REVIEW OF LITERATURE
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2.1 End TB

In 2014 the World Health Assembly adopted the World Health Organisation’s (WHO) “Global strategy and targets for tuberculosis prevention, care and control after 2015. The main aim of this twenty year strategy is to end the global TB epidemic and this is called END TB. Ending TB is defined as an incidence rate of less than 10 people per 100,000 population per year. The incidence rate is the number of new cases of active TB disease in a population in a particular time period.

The main targets in the End TB Strategy are:
- To reduce TB deaths by 95%
- To cut new cases of TB by 90% between 2015 and 2035
- To ensure that no family is burdened with catastrophic expenses due to TB.

WHO quotes that Ending TB is not a public health problem, but a development challenge and opportunity. Ending the TB epidemic is a target under the Sustainable Development Goals that requires implementing a mix of biomedical, public health target and socioeconomic interventions along with research and innovation. There are three pillars which encompasses the End TB strategy.

- integrated, patient-centred care and prevention
  - early diagnosis of TB including drug susceptibility test and screening of contacts and high risk groups
  - treatment of persons with both drug sensitive and drug resistant TB and patient support
  - collaborative between HIV and TB and management of co-morbidities
- bold policies and supportive systems
  - political commitment with adequate resources for TB care and prevention
  - engagement of public services committees, civil society organizations, and public care providers
Universal health coverage policy, and regulatory frameworks for case notification, vital registration, quality and rational use of medicines, and infection control

- Social protection, poverty alleviation and actions on other determinants of TB
- Intensified research and innovation
  - Discovery, development and rapid uptake of new tools, interventions and strategies
  - Research to optimize implementation and impact; and promote innovations

Global elimination of TB as a public health problem, defined as <1 TB case per million population, is a long-term vision of WHO’s End TB Strategy, while the time-bound global target is to “End the global TB epidemic”, defined as bringing down the global incidence from >1,000 per million population in 2015 to <100 per million by 2035 [WHO, 2017].

In connection to achieving END TB strategy, our aim began with exploring the proteome of \textit{M. tuberculosis} and characterizing few antigens proposed to be drug targets from \textit{in silico} analysis.

\subsection*{2.2 \textit{Mycobacterium tuberculosis}}

\textit{Mycobacterium tuberculosis}, the etiological agent of tuberculosis disease is one of the ancient pathogens that have a staggering global impact. It is a fairly large non motile rod-shaped bacterium distantly related to the Actinomycetes. The rods are 2-4 micrometers in length and 0.2-0.5 micrometer in width. MTB is an obligate aerobe. It is for this reason the bacilli are found in the upper lobes of lungs which have proper aeration. They grow inside macrophages as a facultative intracellular pathogen with a slow generation time of 15-20 hr indicating its physiological characteristic in virulence.
Mycobacterium species are impermeable to certain dyes and stains and thus they are classified as acid-fast bacteria. But once stained with acid-fast stain, the bacilli upon heating and treating with acidic organic compounds retain the dye. One of the acid fast stains used to identify or detect MTB is Ziehl-Neelsen stain. In this method, the strain smear is fixed with Carbon-fuchsin dye (pink color) and decolorised with acid-alcohol. The smear is then counter stained with methylene blue or any other stain in order to view. The acid fast bacilli appear pink in the contrasting background. Since the generation time of the bacilli is too long, appearance of even a single organism during the staining process clearly shows that the patient is being “suspicious” of infection with the bacilli.

The impermeability of MTB to different stains is mainly due to the complex architecture of its cell wall. The cell wall is very unique and also the major determination of virulence for the bacterium. Apart from peptidoglycan, the common deposit of the cell wall in all bacteria, the bacilli are made up of complex lipids. Nearly 60% of the cell wall components are lipids and are composed of mycolic acids, cord factor and wax-D.

