1.1 **Background:**

The lack of accurate and rapid diagnostic tests is the limiting factor for the battle against tuberculosis (TB) which is a leading cause of death worldwide ***(Raviglione M et al., 2012)***. Incidences of TB and its mortality rate have been falling since 1990, but the global burden remains substantial due to the slow rate of decline in TB incidence (2% per year) (***Eurosurveillance editorial WHO.2013***).

Sputum smear microscopy of acid-fast bacilli (AFB) and culture of Mycobacterium tuberculosis (MTB) have been widely used for diagnosis of active TB (***Tuberculosis Division International Union 2005***). However, AFB smear microscopy has limited sensitivity (50%-60%) in diagnosing pulmonary TB (PTB) and is time consuming (***Lim J et al., 2014***). The low specificity of chest X-rays used for the diagnosis of smear-negative TB risks over diagnosis (***Kivihya-Ndugga L et al., 2004***). Along with the increased incidences of PTB, the incidence of extra pulmonary TB (EPTB) has also increased. Diagnosis of EPTB by culture is particularly difficult because of the low load of organism in the body fluids of EPTB patients (***Moure R et al., 2012; Tay TR et al., 2013***).The sensitivity of AFB smear microscopy decreases further in detecting bacilli in cerebrospinal fluid (CSF) of Tuberculous meningitis (TBM) patients ***(Thwaites GE et al., 2004b)***. Recently launched Xpert MTB/RIF test have reported promising results in specific population ***(Durovni B et al., 2014***) however, have certain major limitations including high cost, requirement for a continuous electricity supply and short shelf life of consumables ***(Trebucq AD et al., 2011).*** Various immunological and molecular assays have been widely evaluated for the diagnosis of PTB and EPTB cases which

includes majorly TBM, Pleural TB (PlTB) and TB ascites (TBA). Although these methods are quite beneficial for the diagnosis of both forms of TB, however they either suffer from less sensitivity (***Steingart KR et al., 2007a***) or high cost ***(Kashyap RS et al., 2007c)*** in routine diagnostic laboratories. Test such as the adenosine deaminase assay (ADA) has been used as supportive test in TB diagnosis as it is unable to differentiate between TB other infectious diseases (***Ho J et al., 2013; Tuon FF et al., 2010***). Therefore, there is an urgent need to identify new markers which can help in diagnosis and in understanding the pathogenesis of PTB and EPTB.

Proteomics has emerged as a powerful approach for identifying TB disease biomarkers and has become one of the essential tools in biomarker discovery ***(Kumar GS et al., 2012; Kashyap RS et al., 2010d ; Kashyap RS et al., 2005b).*** Therefore, an effort was made to identify new biomarkers which can differentiate between TB (PTB &EPTB) and non TB patients using One &Two Dimension Polyacrylamide Gel Electrophoresis (1D/2D-PAGE) and Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Main objective was to identify biomarker which can discriminate between TB (PTB &EPTB) and non TB patients and can be further developed as diagnostic tool for TB infection.

1.2 MATERIALS AND METHODS

1.2.1 Settings

Central India Institute of Medical Sciences (CIIMS), Nagpur is a 114-bed tertiary referral hospital in Maharashtra, India. It is referral hospital for more than 15000 people per year. Mostly patients from Chhattisgarh, Madhya Pradesh and Vidarbha prefer this hospital for medical examinations. In the year 2014-2015, there were 4469 admissions in the emergency room and 7689 for outpatient department. The number of TB patients admitted with TB has changed over time. In the very first year of the hospital, there were about 125 patients per year with TB, which gradually increased to about approximately 700-800 per year. All the information of TB patients were collected and stored in the hospital record.

1.2.2 Patient selection and samples collection

A total of 999 patients (TBM, TB, PlTB, TBA and their respective controls) were recruited for the entire study. Out of 999 samples, 281 samples were analysed for Proteomic study (Chapter 1), 522 samples were evaluated for Heat shock proteins (Chapter 2), 182 samples were evaluated for Cytokines (Chapter 3) and 14 samples were evaluated for toll like receptor (TLR) induced cytokines (Chapter 5).

A total of 281 TB (PTB &EPTB) suspected patients who were admitted were enrolled in this study. Out of 281, 105 belong to PTB/Non TB and 176 belong to EPTB/Non EPTB group as per the information given at the time of admission in the hospital. Out of 105 samples, 65 belong to PTB group and 40 belong to non TB group. Out of 176 EPTB samples, 75 samples belong to

TBM, 30 samples belong to non TBM, 28 samples belong to Pleural TB (PlTB) , 33 samples belong to non TB, 05 samples belong to Tuberculosis Ascites (TBA) and 05 samples belong to non TB patients.HIV positive patients and other with the evidence of immunosuppressive therapy were not included in the study. Similarly pregnant females and those in lactation period were also excluded from the study.

Details of any prior Tuberculin skin test (TST), presence of underlying illnesses, infections experienced in the last 3 months, medication and history of previous TB or anti-TB treatment were also recorded. Bacillus Calmette–Guérin (BCG) vaccination status was assessed based on examination of BCG scar on left forearm.

