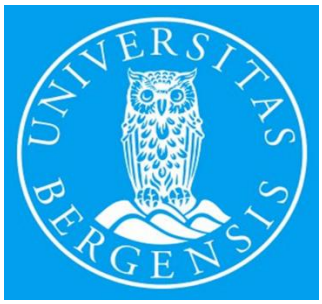
Identification of host biomarkers from dried blood spots for monitoring treatment response in extrapulmonary tuberculosis

Shizza Khalid



Center of International Health, Faculty of Medicine

University of Bergen, Norway

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Contents

Abbreviations and Acronym

WHO	World Health Organization
TB	Tuberculosis
MDR TB	Multidrug resistant tuberculosis
HIV	Human Immuno Deficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
CR3	Complement Receptors 3
MMR	Mannose Receptors
TLR	Toll Like Receptors TLR
TNF-α	Tumor Necrosis Factor-alpha
IL-1	interleukin 1
IL-12	interleukin -12
IL-18	interleukin-18
IL-10	interleukin-10
TGF-β	Transforming Growth Factor-β
IL-23	interleukin-23
Th2	T Helper 2
Th1	T Helper 1
IL-10	Interleukin-10
IFN-γ	Interferon gamma
TLR	Toll like receptors

NK	Natural killer cells
EPTB	Extrapulmonary tuberculosis
DBS	Dried Blood Spot
ELISA	Enzyme linked immunoassay
SDG	Sustainable Development Goals
PRR	Pattern recognition receptors
PAMP	Pathogen associated membrane pattern

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Introduction

1. Burden of Tuberculosis

TB is a major global challenge and remains to be one of the top ten causes of death worldwide from a single infectious agent, *mycobacterium tuberculosis,* ranking above HIV/AIDS (1). The high burden areas can be seen in figure 1, that contribute maximum to the TB burden. Tuberculosis is an epidemic that has affected a total of 10 million people worldwide in the year 2019 (1, 2). A total of 1.2 million mortalities amongst the HIV negative patients have been recorded in 2019 (1). Even though the line graphs in figure 2 show the global incidence and the incidence rate of tuberculosis to be declining, the numbers are still high (3). Majority of the cases reported to have tuberculosis are adults, men accounting for 56% of them followed by women (32%) and then children (1, 4). Significant mortality and morbidity occurs in the developing world due to tuberculosis (1). Eight countries account for majority of the TB burden of the world, Pakistan being one of them (1). Pakistan's TB incidence rate is 130 per 100,000 population and primarily harbors a major burden of multidrug resistant TB (1, 5). Economic instability, malnutrition, poor housing and non-adherence to treatment, are a few key reasons that contribute to the spread of tuberculosis is any population, including Pakistan (1, 3, 4). Studies have shown that, comorbidities like diabetes and HIV contribute to the increased susceptibility to tuberculosis and high mortality rates (3) . Tuberculosis is known to affect primarily young adults in low-income countries, and elderly in high income developed nations. In addition, the ongoing COVID 19 pandemic has affected the progress towards the ending the TB epidemic negatively as there has been a shift of resources and manpower towards combatting the COVID 19 pandemic (1). Due to this, the number of tuberculosis cases are anticipated to increase till 2025 according to the WHO Global TB report (1).

One of the targets of sustainable developmental goal (SDG 3) includes ending the TB epidemic by 2030 along with AIDS and hepatitis (1). In order for the world to reach the End TB Strategy goals and SDG targets, the current rates of TB decline will not be enough to reach the 20% reduction in incidence rate and 35% decline in number of deaths as seen in the circles in figure 2 (1, 5). Accelerated efforts need to be made in the areas of research for developing rapid, low cost, specific diagnostic and disease monitoring tools while ensuring universal health coverage (1, 5).

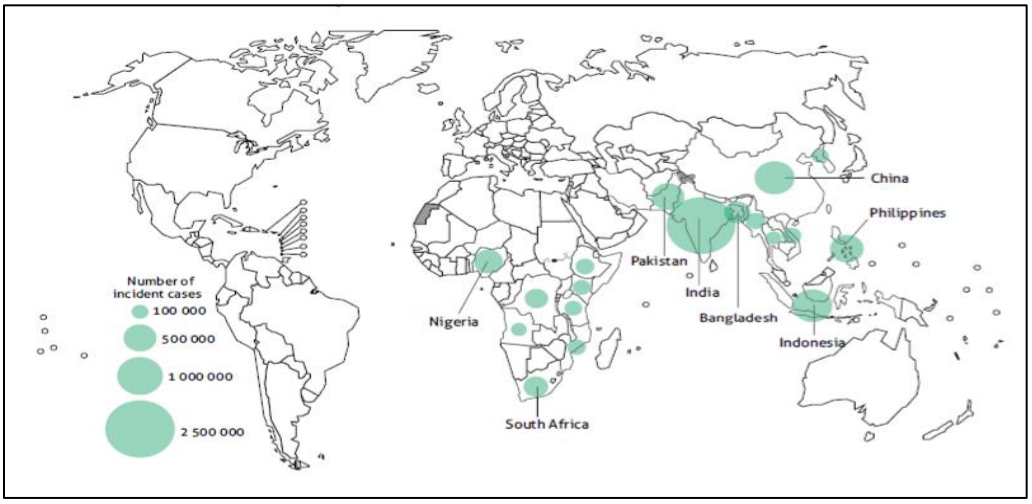

Figure 1. A world map showing countries with at least 100 000 incident cases in 2018(1).

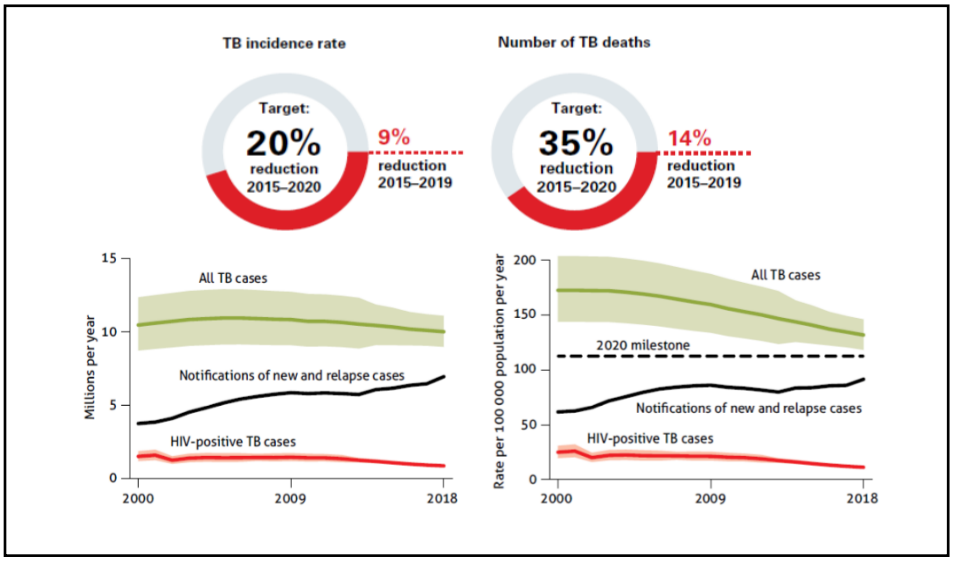

Figure 2. Line graphs showing the trend of the global TB incidence (left) and the incidence rate (right) of between the year 2000 to 2018 (1). Two circles showing the progression of the Sustainable Developmental Goals and End TB Strategy goals.

