, the data was normalized with the maximum value set as 100% and minimum value as 0% for all datasets. The obtained normalized values were graphically represented.