International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Int. J. of Life Sciences, 2018; 6 (1):143-149
ISSN:2320-7817(p) | 2320-964X(o)
UGC Approved Journal No 48951

Original Article Open Access

# THP1 cell proteome library generation using SWATH analysis.

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**Manuscript details:**

Received : 30.11.2017
Accepted : 03.02.2018
Published : 06.03.2018

**Editor: Dr. Arvind Chavhan**

**Cite this article as:**
Hamza Baseerat, Kumar Ajay, Midha Mukul, Sharma Anil, Bupender Kumar Sharma and Rao Kanury (2018) THP1 cell proteome library generation using SWATH analysis, *Int. J. of Life Sciences*, Volume 6(1): 143-149



Available online on
http://www.ijlsci.in
ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

**ABSTRACT**

THP-1 cell line is considered as an important model cell line to study the biology of the monocytes/ macrophages. The cell line is used to study the progression of various diseases such as, diabetes, tuberculosis and cancer. Advent of proteomics has revolutionized the discovery of new proteins by data independent acquisition (DIA) approach as well as the diagnosing the role of the novel or already known proteins in the cell biology by data dependent acquisition (DDA) approach. Recently, Sequential window acquisition of all theoretical mass spectra (SWATH) is introduced as a new Proteomics approach that carries the advantages of both the DIA as well as the DDA.Therefore a reference library has been generated for SWATH analysis and presented in the manuscript.

**Key words**: Macrophages, Mass Spectrometry, Proteins, Reference Library, SWATH analysis, Thp1 cell line.

## INTRODUCTION

The strength of the immune system of a body is an important determinant of the human health. Monocytes and their derivative macrophages are backbone of an immune response. They perform diverse functions ranging from the synthesis of immune effector molecules to the phagocytosis of a pathogen or a cell undergoing apoptosis. In addition to their contribution to the protection of the body, they are contributories to the degenerative diseases and inflammatory pathogenesis.

The macrophages are the center of attention of immunologists not just for pure sciences but also as a therapeutic target. THP-1 cell line is considered as an important model cell line to study the biology of the monocytes/

macrophages. Although a number of differences occur between the macrophages and THP-1 cells (Bruckmeier *et al*., 2012; Schildberger *et al*., 2013), there is no dearth of literature suggesting it as an important model cell line (Gao *et al*., 2000; Schroecksnadel *et al*., 2012; Mehta *et al*., 2010). THP-1 cell line is used to study the disease biology of tuberculosis (Thesus *et al*., 2004; Benjawan *et al*., 2015), diabetes (Nandy *et al*., 2011; Qin, 2012) and cancer (King *et al*., 2006).

Advent of -omics in biological sciences has revolutionized the pace of scientific discoveries. Large scale data production and its analysis enable us to visualize the whole system collectively in a qualitative as well as quantitative fashion. Proteomics has advantage over the genomics and transcriptomics, since all genes do not transcribe RNA and all RNAs may not necessarily code for proteins (Pandey and Mann, 2000) owing to various post transcriptional regulations.

Mass spectrometry (MS) in data-independent acquisition mode, is the method of choice for exploratory proteomics whereas, the Liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) in data-dependent acquisition mode (DDA) has been extensively used for identification of proteins in complex samples. Thp1 cells have been subjected to proteome analysis using Data independent acquisition (DIA) for wide quantification of target protein (Shi *et al*., 2016). Recently, Sequential window acquisition of all theoretical mass spectra (SWATH) is introduced as a new Proteomics approach that carries the advantages of both the DIA as well as the DDA (Schubert *et al*., 2015).

Analysis of Thp1 cell for understanding the biology using SWATH would warrant a reference library of native Thp1 cells. Very recently a comprehensive library of 10000 proteins from human was created (Rosenberger *et al*., 2014). The library was created from various human tissues and cell lines including Thp-1 cell lines. However, So far no reference library is available for Thp-1 cells alone. The present attempt was thus, made to generate the reference library for SWATH analysis of Thp1 cells.