2. Immunological basis of Tuberculosis

Tuberculosis is caused by an air borne transmission of the mycobacterial containing droplet nuclei (3, 6). The transmission depends on the bacterial load of the droplets, the proximity with the infected individual, strength of host immunity and the virulence of the pathogen (2, 4). The location of the infection within the lungs is also a determinant of the clinical outcome of the disease (3). After the inhalation of the *Mycobacterium tuberculosis*, the interaction with the host immune system can result in one of the following outcomes.

a. The host immune system kills the mycobacterium immediately (3, 4).
b. Or the host fails to eliminate the mycobacterium leading to a primary infection (3, 4).

A primary infection may manifest as an active primary disease or remain as a latent infection. In majority (about 90%) of immunocompetent people, M. tuberculosis remain dormant and latent infection does not progress to active disease. Latent infection becomes active tuberculosis when the host undergoes immunosuppression (7). Ultimately the clinical manifestation of mycobacterium infection in the host depends up on the interaction between the host immune system and the evasive power of the mycobacterium (8).

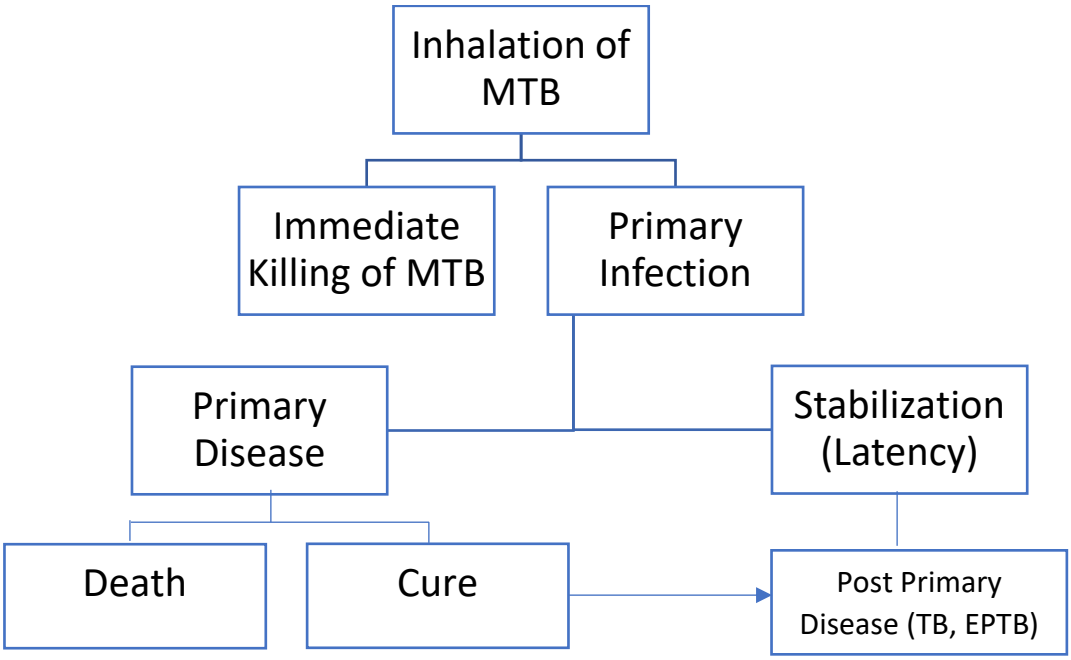

Figure 3. A flow chart showing different clinical manifestation following inhalation of *Mycobacterium tuberculosis*.

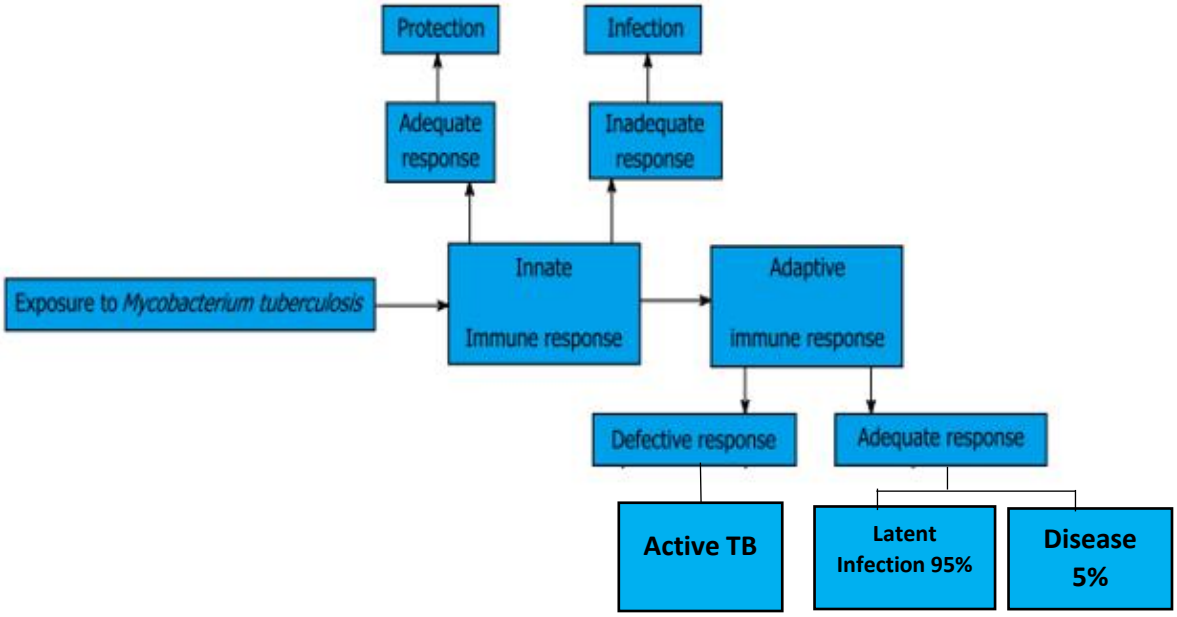

Figure 4. Role of innate and adaptive immunity in various clinical manifestation of *Mycobacterium tuberculosis* (8).

Innate immunity is the first line of defense against *Mycobacterium tuberculosis.* It is mainly mediated by the macrophage and the dendritic cells residing in the alveoli of the lungs (2-4). Pattern recognition receptors (PRR) present on the surface of macrophages and dendritic cells, such as, The toll like receptors, NOD like receptors, scavenger receptors, mannose receptors recognize the pathogen associated membrane pattern (PAMP) on the mycobacterium bacilli (3, 8). This leads to the phagocytosis of the bacilli by the macrophages and the dendritic cells (3, 8). As a result of this interaction, proinflammatory cytokines are released namely, tumor necrosis factor alpha (TNF- α), IL1B, IL 12, nitric oxide, IL 8, MIF and various chemokines (2-4). Chemokines are subfamily of cytokines that are chemotactic. They have an important role to play in recruiting inflammatory cells to the site of infection. They are classified into four subfamilies, namely, C, CC, CXC and CX3C (9). Each family has a distinct role in fighting the infection in the body.