All the recruited patients were classified into following group:

1.2.2.1 Pulmonary Tuberculosis (PTB) cases

Out of 105 collected blood samples, 65 belong to TB group and 40 belong to non TB group.

PTB patients (n=65)

PTB was confirmed if cultures of sputum specimens were positive for MTB. To diagnose active PTB, sputum microscopy was done on two serial sputum samples by staining with Ziehl Neelson (ZN) Stain as per the guidelines of India's Revised National Tuberculosis Control Programme (RNTCP). Final cultures of sputum samples on Lowenstein-Jensen medium obtained after 6 weeks were positive for MTB. When acid fast bacilli (AFB) staining and culture tests were negative, the diagnosis of PTB was made on the following clinical observations: 1) low grade fever for 2-3 weeks, 2) loss of appetite, 3)

abnormal chest X-ray, 4) weight loss, 5) night sweats, 6) chronic cough with or without expectoration/hemoptysis; 7) chronic chest pain for 2-3 weeks, and 8) past history of TB.

Non PTB patients (n=40)

This group includes those patients which were admitted to the hospital with defined acute or chronic non-PTB diseases including neurological disorders, bacterial infections, lung cancer, pneumonia, gastrointestinal infections, bronchitis and non-specific fever.

Serum samples

Venous blood was collected from all the patients. Blood was allowed to clot, and after centrifugation (1000 × g, 10 min) serum was separated and refrigerated at 4°C for further analysis.

1.2.2.2 Tuberculous meningitis (TBM) cases

CSF samples were obtained from 105 patients, including 75 TBM patients admitted to the Department of Neurology and Neurosurgery at the CIIMS, Nagpur. The remaining 30 patients which were included in the study had non-tuberculous nervous system disorders and thus considered as non TBM cases (NTBM).

TBM patients (n=75)

TBM was confirmed by the presence of MTB in CSF by staining and/or culture. When AFB staining and culture tests were negative , TBM was diagnosed on the basis of a) clinical symptoms consisting of subacute onset of mild to moderate fever, headache, vomiting, neck stiffness, and Kernig's sign, b) CSF showing elevated protein and lymphocytes, decreased glucose

and CSF/Blood glucose ratio, and c) good clinical response to anti-tuberculin medication. A history or family history for TB was explored with all patients.

Non TBM patients (n=30)

All other patients who had no evidence of central nervous system (CNS) or extra CNS bacterial or viral infections were grouped in the non infectious disease control group. Patients in this group had chronic intractable headache, epilepsy, stroke and others disorders.

Cerebrospinal fluid (CSF) samples

CSF was collected by lumbar puncture in all recruited patients. Besides the routine microscopic and biochemical analysis, staining was done in all cases with gram stain, AFB, and India ink. Samples obtained from all the patients were stored at -20 °C until further analysis.

1.2.2.3 Pleural Tuberculosis (PlTB) cases

Pleural fluid samples were obtained from 61 patients, including 28 PlTB patients admitted in CIIMS, Nagpur. The remaining 33 patients which were included in the study had non-TB disorders and thus considered as non TB cases.

PlTB patients (n=28)

PlTB was confirmed by the presence of MTB in pleural fluid by staining and/or culture. In the absence of confirmation, the patients with following clinical manifestations suggestive of TB were included. a. presence of productive cough b. low grade fever c. night sweat d. weight loss e. chest pain f. biochemical findings showing increased protein decreased glucose and/or pleocytosis with lymphocytic predominance. g. Ultrasound

Radiographic features supporting the clinical diagnosis considered were lung parenchymal infiltration mainly involving apical and/or mid zone, miliary shadows and pleural effusion. Along with the above mentioned clinical features any one radiological feature was considered sufficient as supportive evidence.

Non PlTB patients (n=33)

All other patients who had no evidence of MTB infection but having non tuberculous pleural effusions were grouped in the non Pleural TB group.

Pleural Fluid samples

Approximately 4ml of pleural effusions were collected under aseptic conditions from the patients enlisted for the study and were subjected to decontamination process. 2ml of the aliquot was analyzed for the presence of blood cells, gram staining, AFB staining, biochemical examinations (protein, glucose) and culturing (BioFM liquid broth, (BioRad) Mames-la-coquette, France),

1.2.2.4. Tuberculosis Ascites (TBA) cases

Ascitic fluid samples were obtained from 10 patients, including 05 TBA patients admitted in CIIMS, Nagpur. The remaining 05 patients which were included in the study had non-TB disorders and thus considered as non TB cases.

TBA patients (n=05)

The diagnosis of TBA was obtained through a combination of several methodologies. First, a microscopic examination was performed of ascitic fluid samples that had been stained with ZN Stain. Where these tests were

negative, the patients were diagnosed by clinical symptoms, such as, a. presence of productive cough b. low grade fever c. night sweat d. weight loss e. abdominal pain f. abdominal mass g. biochemical findings showing increased protein decreased glucose and/or pleocytosis with lymphocytic predominance. h. ultrasound

Non TBA patients (n=05)

The patients recruited in the group were those admitted in CIIMS for acute or chronic defined, non-TBA diseases, including inflammatory bowel disease, various other infectious disorders, malignancy, gastrointestinal symptoms, abdominal tenderness accompanied by non-specific fever, pneumonia, bronchitis, lung cancer, lung infection and others unrelated to TB.