## METHODOLOGY

### Culture media, reagents, buffers, kits and other chemicals

RPMI-1640, Trizol Invitrogen (Carlsbad, USA). Fetal Bovine Serum (FBS) (Hyclone, USA). Middlebrook 7H9/7H11 media, Albumin-Dextrose-Catalase (ADC), Oleic acid-Albumin-Dextrose-Catalase (OADC) supplement, HEPES: Difco-Becton-Dickinson (San Diego, USA). Phorbol 12-Myristate 13-Acetate (PMA), Endoplasmic Reticulum Isolation Kit, #- ER0100: Sigma-Aldrich (St. Louis, MO, USA). Phosphatidyl Ethanolamine – Lot #: LM1-013B, Phosphatidyl Serine – Lot #: LM1-023C, Phosphatidyl Choline – Lot #: LM1-029C, Phosphatidic acid – Lot #: LM1-025E, Phosphatidyl Glycerol – Lot #:LM1-024C, Phosphatidylinositol – Lot #: LM7-130B, Phosphoinositolbisphosphate – Lot#: 181PI (3, 4) P2.

For proteomics work, mass spectrometry (MS) grade material was used throughout the study. Methanol, chloroform, Acetonitrile (CAN) and Water with 0.1% Formic acid were supplied by Sigma (St.Louis, MO, USA).

### Cell lines and culture conditions

THP-1 cell line, a human monocyte leukemia cell line, was derived from a 1-year-old boy with acute monocytic leukemia (Tsuchiya *et al*., 1980). The cells were cultured in RPMI 1640 supplemented with 10% FBS at a density of 2-10 × $10^5$ cells/ml at $37^0$C in a humidified, 5% $CO_2$ atmosphere. This cell pool was harvested and lysed. The lysate was then lyophilized and the proteins were re-suspended in 100 mM ammonium bicarbonate buffer. The proteins were then subjected to denaturation and reductive alkylation prior to digestion with a combination of Lys-C and trypsin. The peptides, thus obtained were analyzed by 2D-LC-MS/MS as described below.

### Strong Cation Exchange

The first-dimensional separation of the peptides was achieved by cation exchange (SCX) chromatography using an SCX Cartridge (5 microns, 300 Å bead from Sciex, USA), using a cartridge holder (Sciex, USA). The pooled and dried peptides were reconstituted in 1 mL Buffer A (5mM ammonium formate, 30 % (v/v) CAN and 0.1% FA; pH= 2.9) and applied to the cartridge using a hand syringe set up. The samples were fractionated using a step gradient of increasing concentration of ammonium formate (50 mM, 80 mM, 100 mM, 150 mM, and 250 mM ammonium formate, 30% v/v CAN and 0.1% formic acid; pH= 2.9) into five different fractions.

**NanoLC-Mass Spectrometry Analysis:** All the five fractions obtained after fractionation on SCX column were analyzed by reverse-phase HPLC-ESI-MS/MS using a Nano LC-Ultra 1D plus (Eksigent), which was directly connected to an SCIEX quadrupole time-of-flight (QqTOF) TripleTOF 5600 mass spectrometer (SCIEX,

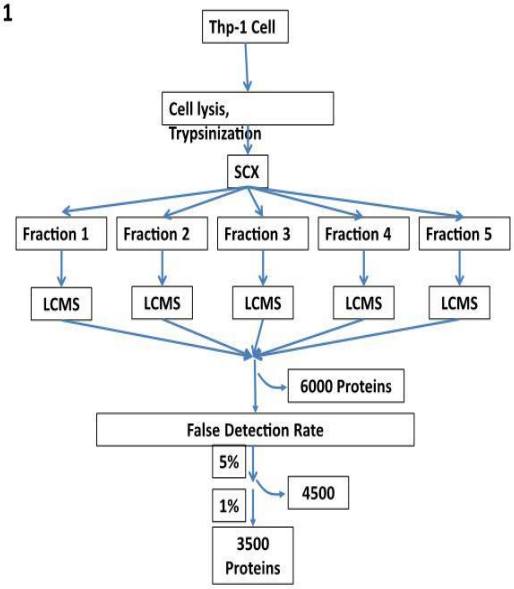
Concord, CAN). Reverse Phase-HPLC were performed via an elute configuration using one Monocap C18 High Resolution (LCGC Sciences, Japan) 200cm long column set up for better separation, with the high spectral counts. Reverse-phase LC solvents included mobile phase A: 2% acetonitrile/ 98% of 0.1% formic acid (v/v) in water; and mobile phase B: 98% acetonitrile/ 2% of 0.1% formic acid (v/v) in water. The auto-sampler was operated in full injection mode overfilling a 1 µl loop with 3 µl analyte for optimal sample delivery reproducibility. Each fraction was reconstituted in 4 µl of mobile phase A, containing 1x iRT (act as Retention Time calibrant) and 200 femtomole β-galactosidase (as an internal standard) and eluted from the analytical column at a flow rate of 400 nL/min for 6 hrs using a linear gradient. The linear gradient mode comprised of 5% solvent B to 50% solvent B over a duration of 275 min. Auto-calibration of the spectra is performed after acquisition of every injection using dynamic LC–MS and MS/MS acquisitions of 50 femtomol β-galactosidase.