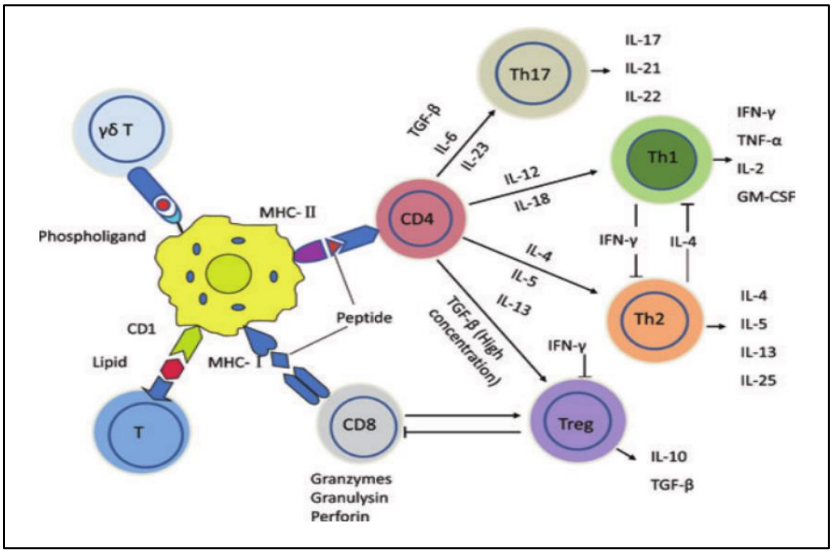

Figure 5. Interplay of the innate and adaptive immunity.

CCL-2, -7, -12 is responsible for the attracting macrophages and T cells in the initial stages of infection (9). CCL20 is specifically involved in increasing the expression of CCR6 on the T cells,

that are specific for Mtb antigens (9). CCL3,4,5 and CCL 19 to CCL21 mediate dendritic cell migration, while CXCL 1-8 attract neutrophils to the site of infection (9). Phagocytosis has an integral role in innate immunity against the pathogen with a subsequent formation of phagolysosome (2, 3, 8). However, *mycobacterium tuberculosis* can sometimes escape destruction from this process. After the engulfment of *M. tuberculosis* macrophages can undergo apoptosis which leads to a successful elimination of the mycobacterium (3, 4, 8), or formation of granuloma, which is the hallmark of tuberculosis (3). It can also lead to elimination of the bacilli or develop latency (4). If the macrophage undergoes necrosis, it leads to further spread and dissemination of mycobacterium tuberculosis bacilli (3, 4). When the innate immunity is not able to eliminate the infection, the adaptive immunity comes into play as shown in figure 5. The infected macrophages and dendritic cells produce several cytokines, including IL12, IL23, IL7, IL15, and TNF alpha (2). The dendritic cells residing in the lungs travel to the regional lymph nodes and play their role in activating B cells, T cells and Regulatory T cells by presenting mycobacterial antigens and mycobacterium to them (3, 4).

Resultantly, the activated B and T cells combat the bacilli by travelling to the infection site in the lungs by producing antibodies and cytokines (3, 4). As seen in figure 5, **T cells** produce Interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF-β). Interferon gamma (IFN- γ), produced by the CD4 T cells activates more macrophages and enhances their ability of phagocytosis (2, 3, 8). Tumor necrosis factor stimulate all three, the dendritic cells, T cells and induces macrophages to form granuloma. It also activates nitic oxide synthase (3). Presence of CD4 T cells play a key role in combatting the mycobacterial infection (3). Lack of CD4 T cells results in delayed immunity. IL10 and tumor necrosis factor beta (TNF-β) produced by the dendritic cells play their role in suppressing the inflammation. These anti-inflammatory biomarkers are produced to prevent

any excessive tissue damage at the disease site (8). Hence, this balance between pro and anti-inflammatory biomarkers, have a protective effect. CD4 T cells further differentiate into T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), and regulatory T cells such as Treg cells. Each type of T helper cells plays a distinct role in defense against mycobacterial infection (8).

Th 1 cells are involved in production of **interferon gamma** (IFN- γ), **tumor necrosis factor alpha** (TNF α), interleukin 2 (IL-2), lymphotoxin, and **granulocyte- macrophage colony stimulating factor** (GM-CSF). All these, then promote the stimulation of Th1 cells and maturation and activation of macrophage (2). On the other hand, **Th2 cells** produce factors that are involved in stimulation of B cells but also suppress the immune response mediated by Th1 cells (2). IL4, IL5, IL10 and IL14. IL23 produced during the innate immunity stimulates the Th 17 cells to produce IL17. Th 17 is the main player in the early phase of defense. **Th17** cells recruit neutrophils, monocytes and CD4 T cells at the site of infection by producing IL 21 and IL22 (2, 3). The stimulation and activation of CD 8 T cells is dependent upon CD4 T cells (3). **CD8 T cells** is involved in cytotoxic killing of the mycobacterium by TNF mediated killing of the infected cells (3, 8) and via the release of granzymes, granulysin, and perforins (2). The regulatory T cells are responsible in controlling the immune response to avoid tissue damage (3).

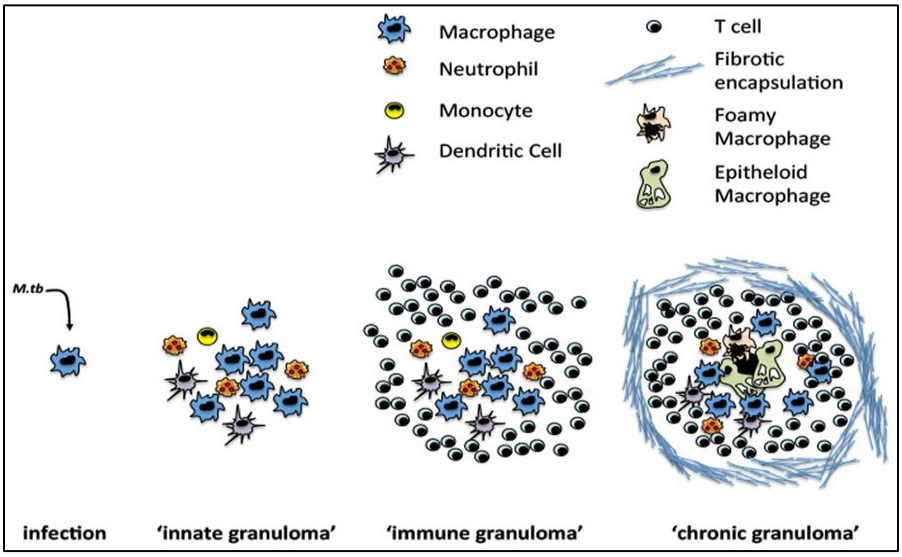

Figure 6. The evolution of granuloma (10).

Granulomas are formed as result of interaction between the human immune system and *M. tuberculosis* (3, 8, 10). Chemokines, CCL17 and 22 have a role in the organization of the granulomas and CXCL13 has a special role in organizing of the T cells in the granuloma in there correct location, as the organization of cells is critical in providing the best protection (9). In the initial phase of infection, the granulomas formed primarily comprise of the immune cells involved in regulating the innate immune response, including, macrophages, neutrophils, dendritic cells and monocytes (10). The center of the granuloma contains infected macrophages or multinucleated giant cells, surrounded by epithelioid cells, monocytes, neutrophils or dendritic cells as visualized in figure 6 (8). At a later stage, during the adaptive phase of the immunity, the granulomas have a macrophage rich center surrounded by B and T cells (10). As the disease progresses, the granuloma undergoes architectural changes. They are surrounded by the collagen fibers that encapsulates the granuloma (8). The center of the granuloma may undergo either caseation, necrosis, followed by

liquefaction or may undergo cavitation (3). These changes occur when there is reactivation of mycobacterium tuberculosis (8).

3. Extrapulmonary Tuberculosis

Infection of *mycobacterium tuberculosis* can occur in a localized or a disseminated form. The disseminated form of tuberculosis is called the extra pulmonary tuberculosis and occurs when inhaled bacteria spreads through lymphatic or hematogenous spread to different organs of the body other than the lungs. The most common sites of infection include lymph nodes, pleura, followed by urogenital system, gastrointestinal track and skeleton as shown in figure 7 (11, 12).