Ascitic fluid samples

For the collection of ascitic fluid, the patient was allowed to lie on his/her back with head at 45°–90° elevation. The area where the needle was to be inserted was cleaned with iodine or similar solution and drapped. The anesthetic was administered to numb the area. The paracentesis needle was carefully inserted into the abdomen. About 1000 to 1500 ml of fluid was removed. 50 ml of the fluid was used for the analysis of different parameters.

1.2.3 One Dimension Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (1D SDS PAGE)

Samples obtained from all forms of TB and non TB cases were subjected to one dimension Sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS-PAGE). SDS-PAGE was performed with a vertical slab gel electrophoresis system (Broviga, India) using the standard Laemmali method, (***Laemmali UK et al., 1970***). 4% stacking gel and 10% running gel were used.

Electrophoresis was carried out at 250 volts/50 mAmps. Gels were developed by staining with Coomassie brilliant blue GR-250 and the protein profiles were then studied.

1.2.4. Two Dimension Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (2D SDS PAGE)

For first dimension, 125 µl (150 ug protein) of each sample from the TB and non- TB groups was applied to a Serva IPG strip (pH 3-10, 7 cm) and then it was subjected to isoelectric focusing (IEF). Briefly, the IPG strips were rehydrated overnight and IEF was then carried out at 20 °C in a Protean IEF unit (Bio-Rad, USA). Prior to second dimension electrophoresis, the IPG gel strips were immersed in equilibration solution for 15 min. The second dimension separations were carried out at 10 °C using SDS slab gels (10%) without stacking gels and a mini-protean tetra cell electrophoresis system (Bio-Rad). The IPG strips were embedded on the top of the gels with 0.5% agarose, and electrophoresis was performed at 30 mA/gel for 1 h. Gels were developed by staining with Coomassie brilliant blue GR-250. Gel images were taken using the gel documentation system (Bio-Rad) and were imported into the PD Quest (Bio-Rad) 2D gel analysis software package. For detection of spots a master gel image was created by combining all of the spots that were present in both the TB and non-TB groups and was used to match each spot.

1.2.5. Liquid chromatography-mass spectrometry/mass spectrometry (LCMS/MS)

An excised protein bands were sent to TCGA, India for LC-MS/MS analysis. TCGA, India characterized these protein using the following protocol: (1) each gel piece was destained and washed prior to in-gel digestion, (2) protein bands were excised, digested and treated with trypsin after reduction and (3) alkylation agents were added prior to the analysis on the LC-MS/MS. In-gel digestion was carried out in 50 mM NH_4HCO_4 buffer, pH 8.5 at 37 °C for approximately 4 hours. An equal volume of the digestion buffer was added, depending on the volume of the gel piece, and usually ranged from 20 to 50 µl. The amount of proteolytic enzyme (Promega trypsin, modified, sequencing grade) that was used depended on both the size of the gel pieces and the estimated amount of protein within the gel band. Typically, 200 ng to 1 µg trypsin was used per gel band. Acetonitrile (ACN), in a volume equal to 3–5 times the volume of the digestion buffer, was then added to the digestion mix to extract the peptides. The samples were then centrifuged at high speed for 5min. The supernatant was transferred to a clean microcentrifuge tube with a gel-loading pipette tip and dried in a SpeedVac on medium heat. For the reduction step, alkylation agents were added prior to analysis with LC-MS/MS. The dried sample was dissolved in 0.5% acetic acid (HOAC) for LC-MS/MS analysis. A Finnigan (ThermoFinnigan, San Jose, CA) LCQ ion trap MS in-line coupled with a high pressure liquid chromatography (HPLC) system was used for LC-MS/MS. A 75 µm (ID) × 10 cm length, 3 µm packing C18 capillary column, which was packed in-house, was connected to a specially designed nanoSpray device, which is capable of delivering a stable electrospray at flow

rates of 100 nl/min to 1500 nl/min. The mobile phases included Solvent A (2% ACN, 97.5% H_2O, 0.1% formic acid) and Solvent B (90% ACN, 9.5% H_2O, 0.1% formic acid). For this analysis, the ion trap MS was set to operate in a data-dependent mode with the Automatic Gain Control (AGC) on. The MS/MS data were first evaluated against several internal quality control (QC) standards. After passing the QC standards, the MS/MS data was loaded into the proprietary ProtQuest search engine to search the most recent non-redundant protein database. The results from the ProtQuest search were then manually analyzed. The endoproteinase trypsin (sequencing grade) was obtained from Promega or Roche (Indianapolis, IN). The ammonia bicarbonate (analytical grade) and HOAC (.99.8% purity) were obtained from Sigma. ACN, methanol (MeOH), and water were each HPLC grade and obtained from Sigma (St. Louis, MO).

