Tuberculosis (TB) caused by Mycobacterium *tuberculosis* (MTB) remains an enormous health problem worldwide. In spite of being a curable disease, 24% cases of global burden were estimated to be located in India. In endemic regions of TB, Tuberculous meningitis (TBM) is a frequently encountered neurological disorder. It is believed that TBM like any other form of TB begins with respiratory infection followed by hematogenous dissemination to extrapulmonary sites. Other forms of extra pulmonary TB (EPTB) including Pleural TB (PlTB) and Tuberculous ascites (TBA) are also common in TB endemic area. Although host immune factors are known to provide protection against the infection, more immunological parameters are to be assessed in order to determine effective biomarkers of protection which will help in diagnosis and also in understanding the pathogenesis of TB.

The lack of accurate and rapid diagnostic tests is the limiting factor for the battle against TB which is a leading cause of death worldwide. Sputum smear microscopy of acid-fast bacilli (AFB) and culture of MTB have been widely used for diagnosis of active TB. However, AFB smear microscopy has limited sensitivity (50%-60%) in diagnosing pulmonary TB (PTB) and is time consuming. Diagnosis of EPTB by culture is also difficult because of the low load of organism in the body fluids of EPTB patients. The sensitivity of AFB smear microscopy decreases further in case of cerebrospinal fluid (CSF) of TBM patients .The low specificity of chest X-rays used for the diagnosis of smear-negative TB risks over diagnosis. Recently launched Xpert MTB/RIF test have reported promising results in specific population, however, have certain major limitations including high cost, requirement for a continuous

electricity supply and short shelf life of consumables. Also the test have limited use in diagnosing EPTB patients. Various immunological and molecular assays have been widely evaluated for the diagnosis of PTB and EPTB cases, however they either suffer from less sensitivity or high cost 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. Therefore, there is an urgent need to identify new markers which can help in diagnosis and in understanding the pathogenesis of PTB and EPTB.

Protein profiling in clinical samples has been done in quite a few studies in which heat shock proteins (Hsps) stand as a promising biomarker for TB disease. Hsps have been shown to share diverse functions such as control of protein degradation, thermotolerance , immunomodulation and regulation of development and evolution . In TB infection, Hsp exhibit different functions, including activation of toll like receptors (TLRs), eliciting immune responses, tool for diagnosis and also serve as a potent vaccine candidate. Clinical and epidemiological studies have suggested a concept of strain–dependent neurovirulence, however, the profile of Hsp in different strains of MTB are only beginning to be elucidated.

The fate of MTB infection is decided by two basic components of Immunity. One is the danger signaling molecules which trigger the immunity and another aspect is host response against that signaling molecule. Hsps are one of the signaling molecule which can be considered as “danger molecule”. Hsps can

be of host origin or of MTB origin. These are well-conserved and immunodominant antigens elicit a cellular and humoral immune response and may play an important role in host defense against invading microorganisms and autoimmune disorders.

The other major factor which decides the fate of MTB is the host response in the form of cytokines. Cytokines are critical for the induction of MTB specific T cell immunity, as the effecter molecules for the stimulation of macrophages and for the formation and maintenance of granuloma, the microenvironment in which macrophages activation occurs. Pathogen killing by macrophages is enhanced and often dependent on additional signals, such as, interferon gamma (IFN-γ) and Tumor necrosis factor (TNF), delivered by other cells of the innate and adaptive immune response, namely Natural killer (NK) cells and T or B cells.

Although few literature is available, however the relationship between Hsps and cytokines are yet be elucidated in TB disease. Bacterial Hsp (s) are highly immunogenic, capable of inducing antibody production and T cell activation. Therefore, increase in the level of MTB Hsp(s) probably alerts the immune system. Recent studies suggest that Hsp(s) may also have potent cytokine-like function independent of peptide binding. It is believed that MTB Hsp(s) act as an endogenous ligand for TLR which upon activation releases pro-inflammatory cytokines through cascade of reactions.