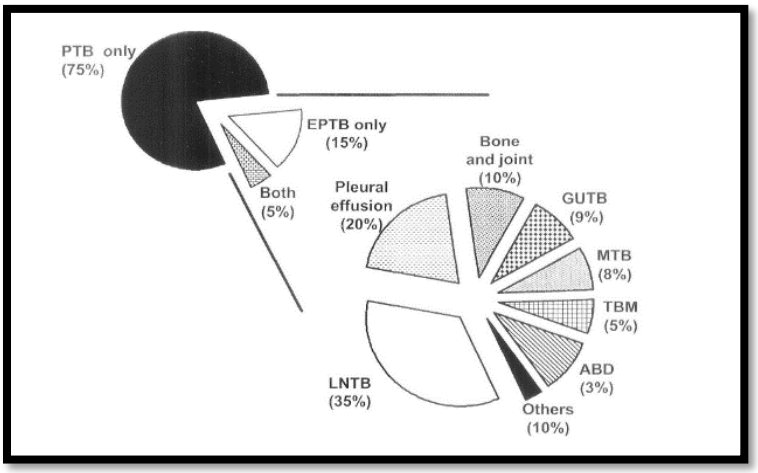

Figure 7. Proportion of different types of extrapulmonary tuberculosis (12).

In 2019, extrapulmonary TB accounted for 16 % of the 7.1 million incident cases, 24% in the eastern Mediterranean region (1). The actual numbers of people getting affected by extrapulmonary tuberculosis are believed to be under reported due to misdiagnosis and underreporting (12, 13). Extrapulmonary tuberculosis presents with symptoms that mimic anomalies of the organs affected. The manifestation of extrapulmonary tuberculosis has a strong association with immunosuppression (12). A paradoxical response to the anti TB treatment has

also been observed to be more in EPTB patients as compared to the pulmonary TB (13). The treatment regimen has to be tailored depending upon the type, severity of the disease and the nature of host response (13).

a. Challenges in the diagnosis of extrapulmonary tuberculosis and empirical treatment

The diagnosis of extrapulmonary tuberculosis comes with its unique set of challenges. Patients with extrapulmonary tuberculosis present with nonspecific signs and symptoms such as anorexia, malaise, weight loss, fatigue, fever and often mimics the anomalies of the organ infected, thus posing a diagnostic challenge (12) (14). All diagnostic methods used for EPTB have low sensitivity (7, 15). As per routine, the initial investigation is done via acid fast bacilli smear, culture and histopathology, but the isolation of the specimen is challenging because of inaccessible disease sites (7, 13) (11). The paucibacillary nature of the disease does not guarantee isolation of the mycobacterial bacilli in the obtained specimen. Due to this, the available methods stand short for making a definite diagnosis of EPTB. Staining and culture have limited sensitivity of 0 to 40 % and 30 to 80% respectively (7, 13). Whereas culture results take up to 4 to 8 weeks to be available which is time consuming, and expensive for the health systems. Other investigations such as fine needle aspiration cytology, histology, biopsy, PCR, endoscopic ultrasounds, or CT scans may follow in order to make the most accurate diagnosis but cannot be used in isolation (13). Evidence of acid-fast bacilli, granulomas, caseation or cavitation can be seen by in an infected specimen through histopathology, still, it cannot be used as a gold standard for diagnosing as granulomas are also present in other disease such as syphilis, brucellosis and other fungal diseases (13). Manipulation of body fluids can also be used in making good clinical judgements, as changes in the levels of the constituent biological molecules can be used as surrogate for an ongoing disease

process. **Lymphocytosis, leukocytosis** and raised protein levels, are observed in EPTB, but the same changes are seen in other infectious diseases (13). Polymerase chain reaction test and GeneXpert/RR are the two tests that involve multiplication of mycobacterium DNA present in the sample and are far less time consuming (7) . These tests have high specificity and sensitivity, but it varies with the type of EPTB (13). Their performance is dependent on the bacterial load of the specimen, which is a challenge in the EPTB (16). Imaging techniques such as X rays, ultrasound, CT/PET scans also aid in making diagnosis of extrapulmonary tuberculosis (12).

In short, due to the lack of a definitive diagnostic tool for EPTB, its diagnosis is made on empiricism (13).

b. Current methods available for treatment monitoring of extrapulmonary tuberculosis

The treatment of extrapulmonary TB includes a two-month intensive phase and a four-month long continuation phase (7) (11). In the intensive phase, patients are treated with isoniazid, rifampicin, ethambutol and pyrazinamide, while in the continuation phase only isoniazid and rifampicin are continued (7, 11). All these drugs have multiple toxic effects such as hepatitis, arthralgia, ototoxicity, that eventually become the reason for nonadherence in many patients (17). Treatment monitoring is a crucial aspect of the management of EPTB as its diagnosis is done empirically (15). Many a times obtaining follow up specimen becomes a challenge because of the inaccessible disease site (18). At times, treatment duration, or treatment regimen needs to be altered too depending upon the type of EPTB or due to drug resistance or a response delay (7, 11, 13). Monitoring is important as it can prevent over or under treatment, which furthers leads to increased mortality and morbidity. There is also a lack of established protocol or laboratory tools, with a satisfactory level of sensitivity and specificity that can be employed for this purpose. In this

scenario, it is hard to manage EPTB with accuracy. Improper management has also lead to an increase in multidrug resistant strains of mycobacterial tuberculosis, which is an emerging challenge for the world (19).

1. Monitoring of presenting signs and symptoms

Due to paucibacillary nature of extrapulmonary tuberculosis, the smear and culture have a limited role in monitoring treatment response. Monitoring via improving signs and symptoms of the patients, during the treatment has yielded to be a reliable and a cost-effective method (15, 20). Even though EPTB often presents with nonspecific symptoms, clinical parameters such as improvement in weight, presenting signs and symptoms such as regression of the enlarged lymph nodes in case of tuberculous lymphadenitis or exudative fluid in tuberculous pleuritis are some examples that indicate an encouraging response to treatment (15). However, it is a subjective method and can be misleading, especially in the presence of concomitant diseases (14). Many a times, the treatment response does not coincide with the resolution of clinical signs and symptoms and thus, cannot be made the basis for the modifications of treatment plans in these patients (18).

2. Smear and culture microscopy

Smear and culture specimen of the infected site, obtained either through FNAC or biopsy can be used to evaluate the response of the patient to treatment (19). It is important to do sputum microscopy in every suspected case of EPTB to rule out concomitant pulmonary TB (21) However, there role is limited because EPTB is a paucibacillary disease, and the test has limited sensitivity and specificity (19). Furthermore, it is unpleasant and costly for the patient to undergo periodic invasive procedures for the sake of monitoring treatment response.