Mass spectra and tandem mass spectra were recorded in positive ion and "high-sensitivity" mode with a resolution of ~35,000 full-width half-maximum. Peptides were injected into the mass spectrometer using 10µm SilicaTip electrospray PicoTip emitter (New Objective Cat. No. FS360-20-10-N-5-C7-CT), and the ion source was operated with the parameters: ISVF = 2200; GS1 = 20; CUR = 25.

**Generating Reference Spectral Library**

All DDA mass spectrometry files were searched in Protein Pilot software v. 5.0 (AB SCIEX, Revision- 4769) with the Paragon algorithm. For Paragon searches, the following settings were used: Sample type- Identification; Cysteine Alkylation- Iodoacetamide; Digestion- Trypsin; Instrument- TripleTOF5600; Species- None; Search effort- Thorough ID; Results Quality- 0.05. Only peptides with a confidence score of >0.05 were considered for further analysis. The search was conducted using a thorough identification effort of a UniProt Swiss-Prot database (January 2015 release) containing Homo sapiens species specification along with iRT peptides and β-galactosidase FASTA sequences. False discovery rate analysis was also performed. The output of this search is a .group file, which may be used as the in-house generated reference spectral library. The .group file contains the following information that is required for targeted data extraction: protein name and UniProt accession, cleaved peptide sequence, modified peptide sequence, relative intensity, precursor charge, unused Protscore, confidence, and decoy result. The parameters used for identification of proteins include: 1) Threshold of 1% accepted Global False discovery rate (G-FDR) proteins; 2) at least one unique peptide with 95% confidence (**Supplementary Fig. 1**). The false positive rates of the aforementioned filter criteria were all below 1%, estimated by using an individual reversed (decoy) sequence database. In brief, false positive rates were calculated by dividing the number of decoy hits by that of hits acquired in search against the forward sequence database.

Figure 1




**Supplementary Fig. 1**

All DIA files were loaded and exported in a .txt format using an extraction window of 10 min and with the following parameters: 3 peptides per protein, 6 transitions per peptide, peptide confidence of >95%, 10% G-FDR, exclude shared peptides, and XIC width would be set at 50 ppm. This export would results in the generation of three distinct files containing the quantitative output for (1) the area under the intensity curve for individual ions, (2) the summed intensity of individual ions for a given peptide, and (3) the summed intensity of peptides for a given protein. This protein data may be used for all data analysis in the Marker view software. The reversed sequences were removed from the dataset prior to further analysis.

## RESULTS

THP-1 cell lines are widely used model to study the biology of macrophages (Auwerx, 1991). Proteome

study of the Thp-1 cells using SWATH analysis would need a reference library for comparison. A comprehensive in-house protein library of nearly entire expressed THP1 cell proteome was, thus, created with the help of discovery-driven MS (Figure 1). For the purpose, the proteins were extracted fron the Thp1 cells and were trypsin digested. The peptides, thus, obtained were purified by 2-dimensional strong cation exchange (SCX) to capture broad spectrum. Purified proteins in five different fractions were then subjected to reverse-phase HPLC-ESI-MS/MS coupled with triple TOF.

Each fraction was analyzed repeatedly to increase the proteome coverage since the protein coverage was very less in a single run owing to the stochastic nature of discovery of MS. The repetitive analyses led to the increase in the number of the proteins identified. A total of 5281 proteins were identified. The data analysis acquired on the five fractions of THP1 proteins confirmed predicted that 95% of the MS-observable proteome was reached.