1.2.6 Electro elution of protein bands:

Identified protein bands were sliced out of the gel and pre-equilibrated in elution buffer [0.15 M phosphate-buffered saline (PBS), pH 7.4] and then electro-eluted in a whole gel eluter system (Biotech, India) for 90 min at 30 volts ***(Mudaliar AV et al., 2006).***

1.2.7. Antibodies

After characterisation bands of host Hsp 60, MTB Hsp 16, Hsp 65 and Hsp 71 were eluted from the gel and polyclonal antibodies against the same Hsp were rose in rabbits (Chromus Biotech, India). Corresponding IgG was purified from whole serum using column chromatography (Bangalore Genei).

Goat anti rabbit IgG HRP was used as secondary antibody which was purchased from Bangalore Genei.

1.2.8 Enzyme-linked immunosorbent assay (ELISA)

Each set of micro titer wells was coated with 100µl of samples (CSF/Serum/Pleural fluid/Ascitic fluid) in different dilutions for different Hsps and incubated at 37°C for 90 min. The plates were then washed once with PBS and 100µl of blocking buffer (BSA) was added per well and were subjected to incubation at 37°C for 45 min. After incubation the plates were washed three times with PBS and 100µl of primary antibody solution i.e. antibodies generated against Hsps were separately added to each well followed by incubation period of 37°C for 45 min. The wells were then subjected to washing with PBS thrice and 100µl of secondary antibody (Goat anti rabbit) solution was then added followed by incubating at 37°C for 45 min. Wells were washed 3 times with PBS followed by addition of the substrate TMB/ H_2O_2 100µl per well. The wells were incubated for 5 to 10 min after which the reaction was stopped with the addition of 100µl of 2.5N H_2SO_4. Absorbance of each well was read at 450nm on an ELISA reader.

CSF sample dilutions used for the assay were 1:5 (host Hsp 25, host Hsp 90 and MTB Hsp 16, MTB Hsp 65, MTB Hsp 71) and 1:20 (host Hsp 60 and host Hsp 70). Serum/Pleural fluid/Ascitic fluid sample dilutions used were 1:200 (Host Hsp 25), 1:400 (Host Hsp 60, Hsp 70 and MTB Hsp 16, Hsp 65, Hsp 71) and 1:100 (Host Hsp 90). The dilutions used for primary antibodies were 1:8000 (Host Hsp 25), 1:1000 (Host Hsp 60), 1: 10000 (Host Hsp 70), 1:4000

(Host Hsp 90) and 1:5000(MTB Hsp 16, Hsp 65 and Hsp 71) and secondary antibody dilutions were 1:2000 (Host Hsp 25 and Hsp 60), 1: 4000 (Host Hsp 70 and Hsp 90) and 1:10,000 (MTB Hsp 16, Hsp 65 and Hsp 71) for all the analysis.

Institutional Ethics Committee

An informed consent was obtained from all the patients. The Institutional Ethics Committee of Central India Institute of Medical Sciences, Nagpur, India approved the study.

1.3 RESULTS

A total of 999 samples were collected throughout my work and then evaluated for various analysis including Proteomics, Hsps evaluation, cytokines as well as TLR induced cytokines evaluation as shown in Figure 1.1.

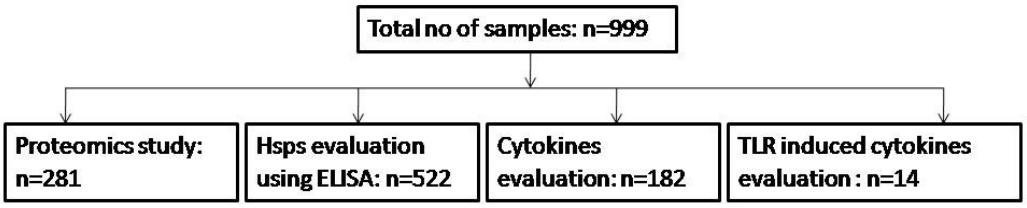


Figure 1.1 *Flow Chart of the entire work.*

For Proteomic analysis , a total of 281 samples were collected and evaluated using 1D and 2D SDS PAGE for the identification of new biomarkers for PTB and EPTB disease.