3. Imaging Techniques

Since extrapulmonary tuberculosis is a multiorgan disease, the use of imaging techniques has their due importance in surveillance of the disease. Xray, Ultrasound, PET/CT scans and F 18 fluorodeoxyglucose positron emission tomography/emission tomography, all are being used in clinical practice in monitoring the treatment response. Ultrasonography is performed in patients with suspected pleural effusion, or abdominal lymphadenopathies or micro abscesses (22). It has been useful in monitoring the resolution of abdominal ascites with successful treatment (22). F 18 fluorodeoxyglucose positron emission tomography/emission tomography is useful in locating and gauging the extent of EPTB in the body (20). This method uses the hypermetabolic activity of the lesion to its advantage (20). It is also helpful in measuring the response of the tuberculous lesion to treatment (20). However, this method is expensive, it cannot be used in low resource settings. It also cannot differentiate between other inflammatory conditions and even though imaging techniques are important in following the response to treatment, they cannot be used in singularity (20).

c. Role of biomarkers in treatment monitoring

Relying on the current tools and techniques for treatment monitoring in extrapulmonary tuberculosis management have their obvious limitations (7, 23). No one method has enough sensitivity or specificity to be used in singularity and many are high-cost methods that cannot be employed in a low resource setting with limited infrastructure and facilities. That is why, the monitoring of EPTB treatment is done subjectively with the aid of a combination of therapy monitoring tools, especially in the resource constraint parts of the world. Keeping these challenges of the current methodologies in mind, there is a need of a tool that is objective, robust, inexpensive,

and non-invasive (19). Use of biomarkers can solve many challenges in the effective management of EPTB as they possess all the above-mentioned characteristics. An abundant number of inflammatory biomarkers such as cytokines and chemokines are produced during the disease process. These inflammatory mediators are then released in blood and can easily be measured to gauge the treatment response. Biomarkers have a potential to be objective and highly sensitive, and there use in EPTB management is crucial in decreasing mortality (19, 21). In contrast to pulmonary tuberculosis, a limited number of studies have been done regarding the role of biomarkers as potential surrogate for treatment response in extrapulmonary tuberculosis. Some of the biomarkers that have been investigated are.

1. IP 10

IP 10 is a proinflammatory chemokine that is viewed to have a great potential to be used in EPTB management. It is secreted by the antigen presenting cell in response to interferon gamma produced by the T cells and is responsible for leukocyte activation and migration in TB infection. Many studies have shown its level to decline significantly in blood, plasma and urine with effective TB treatment (19). It has proved to be a robust candidate biomarker that remains stable in dried blood and plasma spots, and it has the potential to be used as a point of care test for treatment monitoring of EPTB (19) . IP10 is expressed at high levels in the body fluids which makes it a good candidate for being used in commercial ELISA (24). In some instances, the levels of IP10 have also been observed to increase in case of treatment failure, or paradoxical reaction, but the data is limited in this regard (19, 21). More studies with larger study groups are required to understand its role better, especially in HIV co infected, pregnant patients and patients with limited disease (19, 21).

2. Interferon gamma response assay

The interferon gamma release assay has been reported to show change in its levels during treatment in extrapulmonary tuberculosis (7). It is produced in response to two secretory protein of mycobacterium tuberculosis bacilli, ESAT- 6 and CFP – 10 (7). However, many individual variations have been reported in the previous publications and more studies are required to validate its use in therapy monitoring and as a potential biomarker (7, 25).

3. T cell response

It has been suggested that the mycobacterial load influences the phenotypic expression of the MTB specific CD 4 T cells (7). Successful anti TB treatment decreases the mycobacterial load and therefore restores the T cell proliferation capacity (7). Studies have also revealed a decline in the proportion of regulatory T cell subsets (7). However, the use of cellular analysis is expensive and cannot be carried out in all settings, as the cellular viability is highly vulnerable to temperature change and storage conditions (7).

4. Acute phase proteins

The acute phase proteins are produced in the body as a result of an ongoing infection or inflammation (25). These proteins, particularly, **C reactive protein** have a potential of being used as a biomarker for monitoring treatment (25). However, CRP is not raised in many cases of EPTB and hence not reliable for treatment monitoring (25, 26).

5. Biosignature of MIG, IP10, CCL23 and CCL22

In the sister study of my project, forty inflammatory biomarkers are investigated in an unstimulated plasma, for their potential to be used as a tool for therapy monitoring in extrapulmonary tuberculosis. Bio-Plex Pro TM Human Chemokine Panel reveals the detection of all the forty inflammatory biomarkers in plasma. According to the study, levels of MIG/CXCL9, IP-10 and

CCL23 significantly decline with successful treatment amongst tuberculous lymphadenitis and tuberculous pleuritis. A biosignature comprising of MIG, IP10, CCL23 and CCL22 has the potential to predict treatment response in 97% of patients after 2 months and 99% of patients after 6 months of treatment (27). It has been observed that the use of a biosignature instead of a single biomarker can be more reliable for therapy monitoring in EPTB (27).

Plasma sampling is an invasive procedure which requires trained technical staff, and fully equipped laboratories to be carried out (28). It is important that the standard operation procedures are adhered to, in order to prevent incorrect sampling or contamination (28, 29). Plasma samples are susceptible to change in their chemistry if a strict cold chain is not maintained for their storage and/or transport (29). For our study to be relevant to the low resource settings, and for the purpose of establishing a point of care test, we have investigated the same forty biomarkers in dried blood spots.

Study Objective

The objective of this study was to explore the possibility of identifying novel host biomarkers in the dried blood spots of the patients with extrapulmonary tuberculosis for monitoring treatment efficacy with a potential to be developed as a point of care test.

The specific objectives were.

a. To identify novel host biomarkers in dried blood spots
b. To identify a combination of biomarkers which can predict response to treatment in maximum number of patients.

Simple ELISA versus multiplex ELISA

As the name suggests, sandwich ELISA is an assay procedure which involves wrapping an analyte of interest between a primary antibody and a secondary antibody (30). These tests are carried out in a 96 well plate with a flat bottom. ELISA test is rendered to be more sensitive and specific in detecting and quantifying analytes as compared to preceding bioassay tests (30). The mechanism of coupling of the antigen and the antibody is the main reason for its specificity (30). In sandwich ELISA, the quantity of the analyte in the specimen is measured indirectly via the enzyme conjugated with the secondary antibody. This can be in the form of fluorescence, change of color or light emission produced as a result of a reaction between the substrate and the enzyme (30). It is a sensitive test, because the analyte of interest is already attached to the well of the 96 well plate. The popularity of ELISA over the years is primarily because of the wide variety of analytes it can detect and quantify such as antibodies, RNAs, DNAs, hormones and tumor markers (30).

However, ELISA has its set of limitations in the sensitivity and specificity it offers. Firstly, there is always a chance that the protein of interest does not attach to the primary antibody, and the interaction between primary and secondary antibody produces a false positive signal. There are higher chances of this when working with plasma samples that constitute multiple proteins (30). Secondly, there is a greater margin of intertest and inter-plate variability (31). The quantity of reagents, buffers, chemicals and sample dilution may differ from one plate to the other. It is crucial for the accuracy of the result that the assays are consistent (30). Readout of the results may differ depending upon the technique used or the material, color and the cleaning of the well plate (30). ELISA tests are time consuming with lengthy assays, that require large amount of

reagents and samples (30, 31). Ultimately, rendering them to be more expensive, especially for large scale scientific studies (30, 31). Even with extra precautions, many errors can arise during performance of ELISA, resulting in its limited reliability and reproducibility (30).

Over the years, efforts have been made to modify the ELISA to counter its limitations. One of the modifications includes the introduction of microbeads (30). Use of microbeads have many advantages attached to it. Firstly, it has made multiplexing possible with minimum margin of error (30). Moreover, the spherical shape of the bead provides mores surface area for analyte to bind and improves antigen and antibody interaction, leading to a higher detection signal for an analyte of interest (30). Due to the freedom of mobility that comes with use of micro beads, they can be applied to many varieties of detection systems (30).

Further results of the study are in the manuscript.

