Summary
Leprosy is an infectious disease which manifests itself in a broad clinical and immunological spectrum. The events which lead to the development of this multispectral disease are poorly understood. An individual infected with *M. leprae* may self-cure or may develop clinical signs and symptoms for paucibacillary or multibacillary leprosy after a prolonged incubation period [Reviewed in Scollard *et al.*, 2006a]. The subclinical phase, occurring between infection and the slow development of the earliest detectable clinical symptoms, might control the key host response mechanisms that determine subsequent progression of infection into self-cure or clinical disease [Mira *et al.*, 2004; Krutzik *et al.*, 2005; Scollard *et al.*, 2006a]. Identification of *M. leprae* infection in the subclinical stage will help in initiating the treatment at the early stages itself, which in turn might help in reducing the severity of disease and control the possibilities of transmission [Aseffa *et al.*, 2005]. However, identification of subclinical stage in human subjects is rather difficult since any detectable signs of infection such as the presence of skin lesions, nerve enlargement or anesthetic patches are absent at this stage. People living in leprosy endemic areas and healthy household contacts of leprosy patients might not prove to be an ideal experimental group to examine subclinical infection since the exposure to other pathogenic or non-pathogenic organisms cannot be ruled out. In this study, plasma proteome analysis of mice experimentally infected with *M. leprae* in the footpads was carried out using proteomic approaches to identify differentially expressed proteins in response to infection. Even though armadillo recapitulates the disease, mice were preferred to armadillos as model organisms for leprosy considering the ease of handling and local availability in large numbers.

Gel-based proteome profiling of *M. leprae* infected mice plasma was carried out and sample preparation and 2D analytical conditions were optimized to improve the separation of proteins in the gel. Removal of ions and interfering substances by acetone precipitation was found to be an ideal desalting method for mice plasma as it improved the resolution of closely located proteins and isoforms and resulted in minimum sample loss compared to the other methods tested. Removal of albumin by
immunoaffinity depletion was found to be an ideal method to enrich the moderately abundant proteins in mice plasma. Immunodepletion using Seppro® IgY anti-mouse albumin spin column was adopted as the method of choice considering the reproducibility of the method and reusability of the column. Moreover, it was a less laborious and cost effective method. Attempts were also made to deplete top7 abundant proteins in mice plasma to enrich the low abundant proteins. However, the number of new proteins that could be resolved from the top7 depleted plasma using 2D PAGE technique was modest since the resolution is subjected to variabilities such as sample concentration, staining method, etc. The 2D analytical conditions such as the ideal pH range to enable the maximum separation of proteins, type of rehydration methods for IEF, type of gels for second dimensional separation and the staining methods were also optimized for the mice plasma proteome analysis.

Two dimensional separation of mice plasma was carried out under the optimized conditions and the separated proteins were identified by mass spectrometry to construct a 2D reference map. Availability of a reference map is a prerequisite in biomarker-oriented proteomic studies. However, published reports on the proteomics of murine plasma/serum were limited [Gianazza et al., 2012] and the maximum number of spots annotated from two dimensionally separated mice plasma/serum, until this study, was 103 spots corresponding to 90 proteins and its isoforms. In this study, using peptide mass fingerprinting by MALDI-TOF and MS/MS analysis by nanoLC-QTOF, identity of 142 spots corresponding to 63 proteins and its isoforms could be ascertained from albumin depleted mice plasma. The differentially expressed proteins in M. leprae infected mice plasma were studied further.

Quantification of differential expression of plasma proteins in M. leprae infected mice by 2D DIGE identified 111 spots showing ±1.5 fold change (p>0.01) in expression levels. This indicated that even though the infection of M. leprae in the footpads of mice does not lead to a clinical disease, the host elicits a generalized response to infection. The effect of these responses could be determined as variations in plasma protein profile as early as six months post infection. Most of the 35 differentially expressed spots identified by mass spectrometry represented acute phase proteins indicating that the localized infection of M. leprae in the footpads indeed induce an acute phase response in mice. Moreover, these changes could be
observed even in the cases where the active multiplication of the bacilli was not observed in the footpads, indicating that the variations in the plasma proteome profile were mainly due to the invasion of the bacilli rather than its multiplication. Thus, the application of footpad inoculated mice as a model organism to conduct proteomic studies to identify early host responses has been demonstrated in this study. However, these findings need to be ascertained in human subjects for validation. Even though the differential expression of plasma proteins could be demonstrated, the role of these proteins in *M. leprae* infection could not be explained with existing data.

Haptoglobin is a highly conserved plasma acute phase protein secreted in response to infections and inflammations by hepatocytes. Biological variation analysis by 2D DIGE demonstrated approximately 22 fold upregulation of haptoglobin in *M. leprae* infected mice plasma. Variation in the expression levels of haptoglobin had been reported in leprosy patients [Sriharan et al., 1981; Gupta et al., 2007]. This study demonstrates that variation in haptoglobin expression in response to infection could be identified in the subclinical stages in *M. leprae* infected mice. Immunoblot analysis and ELISA could validate the proposed application of haptoglobin as a candidate screening marker for the subclinical stages of leprosy. The role of haptoglobin as an anti-inflammatory agent and as a bacteriostatic agent had been suggested in humans [Langlois and Delanghe, 1996]. However, similar studies are very limited in mice. Time course analysis for plasma proteome profiling of *M. leprae* infected mice demonstrated an increase in the expression of acidic isoforms of haptoglobin β chain during the course of infection. Since glycosylation of haptoglobin β chain could be demonstrated by whole plasma deglycosylation assay, the increase in expression levels of acidic isoforms were attributed to an increase in glycosylation during infection which in turn is believed to enhance the half life of plasma proteins in circulation [Ashwell and Morell, 1974]. Even though the functional implications of acidic isoforms of haptoglobin in leprosy cannot be explained with the existing data, it can be suggested that an increase in glycosylation might be an approach to enhance the stability of this bactericidal protein during infection.
Haptoglobin is a major acute phase protein and responds to various stimuli and hence the upregulation of this protein might not be a specific indicator for *M. leprae* infection. However, it could be used as a complimentary method along with more specific approaches such as IFN-γ release assays to identify subclinical infection in healthy contacts and people from endemic areas.