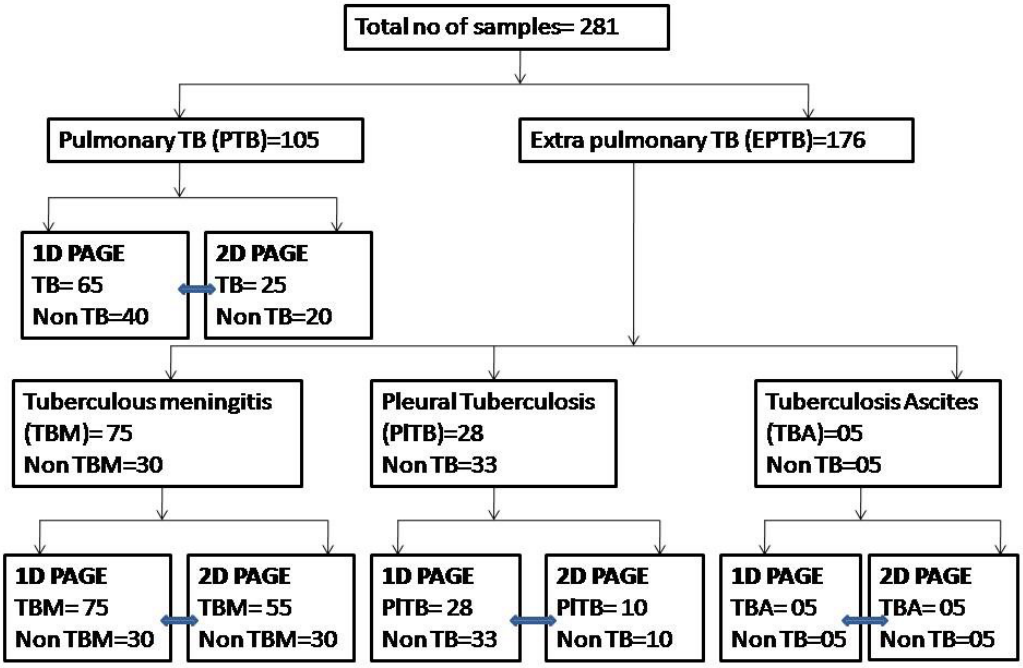


Figure 1.2: *Flow Chart of the Proteomic analysis.*

Flow chart (Figure 1.2) for classification of patients in different groups and their proteomic analysis is as follows:

Samples were selected (as per the flow chart **Figure 1.2**) for one dimensional electrophoresis to obtain differential electrophoretogram . A marked increase in the intensity of 65 kD, 45 kD and 30 kD bands in the clinical samples of TBM, PTB, PlTB, TBA patients were observed when compared to their respective controls as shown in **Figure 1.3.**

To generate a sample protein signature that was associated with the PTB and EPTB, further selected samples were subjected to 2D SDS-PAGE (as shown in **Figure 1.2**). Proteins that were up-regulated in TBM, PTB, PlTB, TBA patients, compared to their levels in non-TB patients, respectively are shown in **Figure 1.4**. Proteins that were highly expressed in the TBM, PTB, PlTB, TBA patients when compared to their respective control patients were

selected for further characterization using LC-MS/MS. The characterized proteins are tabulated in table 1.1.

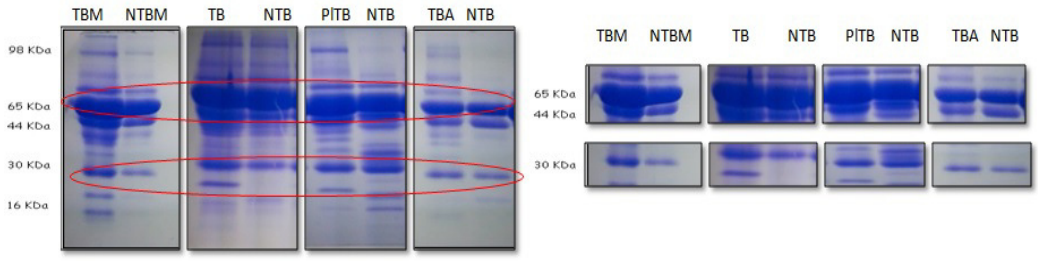


Figure 1.3: *One dimensional gel electrophoresis of the samples collected from a) TBM (n=75) & non TBM (n=30) b) TB (n= 65) & Non TB (n=40) c) Pleural TB (PlTB) (n=28) & non TB (n=33) d) TB ascites (TBA) (n=05) & non TB (n = 05) patients. Proteins which are up regulated in the TB patients are indicated with red circle*

Increased levels of MTB heat shock protein (Hsp) 65 was identified in the CSF samples of TBM patients, serum samples of PTB patients and ascitic fluid of TBA patients when compared to their respective controls. Similarly, increased levels of MTB Hsp71 and MTB Hsp 16 were identified in serum samples of PTB patients and ascitic fluid of TBA patients when compared to their respective controls. Increased levels of host Hsp 60, protein unc-45 and E3 ubiquitin ligase SMURF2 molecule were detected in the CSF samples of TBM patients when compared to non TBM patients.