Biorad 40 plex Bio-Plex Pro TM Human Chemokine Panel

Biorad 40 plex Bio-Plex Pro TM Human Chemokine Panel used on Luminex xMap TM (seen in figure 8) is an extension of ELISA, that employs microbeads in a suspension array system to simultaneously qualify and quantify analytes using flow dual laser system. The Bio rad bio plex suspension array system uses the principle of sandwich ELISA to measure molecules bound to the surfaces of the fluorescent microspheres, providing real time digital analysis It can detect and quantify up to 100 different analytes in less than 30 minutes (32). It is a multiplex immunoassay system that consists of a fully integrated array reader, microplate platform, micro- beads and assays. The validation and calibration kits are provided with the bio plex. It uses the feature of multiplexing, that decreases the sample volume, reduces the volume of assay reagent, expenditure, labor, and generates more information about the inter relation of the analytes (33, 34). The samples

can be serums, or culture medias and the volume of the sample can range from 15 to 50 µl. The assay specific microbeads are coupled with capture monoclonal antibodies that bind to a specific analyte to be detected. (31-34).

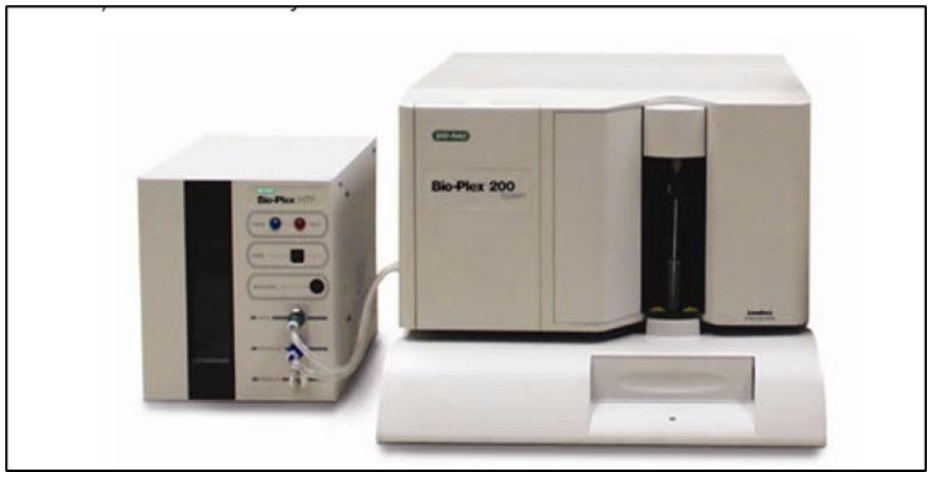

Figure 8. Biorad 40 plex Bio-Plex ProTM Human Chemokine Panel (33)

As visualized in figure 9, the microsphere beads are conjugated with two different types of dyes. Each bead is conjugated with a different ratio of dyes. Every bead is distinguished by its own signature fluorescence, and provides real time data, with no spectral overlap (33, 35). Multiple capture sandwich immunoassays are formed on their surface, as seen in figure 10 (31, 33). Multiple independent measurements within each microsphere population for a single biomarker is obtained. This is the main reason for the high precision in a multiplex ELISA (36).

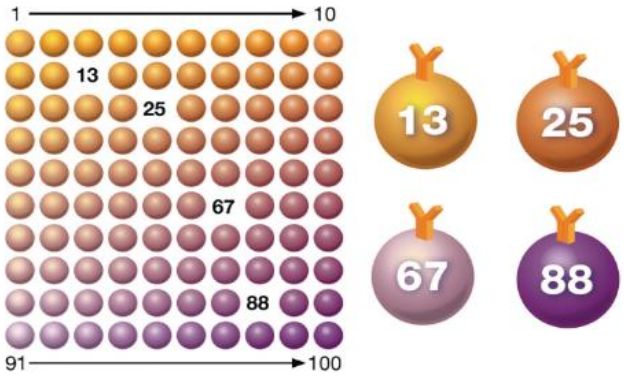

Figure 9. Microbeads conjugated with two different dye ratios

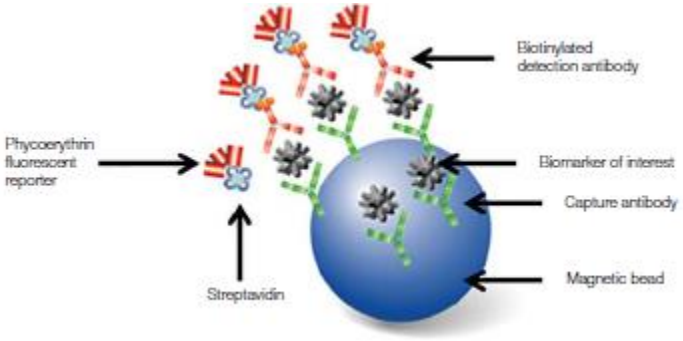

Figure 10. Sandwich immunoassay on a microbead

Methodology

In my study, we used the bio plex chemokine panel to analyze forty different analytes in the elutes of dried blood spots. A protocol was established to make dried blood spots from the plasma samples of 105 EPTB patients. Each bead was conjugated individually, with a different analyte (35). After the coating was done, the additional sites on the beads were blocked, and the beads were plexed together in a single well as seen in figure 11 (31). The elutes of the dried blood spots were added in the vile with forty beads coupled with forty different analytes. The antibody's

present in the elute sample then bind to the analytes (33, 35). After that detection antibody was added, which is also coupled with phycoerythrin fluorescent reporter (34).

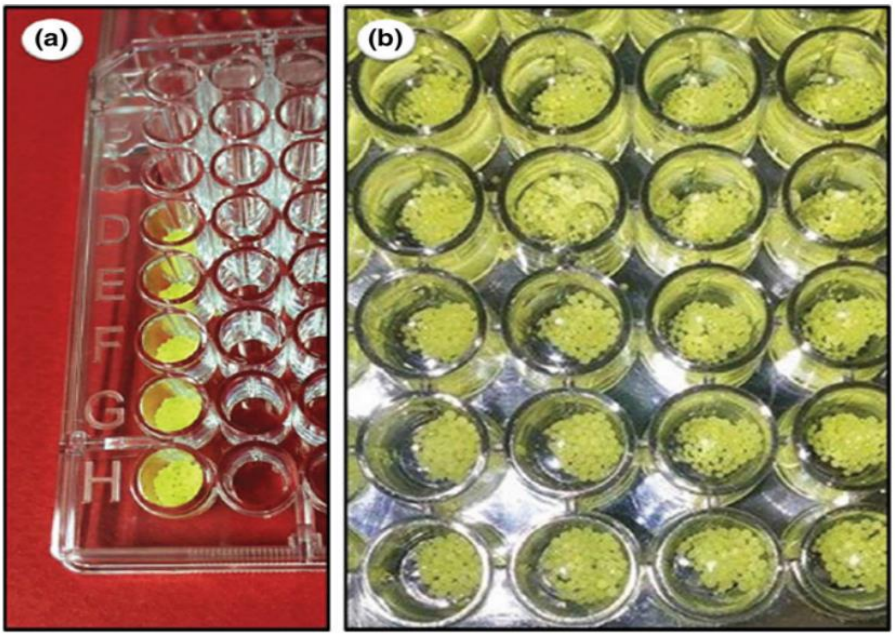

Figure 11. A 96 well plate with integrated microbeads

Bioplex chemokine panel also makes use of a 96 well plate. Picture of a well plate can be seen in figure 12. Plate formatting is done as per the instruction's manual. Figure 13 shows how the plate formatting is done. There is a separate well for standards, controls, sample, unknown and some are blank wells. Assay was performed as per the manufacturer's instructions manual. Each well and its contents serve a unique function. The sample containing the unknown quantity of analyte is added to the wells labelled as unknown. Standard solution is added in wells designated as standard wells. A standard is a solution whose analyte concentration is known. The standard solution is used to qualify and quantify analytes in the sample under investigation and are used to generate a standard curve, which is used as a reference to measure analytes in the sample. A minimum of eight standards of different concentrations are added in the wells. The lowest and

highest concentration of standards are achieved by serial dilution and to produce a standard curve in the display (33, 35). Based on the concentration of the standard, a regression curve equation is created by the system to measure the concentration of the standards versus the analyte. The nonlinear standard curve is created by the software based on the concentration of the standards (33). The data points that fall on the standard curve, within the lowest and highest detection limit of the standard curve, are considered a valid reading (35). The control wells contain **samples** of known concentration. They help to calculate the expected versus the observed concentration of the analytes (33). The blank wells contain all the components of the assay apart from the analyte. They are important as they help to reduce the effect of auto fluorescence on the final reading (33).