The peptides with a confidence score of >0.05 in Protein Pilot were considered for further analysis. False discovery rate (FDR) analysis was also performed. The 5% FDR analysis filtering of the proteome the proteins reduced the number to 4500. Nearly 1000 proteins were further filtered out by employing more stringent analysis of 1% FDR. This strategy allowed us to assign fragment ion spectra to peptides mapping to (77%) of the annotated 3765 proteins of THP1 cells at a protein false discovery rate (FDR) of < 1%.

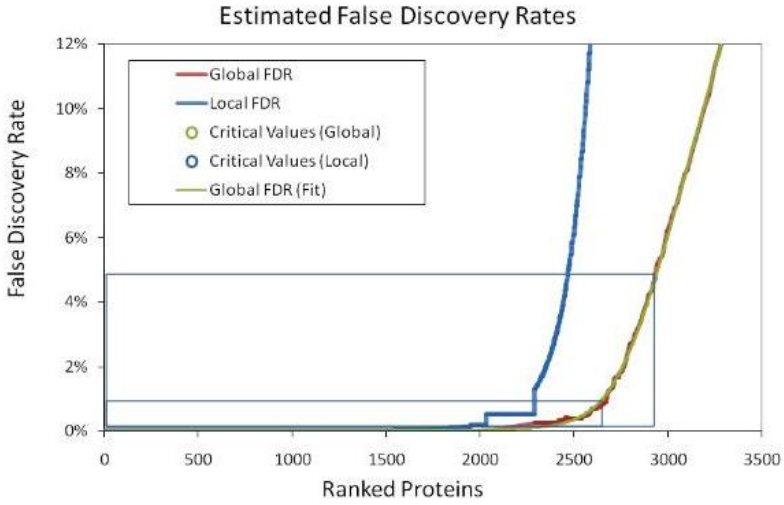



**Fig. 2:** Estimated discovery rates

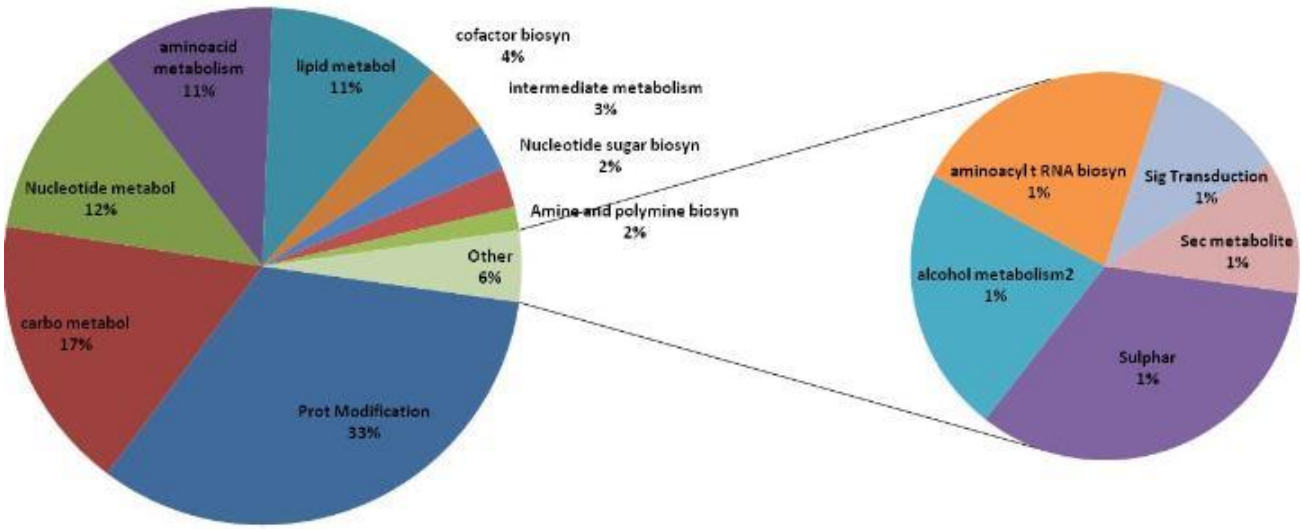



**Fig. 3:** Pathway analysis of total proteins mapped in THP1 cell

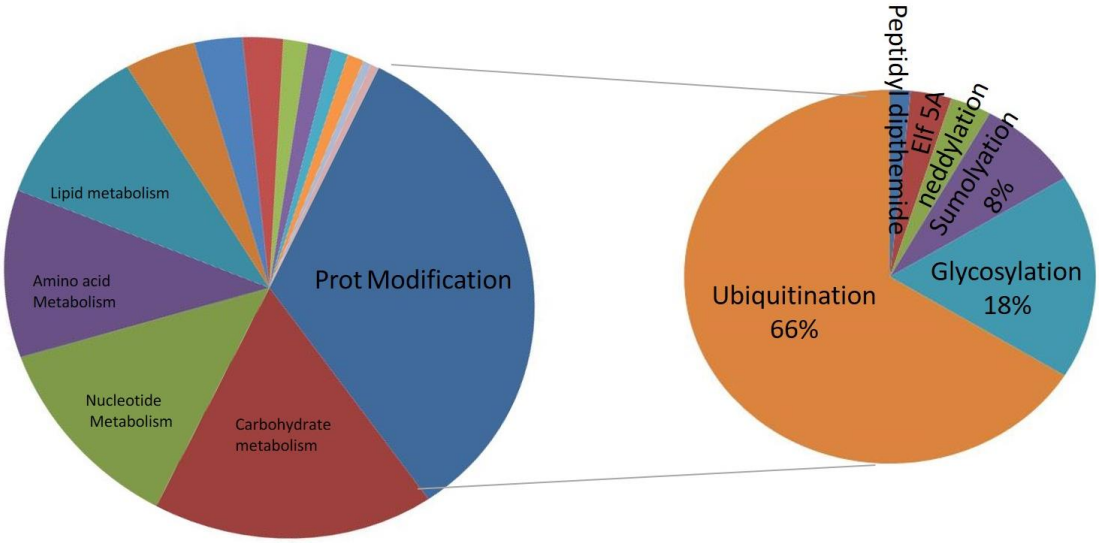



**Fig. 4.** Protein modification breakup

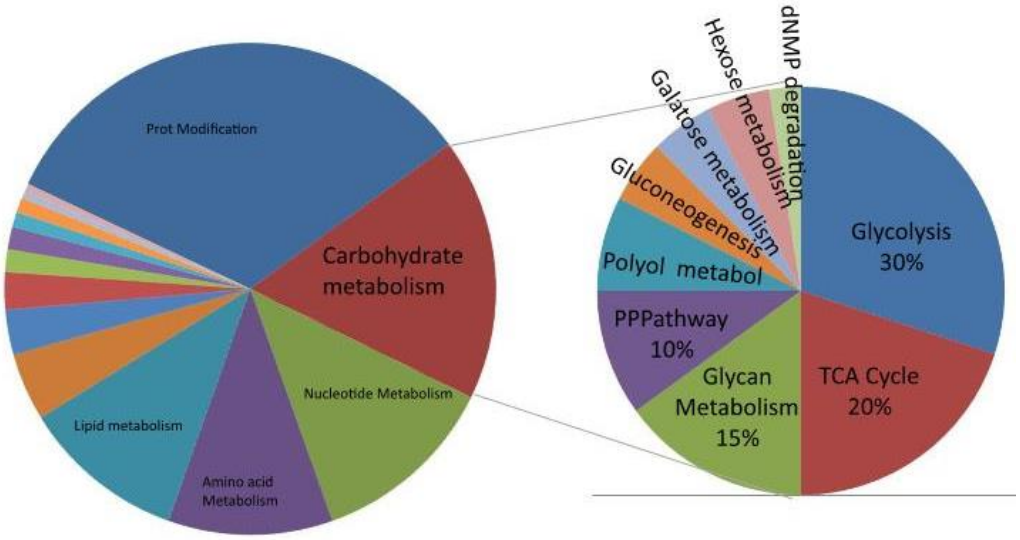



**Fig. 5:** carbohydrate metabolism breakup

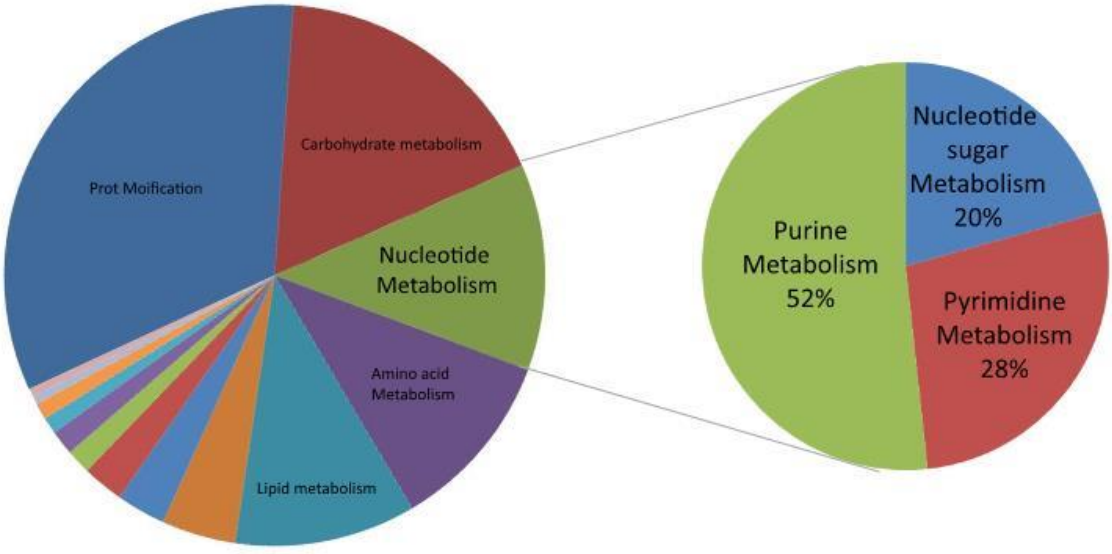



**Fig.6:** Nucleotide metabolism breakup

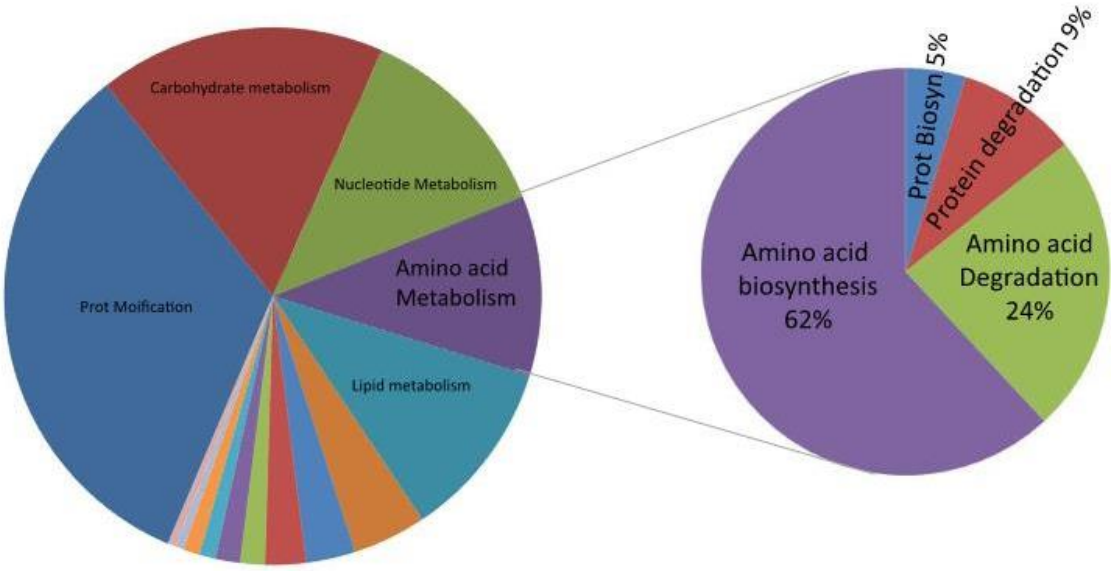



**Fig.7**: Amino acid metabolism breakup

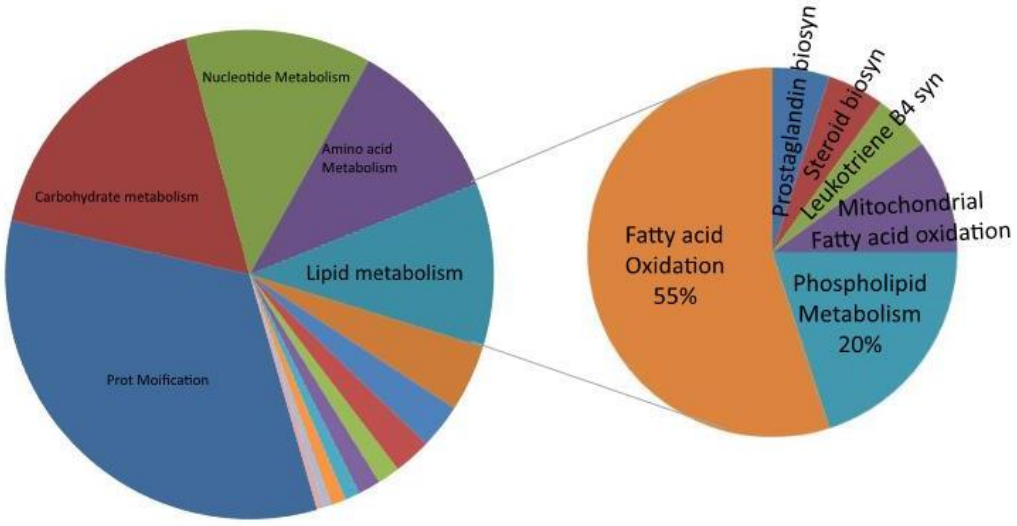