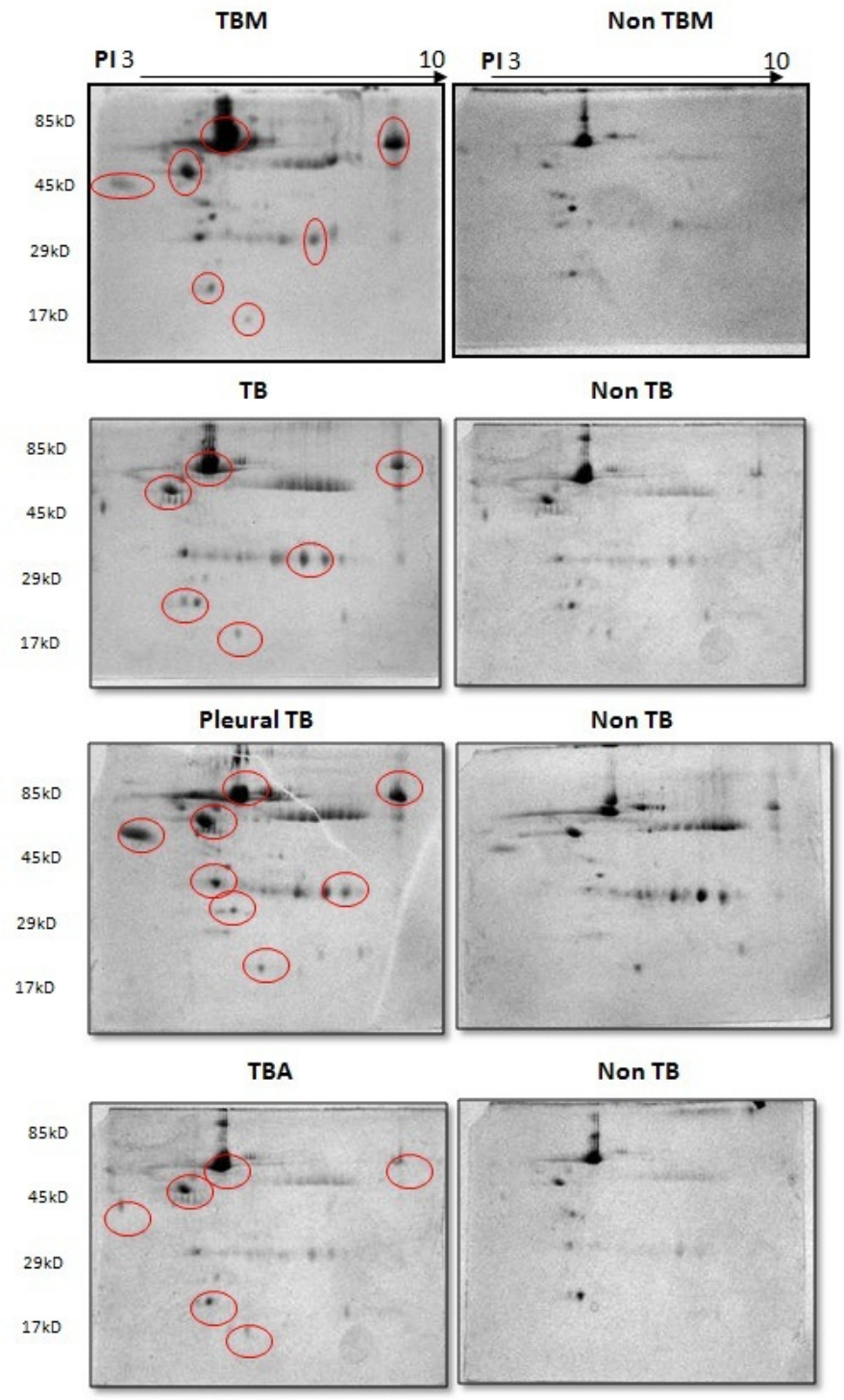
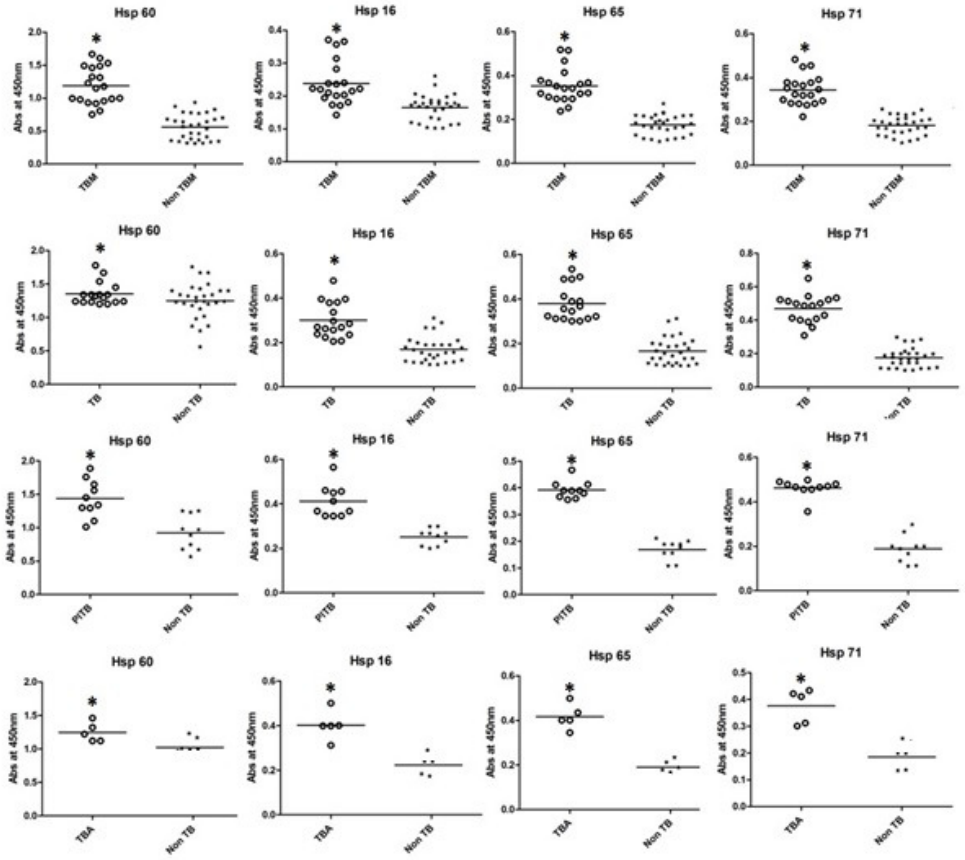