Figure 12. 96 well microtiter well plate

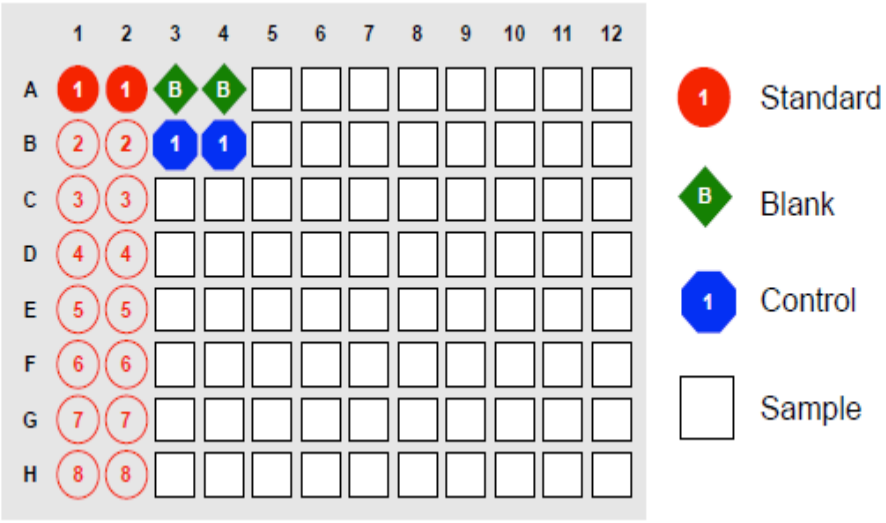

Figure 13. The plate formatting

After the plate formatting, the plate was placed in the machine and the system was run. The beads were then drawn up into a suspension array and passed through red and green laser beams one by one(35) (33). The red laser is responsible for **identifying** the cytokine, while the green laser excites the phycoerythrin bound to the detection antibody for **measuring** the amount of fluorescence by each bead (33). As many beads as can be added for each analyte. Resultantly, a standard curve is generated for each cytokine, corresponding to the fluorescence generated.

Data Analysis in Bioplex suspension array system

The fluorescence is directly proportional to the amount of analyte in the sample (34) .The results are displayed in the form of a histogram and bead maps (33). The bead map shows different bead regions or bead types of different coatings. Each bead is recognized as a distinct location in the bead map as seen in figure 14. They are formed due to the raw fluorescence data from the phycoerythrin that is conjugated with the detection antibody. The numerical values of the standards and the unknown are also based on the raw fluorescence. The value of the unknowns depends upon

the quality of standard curve. We get the standard curve based on the values we get after comparing the observed with the expected concentration of an analyte. Multiple standard curves are formed for each analyte. The concentration of the analyte has a linear relation to the fluorescence generated by that analyte. The lowest value of the standard curve corresponds to the most diluted standard and the highest value corresponds to the least diluted standard. A typical standard curve generated by the system can be seen in figure 15. Ideally, the values of the unknown should fall on the standard curve (33). The bioplex software has the function to optimize the standard curve, and automatically fit all the data points on it. There are certain readings (observed concentration) that are within the detection limit of the instrument but above or lower than the standard detection limit and the concentration range of the standards. They are seen as <OOR or >OOR in the raw data (33). These values appear with the aster in the observed concentration values row. Only the values that are within the range of standard curve are reported with a numerical value.

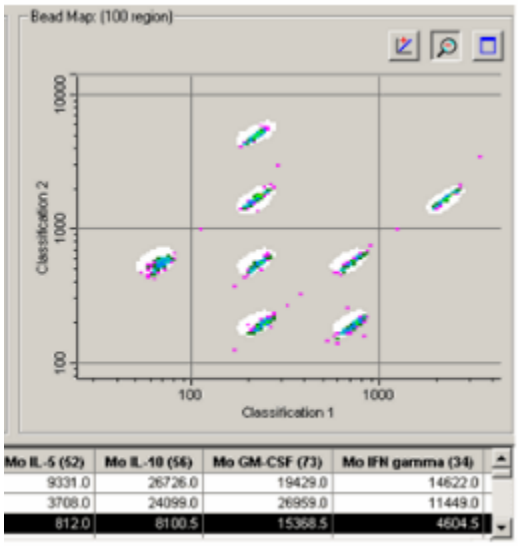

Figure 14. A bead map showing distinct locations of bead regions (33)

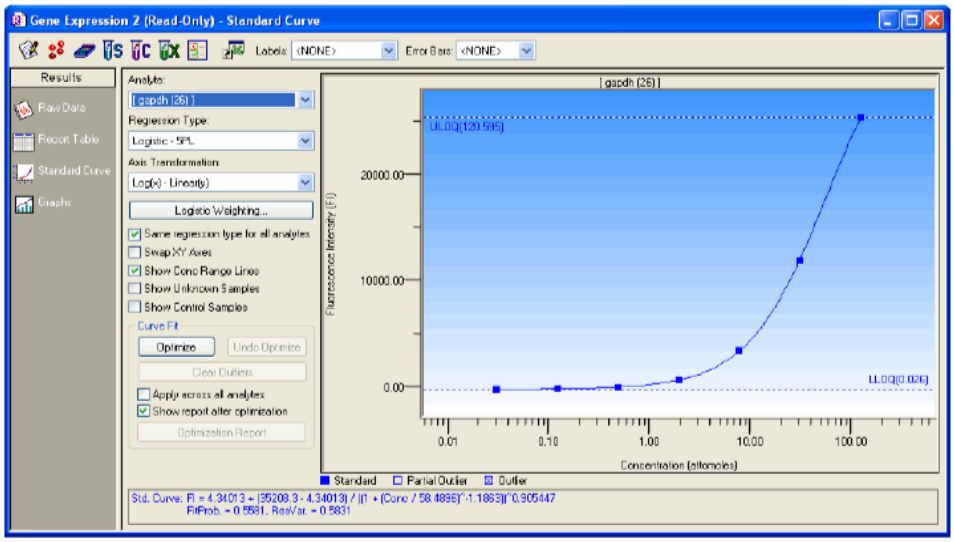

Figure 15. Standard curve (33)

Limitations of Multiplex assays

Bioplex multiplex array system offers more sensitivity and specificity as compared to the conventional sandwich ELISA method (31, 34). However, like any other technology, bioplex has also its set of technical and operational limitations (36).

Firstly, there is always a chance of cross reactivity in multiplex ELISA. The presence of multiple analytes, proteins and antibodies in a sample/assay solution in a mobile state (because of the presence of microbeads) increases the possibility of cross reactivity amongst the molecules (36). While working with plasma and serum, the chances are higher because they already have a high protein cellular content (37).