**Fig. 8:** Lipid metabolism breakup

From the pathway map (Figure 2), it becomes apparent that majority of the proteins (33%) are involved in the modifications of other proteins followed by the proteins involved in the central carbon metabolism, including the carbohydrate metabolism (17%), lipid (11%) and amino acid (11%) metabolism. A fair share of proteins belonged to the nucleotide metabolism (12%). Rest of the proteins belonged to the cofactor biosynthesis, intermediate metabolism, amine-polyamine biosynthesis, aminoacyl tRNA biosynthesis, alcohol metabolism, sulphar metabolism, secondary metabolites and signal transduction.

Among the proteins involved in the modification of other proteins, protein involved in ubiquitination dominated the group with nearly 66% abundance, followed by those involved in the glycosylation and sumolyation of proteins. Similarly, Carbohydrate metabolism breakup revealed that majority of proteins belonged to the Glycolysis pathway (30%) followed by the TCA cycle (20%), Glycan metabolism (15%) and Pentose phosphate pathway (10%). Other proteins in carbohydrate metabolism belonged to the polyol metabolism, gluconeogenesis, galactose metabolism, hexose metabolism and dNMP degradation. Among the proteins involved in the carbohydrate metabolism important proteins such as Citrate synthase, involved in mitochondria dysfunction due to diabetes (Sivitz and Yorek, 2010) and fumarate hydratase and succinate dehydogenase involved in tumor suppression (King *et al*., 2006).

Among the proteins involved in the amino acid metabolism 62% were involved in the amino acid

biosynthesis while 24% belonged to the amino acid degradation. Protein degradation and biosynthesis contributes 9% and 5% of the proteins identified. Among the proteins involved in the amino acid metabolism, vimentin is reported to be involved in the binding of NKp46 to Mtb H37Ra infected monocytes (Garg *et al*., 2006).

Among the proteins involved in the lipid metabolism, 55% have role in fatty acid oxidation and 20 % in phopholipid metabolism. Among proteins involved in nucleotide metabolism majority (52%) were involved in the purine metabolism while 28% were involved in the pyrimidine metabolism. The rest of the 20% proteins were mapped to the nucleotide sugar metabolism.The Thp1 cell line is an important model for understanding the biology of macrophages. This library would open the avenue for the proteomics studies of the Thp1 cell line. Very recently a comprehensive library of 10000 human proteins was created (Rosenberger *et al*., 2014). The library was created from various human tissues and cell lines including Thp-1 cell lines. However, So far no reference library is available for Thp-1 cells alone. The present reference library is already in use for in-house studies in our laboratory to understand the interaction of Thp-1 with infected diseases such as Mtb using SWATH technique (Communicated elsewhere).

## CONCLUSIONS

The one time generation of MS reference maps for each component of whole canonical or proteomes will help in the successful application of SWATH-MS and facilitate the analysis of multiple pathways for any given disease.

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