Figure 1.4: *Two dimensional gel electrophoresis of the samples collected from a) TBM (n=55) & non TBM (n=30) b) TB (n= 25) & Non TB (n=20) c) Pleural TB (PlTB) (n=10) & non TB (n=10) d) TB ascites (TBA) (n=05) & non TB (n = 05) patients. Proteins which are up regulated in the TB patients are indicated with* ⬭

Sr. No	Identified Protein	pI	Detected in Samples with peptide sequence
1	MTB Hsp65	4.5	CSF (EKIGA) , Serum (VAAGA), Ascitic fluid (AKEVETKE)
2	MTB Hsp71	7.7	Serum (RHMGSDWSIE IDGKKY), Ascitic fluid (PYITVDADKNPLFLD, RKRREEADVR NQAE)
3	MTB Hsp 16	4.7	Serum (PEFSE) , Ascitic fluid(FDGR SEFAYGSFVR)
4	Host hsp60	4.65	CSF(DPGMGAMGGMGGGMGGGMF)
5	protein unc-45	5.8	CSF(DGGDVKALYR)
6	E3 ubiquitin ligase SMURF2	8.31	CSF(FLRGIEAQFLALQK)

Table 1.1: *Identification of the protein(s) bands in TB patients. The peptides were detected by liquid chromatography- tandem mass spectrometry (LC-MS/MS) analysis.*

After the identification of host and MTB Hsps in the clinical sample of PTB and EPTB patients using 1D& 2D PAGE and LC-MS/MS, host Hsp 60, MTB Hsp 16, MTB Hsp 65 and MTB Hsp 71 were eluted from the gel and sent for antibody production. The produced antibodies were then evaluated in the clinical samples of PTB and EPTB patients. It was observed that the levels of host Hsp 60, MTB Hsp 16, MTB Hsp 65 and MTB Hsp 71 were significantly increased in PTB and EPTB (TBM, PlTB & TBA) patients as compared to their respective controls as shown in **Figure 1.5.**



TBM:Tuberculous meningitis, PM: Pyogenic meningitis, VM: Viral meningitis, PTB: Pulmonary TB, PlTB: Pleural TB, TBA: Tuberculous Ascites

Figure 1.5: *Demonstration of host and MTB Hsps antigen in PTB and EPTB patients using the antibodies generated against host Hsp 60, MTB Hsp 16, MTB Hsp 65 and MTB Hsp 71. a) CSF sample of TBM (n=20) and Non TBM (n=30) b) serum sample of PTB (n=17) and Non TB (n=29) patients c) Pleural fluid sample of PlTB (n=10) and non TB (n=10) d) Ascitic fluid sample of TBA (n=05) and non TB (n=05).*indicates significant p value (p<0.05).*

1.4 DISCUSSION AND CONCLUSIONS

Although significant advances have been documented in the field of TB, still the incidences of TB are increasing. Therefore, there is a need to identify new markers for diagnosing TB so that the patients can be benefitted with the early treatment. Proteomics has emerged as a powerful approach for identifying biomarkers and hence become one of the essential tools in biomarker discovery ***(Kumar GS et al., 2012; Kashyap RS et al., 2010d: Kashyap RS et al.,2005b)***. Therefore, in the study using proteomic approach (1D/2D PAGE and LCMS/MS), I identified several markers which are differentially expressed in PTB and EPTB patients when compared to their respective controls. Later, the proteins were eluted out and antibodies were produced against them. These produced antibodies were then evaluated in the clinical sample of PTB and EPTB patients to obtain the Hsp antigen profile in the same patients.

In my Institute, there has been a constant demand for identification of new biomarkers of TB disease which can be further used in diagnosis and in understanding the pathogenesis of PTB and EPTB disease. Therefore, I collected clinical samples from TBM, PTB, PlTB and TBA patients along with respective controls and performed 1D & 2D SDS PAGE. An increase in several protein bands were observed in TBM, PTB, PlTB and TBA patients when compared to their respective controls. These differential bands were then sent to TCGA, India for characterisation using LCMS/MS. The identified proteins which were increased in PTB and EPTB patients are MTB heat shock protein (Hsp) 65, MTB Hsp 71, MTB Hsp 16, Host Hsp 60, protein unc-45 and E3 ubiquitin ligase Smad ubiquitination regulatory factor 2 (SMURF2). I selected bands of host Hsp 60, MTB Hsp 16, MTB Hsp 65 and MTB Hsp 71

and eluted these proteins from the gel. The eluted proteins were then sent for antibody production. The produced antibodies were used to detect the respective Hsp antigen in the clinical samples of PTB and EPTB patients. An increase in the levels of host Hsp 60, MTB Hsp 16, MTB Hsp 65 and MTB Hsp 71 was observed in PTB and EPTB patients as compared to their respective controls indicating the probable use of the selected proteins as diagnostic biomarkers in TB disease.