Thus, the aim of the thesis is to study the role of Hsps and cytokines in the diagnosis and pathogenesis of PTB and EPTB patients. To achieve the objective, clinical samples were collected from PTB and EPTB patients. EPTB patients were further classified into TBM, PlTB and TBA patients. A total of 999 patients (TBM, TB, PlTB, TBA and their respective controls) were recruited for the entire study. In **chapter 1**, 281 (PTB & EPTB) (out of 999) clinical samples were subjected to proteomic analysis (1 D and 2D PAGE). Out of 281 selected samples, 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. Selected samples were subjected for one and two dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D and 2D SDS PAGE) to obtain differential electrophoretogram. Based on results 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. These expressed proteins were selected for further characterization using Liquid chromatography mass spectrometry (LC-MS/MS).

Increased levels of MTB Hsp 65 was identified in TBM, PTB and TBA patients when compared to their respective controls. Similarly, increased levels of MTB Hsp71 and MTB Hsp 16 were identified in PTB patients and TBA patients when compared to their respective controls. Increased levels of host Hsp 60 was detected in the TBM patients when compared to non TBM patients. The identified host Hsp 60 and MTB Hsps (Hsp 16, Hsp 65 and 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 patients as compared to their respective controls.

In **chapter 2** a panel of Hsps identified using proteomic tools (host Hsp 60, MTB Hsp 16, MTB Hsp 65 & MTB Hsp 71) and Hsps available in literature(Hsp 25, Hsp 60, Hsp70 and Hsp 90) were further evaluated in the clinical samples of PTB and EPTB patients**.** The assay was developed using different concentrations of the Hsp antigen. A total of 522 samples were evaluated using the developed in house ELISA method. The levels of all the evaluated Hsps were increased in the clinical samples of TBM, TB, PITB and TBA patients as compared to their respective controls. Among host Hsps, Hsp 70 and Hsp 90 show reasonably good positivity and negativity in TBM, PITB and TBA patients. MTB Hsp 65 and Hsp 71 showed good positivity and negativity in PTB and EPTB patients. During follow up studies, it was observed that level of Hsps increases when a patient develops TB and decreases when the patient is started on anti TB treatment (ATT). Also it was observed that the levels of Hsps increases progressively in follow up samples as compared to original samples. The developed assay was compared with P polymerase chain reaction (PCR) targeting IS6110 gene and a good concordance was obtained between both the assays. Results suggest us that host Hsp 60, host Hsp 70, MTB Hsp 65 and MTB Hsp 71 can efficiently discriminate between PTB & EPTB (TBM, PITB and TBA) patients with their

respective controls. These evaluated Hsps might be useful in designing better point of care diagnostic methods for PTB and EPTB disease.

In **chapter 3,** cytokines (IL-6, TNF-α and IFN-γ) were evaluated in the clinical samples of PTB (n=40) and EPTB (n=142) patients. On evaluation, it was observed that the levels of interleukin-6 (IL-6), TNF-α and IFN-γ were significantly ($p<0.05$) increased in PTB patients as compared to non TB patients. In TBM patients, the level of IL-6 and IFN-γ were significantly ($p<0.05$) increased as compared to non TBM patients. Levels of TNF-α, and IFN-γ were significantly ($p<0.05$) increased in PITB patients as compared to non TB patients. Similar profile was observed in TBA patients when compared to non TB patients. IFN-γ remains the only cytokines which was significantly increased in all the forms of TB therefore considered as the promising diagnostic marker for all forms of TB. Along with IFN-γ, IL-6 also shows promising results for the diagnosis of TBM. All the three evaluated cytokines can be used to diagnose PTB patients and TNF-α along with IFN-γ seems to be ideal candidates for the diagnosis of PITB and TBA.

The increased proinflammatory cytokine levels in TB patients signify protective immunity against MTB. Follow up studies support my hypothesis and suggest cytokines may have prognostic significance for pathogenic outcomes and thus open the avenues for therapeutic research. The relationship between Hsps and cytokines suggest that both are key regulatory molecules of immunity and Hsps have immunomodulatory action and thus may be further evaluated as a candidate for vaccine development in near future.