Secondly, even though use of multiplex ELISA is cost effective as compared to the simple ELISA. The purchase amount of the multiplex technology is quite high. There is also an additional cost of

the assay kits that need to be obtained by manufacturers to run the assays. This limits its use in a resource constrained laboratory setting (37).

Thirdly, the quality of standards and controls is crucial in avoiding any intra assay variation. The commercially available ELISA kit obtained from different manufacturers may have some variations and affect the accuracy of the results (36). Hence the standard curve that is generated might differ with each manufacturer. It is also important to recheck the regression analysis to ensure accuracy. As values calculated based on a suboptimal curve will affect the values obtained of the unknown analyte (37).

Another challenge faced by many scientists is the interpretation of very low and high values that do not fall on the standard curve and many a times reporting these values can be highly problematic. All the above-mentioned limitations can be a potential source of imprecision and inaccuracy in multiplex ELISA, but their effect can be minimized with gaining more experience in operating the multiplex technology and by adapting good laboratory and quality control practices (37).

Study Setting and Participants

This was a prospective cohort study that was carried out at Gulab Devi Hospital, Lahore, Pakistan. Pakistan has ranked fifth amongst the high TB burden countries of the world. In recent years, an increase in the incidence of EPTB has been reported in Pakistan (38). According to the Global TB report 2019, currently Pakistan has TB incidence rate of 570 000 per 100 000 population, as indicated in figure 16. There is a high loss to follow up rate, resulting in treatment failure, development drug resistance and further transmission of the disease (39). Currently the

incidence of multidrug resistant cases is 25 000 per 100 000 population (1). The rate of treatment success in Pakistan is around 70% which is far less than the target set by the WHO (39).

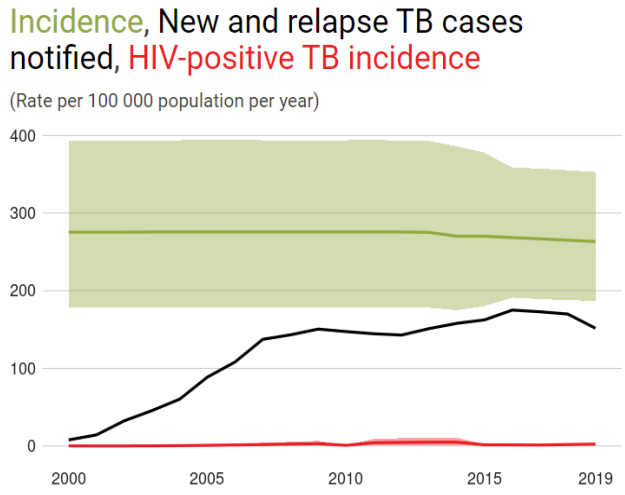

Figure 16. A line graph showing the overall incidence rate of TB, rate of new and relapsed TB cases notified and the HIV positive TB incidence in Pakistan.

Tuberculous pleuritis and tuberculous lymphadenitis have been reported to be the most common form of EPTB in Pakistan, followed by abdominal TB and tuberculous meningitis (38, 39). A female predominance was reported amongst the tuberculous lymphadenitis patients in a nationwide multicentric study (38). Similar statistics were mirrored in my study, where TB lymphadenitis and tuberculous pleuritis were only types of EPTB that we had in our study group. Fifty-seven patients out of the total 105 patients had tuberculous lymphadenitis, with 42% of them being female patients. Figure 17 summarizes the composition of the study cohort.

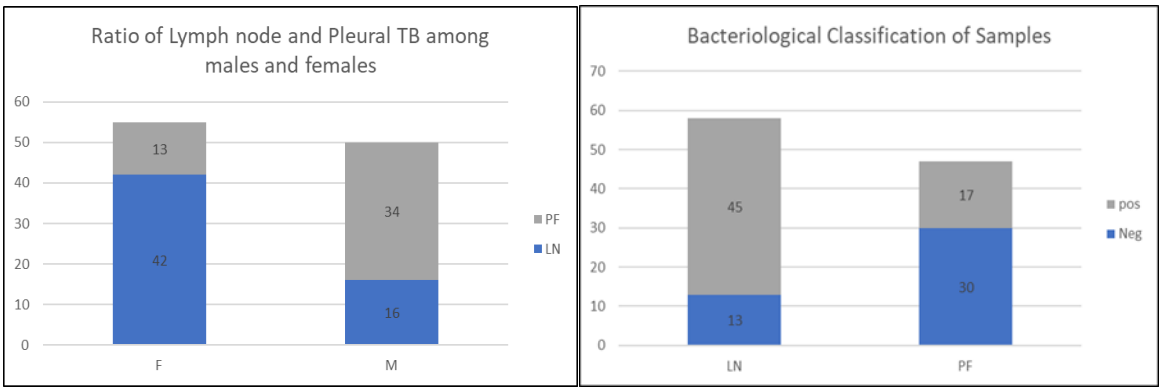

Figure 17. a. Bar charts showing ratio of males and females amongst the TB pleuritis cases, b. showing the ratio of bacteriologically confirmed cases in both types. PF; pleural fluid, LN; lymph node, pos; positive, neg; negative, M; male, F; female

Results

The results of the study can be reviewed in the attached manuscript.

Discussion

The results and findings have been discussed in the attached manuscript.

Implications of my findings in low resource setting for TB control

It is possible to translate the findings from this study into a simple ELISA based method for monitoring response to treatment. Blood sampling from a finger prick and preservation of samples on the protein saver cards makes it feasible to implement the method in the low resource settings where the samples can be transported from small centers to a central lab. However, development of a point of care test by using dried blood samples in low resource settings will not give us the desired control over TB if the health care is not supportive. Ones the dried blood

spots are taken from the patients, their proper handling, storage and timely transport to the centralized laboratory is necessary for achieving the desired goals in disease management.

Every country has a national TB control program whose aim is to combat the growing TB burden in their respective countries. Many of these countries have not performed well enough to reduce the incidence and prevalence of TB to an acceptable level. There are many contributing factors for this failure. One of the reasons is the lack of a well-structured and a well-integrated health care system. For many health interventions to be effective, proper disease management facilities need to start at the grass root level. Many a times patients can be saved from the adverse effects of the anti TB treatment and repeated cycles of treatment, if they are offered effective, inexpensive and timely treatment monitoring tools. This also ultimately reduces burden on the health care system (40).The larger burden of TB is present in peri urban and rural areas of the developing world. These areas, lack well equipped and functional health care centers that can be useful in providing timely basic health services (40). An open channel of communication, between the health facilities at every level is important to ensure that the EPTB are notified, and a proper call re call system should in place for the treatment monitoring of the EPTB patients. It is also important to ensure the attendance of a medical staff in health facilities.

In many settings, such as Pakistan, ones the diagnosis is made, the follow up and disease management is quite poor (41). Diseases such as diabetes, HIV and other immune compromising diseases are a risk factor for tuberculosis. Strengthening of the basic infrastructure is imperative to enable timely screening and referral of patients at the risk of developing TB. This can lead to an early detection of cases. The availability of better and user-friendly diagnostic and patient management tools can ensure a holistic approach to patient care.

In summary, no intervention can bring the desired results in singularity. The use of dried blood spots, as a point of care test for treatment monitoring in EPTB will not be able to reduce disease mortality or morbidity if a well-integrated health system is not in place. There needs to be a strong commitment by the government, to provide health services at every level. Research and improvement in the delivery of health services needs to go hand in hand. Our efforts will render useless in the absence of health accessibility, surveillance and monitoring at every level.

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