Hsps are conserved molecules that play an important role in protein folding, assembly and in translocation of proteins between different compartments. Under stress, Hsp synthesis is drastically increased, representing a mechanism essential for cell survival. In infection the major role of Hsps is the prevention of protein aggregation and reversion of polypeptide unfolding ***(Becker J et al., 1994; Craig, EA et al., 1994).***

Role of Hsps are well documented in various infectious diseases including Schistosomiasis ***(Hedstrom R et al., 1988),*** Malaria (***Mattei D et al., 1988***) , Onchocercosis (***Rothstein NM et al., 1989***), Filiariasis (***Selkirk M E et al., 1989***) , Chagas' disease (***Dragon EA et al., 1987***), Leishmaniasis (***Amorim AG et al., 1996***) , Toxoplasmosis (***Himeno K et al., 1996***), Candidiasis (***Matthews RC et al., 1991***), Histoplasmosis (***Gomez FJ et al., 1992***), Leprosy (***Young DB et al., 1988a***), Trachoma (***Morrison RP et al., 1989***) , Lyme disease (***Anzola J et al., 1992***), Yersiniosis (***Noll A et al., 1994***) , Legionnaires' disease, Syphilis (***Hindersson P et al., 1987***), Pertussis (***Del Giudice G et al., 1993***) and Listeriosis (***Kimura Y et al., 1998***). Therefore, in the light of literature survey it seems that Hsps play important role in infectious diseases.

All the proteins which are identified in PTB and EPTB patients are either Hsps or are associated with the functions of Hsps. MTB Hsp 65 which was identified in the CSF, serum and ascitic fluid sample of TB patients is a membranous protein expressed at high levels by bacterial pathogens during adaptation for intracellular survival. The 65-kD antigen has been reported to be a major immunologically active MTB antigen following infection and is expressed at high levels during TB infection (***Mudaliar et al., 2006; Rajan et al. 2007***). Similarly, MTB Hsp 70 has a specific role in virulence of the pathogen and is an immunodominant antigen in TB infection (***Stewart et al., 2001; Shekhawat et al., 2012***).

As per the reviewed literature, MTB Hsp16 is required to overcome hypoxia and starvation for the survival of bacteria in stationary phase ***(Voskuil et al., 2004; Kashyap et al., 2011a; Young DB et al., 1991b)***. The higher level of the 16 kD Hsp protein during stationary phase and the constant cellular level of this protein is an important strategy for MTB to survive in dormant state ***(Yuan et al., 1996).*** The increase level of Hsp 16 in the serum and ascitic fluid of TB patients is therefore well supported by cited literature.

Another Hsp which was identified to be increased in the CSF samples of TBM positive patients when compared to non TBM patients was human Hsp 60. Hsp60 has been identified as a ligand for the innate immune system ***(Cohen-Sfady M et al., 2005)*** and thought to stimulate cytokines secretion (***Flohe S et al., 2003)*** thus it might be possible that the increased level of Hsp 60 in CSF sample of TBM patients might trigger immune system to produce cytokines to evade MTB.

In the CSF sample of TBM patients increased amount of UNC-45 protein was also observed when compared to non TBM patients. The UNC-45 chaperones play an evolutionarily conserved role in promoting myosin-dependent processes, including cytokinesis, endocytosis, RNA transport, and muscle development. Literature also suggest that Hsp70 and Hsp90, bind to the TPR domain of UNC-45, and act in concert and with defined periodicity on captured myosin molecules ***(Gazda L et al., 2013).*** However, the role of UNC-45 in TBM patients is yet not been elucidated in details. The presence of this protein in the CSF sample of TBM patients indicates its probable importance in TBM. However, the result needs further confirmation.

SMURF 2 was highly expressed in the CSF sample of TBM patients when compared to non TBM patients. It is a crucial part of the ubiquitin-proteasome pathway (UPP) that regulates cellular signal transduction via ubiquitin-dependent degradation of some substrates and receptors ***(Sun Y et al., 2011).*** Sun Y et al showed that over expression of Smurf2 altered the sub cellular localization and distribution of Hsp 27, and induced a decrease of Hsp 27 protein levels through Hsp 27 degradation by the UPP in human lung adenocarcinoma epithelial cell line A549 ***(Sun Y et al., 2011).*** Wrighton K H et al showed that SMURF 2 have association with Hsp 90 as inhibition of Hsp90 compromises TGFβ-mediated transcriptional responses by enhancing TβR ubiquitination and degradation in a SMURF 2 ubiquitin E3 ligase-dependent manner ***(Wrighton KH et al., 2002).*** Thus, it seems that SMURF2 has association with Hsps however direct role in TBM is to deciphered.

The results obtained have confirmed the putative involvement of Hsps in the PTB and EPTB patients. It is confirmed that due to stress, MTB and host

Hsps are released in the clinical samples of PTB and EPTB patients when compared to their respective control patients. Our ELISA results indicate that the identified proteins might be useful for the development of either diagnostic tool or have potential role in pathogenesis of PTB and EPTB patients. Therefore, these proteins along with other Hsps were evaluated and reported in Chapter 2.