In **chapter 4**, studies from bed side (clinical samples) to bench sideare planned to ascertain role of Hsps and cytokines as diagnostic and prognostic marker in PTB and EPTB patients. The ascertain above objective an in vitro a cell line model was developed using monocytes (THP-1 cell line) to study both the parameters. The monocytes were infected with MTB isolated from clinical isolate. After infection of 48 hrs supernatant as well as cells were isolated and used for evaluation of Hsps (Host Hsp 25, Hsp 60, Hsp70, Hsp90 and MTB Hsp 16, Hsp 65 and Hsp71) and cytokines (IL-6 and TNF-α). The level of same panel of Hsps except Hsp 60 was increased in the cell lysate of monocytes infected with MTB when compared to uninfected cells. Also the levels of IL-6 and TNF-α were increased in the cell supernatant of monocytes infected with MTB when compared to their respective controls. Proteomic analysis 1D and 2D SDS PAGE profile of cell lysate showed a significant increase in the 65 kD region of infected cells when compared to control. This protein was identified as MTB Hsp 65 using LCMS/MS analysis, the results of proteomics analysis indicate an similar levels of HSPs profile on MTB infection and support our ELISA results as discussed in previous chapters Thus, the results of bed side (clinical samples) and bench side (in-vitro model) suggest that Hsps and cytokines may serve as potential biomarkers for the diagnosis and in understanding the pathogenesis of TB disease.

Hsps plays important role in both innate and adaptive host responses in TB. During TB infection, high levels of Hsp(s) confer protection against MTB by signaling through several Pathogen Recognition Receptors (PRRs). TLRs are a crucial family of conserved PRRs well known for their roles in recognizing

specific microbial patterns and allowing the host cells to distinguish between self and non-self molecules. Hsps can activate TLRs and thus triggers innate immune responses and primes antigen-specific adaptive immunity toward exogenous bacilli. Increased level of Hsps leads to NF-κB activation, and thus TNF-α, IL1-β and IL-6 production. To improve the understanding of host defenses and pathogenesis, I investigated differences in immune parameters including TLR induced cytokines and Hsps in patients with PTB or EPTB in **chapter 5.** It was observed that TLR induced TNF-α, IL-1B, IL-6, IL-8, MCP-1, MIP-1α, MIP-1β cytokines were significantly increased in TBM cases when compared to non TBM cases. Similarly, TNF-α, IL-6, IL-8, MCP-1, RANTES, MIP-1α, MIP-1β and MDC were significantly increased in the serum samples of TB patients as compared to non TB patients. I observed a positive correlation between MTB Hsp 65 and MDC in the CSF sample of TBM patients. Also, a positive correlation was also obtained with MTB Hsp 16 and TNF-α & IL-17a in TB patients indicating their role in modulation of immune response in TBM/TB and can be further evaluated as a candidate for vaccine development in near future. Similarly, a very significant positive correlation (r=1) was obtained between host Hsp 70 and Eotaxin in TB patients.

Differential levels of Hsps and TLR induced cytokines were obtained in PTB and EPTB patients. Further the relationship between Hsps and cytokines were also different in both the biological fluids. The differential levels of these two immunological parameters could be because of different strains of MTB which causes PTB and EPTB in patients. Also the immunomodulatory role of Hsps supports its importance in vaccine development.

The study presented in the thesis thereby shows the significant role of Hsps in the diagnosis and pathogenesis of PTB and EPTB patients. Host Hsp 70, host Hsp 90, MTB Hsp 65 and MTB Hsp 71 shows promising results and could be used in the diagnosis of PTB and EPTB patients. The developed assay has an advantage of being rapid and cost effective and can be further developed in point care diagnostic test. Additionally, Hsps can be used to study the progression of the disease as suggested by the obtained results. Characteristic profile of cytokines (IL-6, TNF-α and IFN-γ) was observed in PTB and EPTB patients and IFN-γ seems to have diagnostic potential for PTB and EPTB patients. The association of Hsps with cytokines via TLR plays a significant role in TB and thus added to the existing knowledge of immunological processes induced by MTB and the host-MTB interactions. A better understanding of MTB Hsps with TLR is necessary to the development of effective vaccines and more efficacious treatments for TB. The interaction between host Hsps, TLR and cytokines could help in boosting the immune response against TB and other infections.