Mycolic acids are unique alpha-branched lipids making up 50% of the dry weight of the cell wall envelope. They form a hydrophobic shell around the bacterial cell wall making the cell impermeable to cationic proteins, lysozyme and oxygen radicals in the phagocytic granules. The cord factor is a glycolipid molecule (trehalose dimycolate). It influences the arrangement of MTB cells into long and slender structures and plays an important role in the virulence to the host. On the other hand, wax-D, a mixture of peptidoglycan and mycolic acid, possesses biologically important properties such as Freund’s adjuvant effect.
This intracellular pathogen has high GC content in its genome of 4.4 million base pairs that contains 4018 protein coding genes. The genome was sequenced in 1998 [Cole et al., 1998]. 26% of the genes of MTB, called leaderless genes, lack 5' UTR and hence the Shine-Dalgarno sequence used for ribosome engagement is commonly used among them [Cortes et al., 2013]. The organism lacks horizontal gene transfer unlike E.coli and also genetic recombination thus keeping its genome in complete linkage [Rose et al., 2013; Portevin et al., 2011]. Technological advancements of high throughput screening paved way in exploring the diversity and evolution of MTB with respect to the host. Since their origin, the bacilli have co-evolved, migrated and expanded within the human host [Comas et al., 2013]. To date, there are approximately seven lineages of strains infecting humans. Each lineage is associated with the geographical regions and on an average; any two strains differ by 1200 SNPs [Gagneux et al., 2006]. As a contrast to many bacteria, these SNPs occur in the coding region of the genome and more than half fall into highly conserved positions.
thereby causing functional changes and consequently implicated in the phenotypic diversity of the pathogen [Comas et al., 2010; Hershberg et al., 2008].

### 2.3 History of tuberculosis

Tuberculosis, the contagious and infectious disease, caused by *Mycobacterium tuberculosis*, long lasts inside the host and forms tubercles in different parts of the body. The bacilli are of very ancient origin and survived over 70,000 years. Though not proceed to active disease, nearly one third of the human population contain the infection.

The bacillus was first ever founded on the skeletal deformities of Egyptian mummies way back to 2400 BC. The term tuberculosis is coined from Latin named tuberculata which means small lumps and refers to the small scars seen in the tissue of infected persons. Scientific understanding of the disease and the bacilli advanced considerably in early 19th century. It was in the year 1882, Robert Koch revealed that the disease was caused by an infectious agent. He proposed a staining technique to detect the presence of bacilli in the sputum of the infected person. In 1908, the French Scientists Albert Calmette and Camille Guerin developed the first genuine vaccination against TB and it was introduced to the world in 1921. It was called Bacillus Calmette-Guerin or simply BCG. The discovery of antibiotic streptomycin against tuberculosis in 1944 was considered as the beginning of modern era of tuberculosis. Further improvements in the treatment regimen- the discovery of isoniazid and rifampin hastened recovery times and decreased the mortality rate in those time period.

### 2.4 Etiology and epidemiology of tuberculosis

TB, caused by MTB is transmitted from one person through air by inhalation of airborne particles containing *M. tuberculosis*. This contagious disease is disseminated primarily through coughing or other forceful operations leading to respiratory outcome by a person having active pulmonary tuberculosis disease. The spread of the infection is more prone from a person detected to be sputum smear positive in comparison to smear negative and also from a person having cavitary disease than those without. This transmission of infection happens only upon inclusion of certain factors:
a) presence of viable bacilli in the sputum,
b) concentration of bacilli in air,
c) immunity of the host,
d) duration of exposure to the infected person.

The high TB burden in low income countries are indicated by environmental and nutritional factors. Similarly, environmental and socio-economic factors contribute to high risk of acquiring and developing TB. For example, homeless and under-house groups are expected to be at high risk of acquiring TB infection and disease [Bamrah et al., 2013; Feske et al., 2013; Haddad et al., 2005; Khan et al., 2011]. Besides all these factors, it is the intrinsic virulence of the organism which always determines the factor of infection and disease.

Tuberculosis disease continues to be a major health concern worldwide despite administering proper vaccination and treatment. It is said that about one-third of the world’s population is infected with the bacilli and new infections occur at the rate of one per second. Though not all the infections with MTB are converted to active disease, many infections are asymptomatic. TB is the second most common cause of death from infectious disease after HIV. Despite observing a decline in the disease incidence, prevalence and mortality over the last decade, elimination of the disease in global level is still challenging. It is estimated that about 10.4 million cases worldwide in 2015 and nearly 1.5 million died.

WHO has estimated that in 2016 [WHO, 2016], the accountability of TB cases in South East Asia Region is 45%, Africa is 25% and Western Pacific region is 17% with smaller proportions of the cases in Eastern Mediterranean Region (7%), European (3%) and American region (3%). In 2016, the number of TB cases in a year in relation to the population size was found to be varied from under 10 per 100 000 population in most high-income countries to 150–300 in most of the 30 high TB burden countries. Regionally, the fastest decline in TB incidence is in the WHO European Region (4.6% from 2015 to 2016). It was found that among the HIV-negative people, about 82% of TB deaths occurred in 2016 which was found prevalent in the WHO African Region and the WHO South-East Asia
Region. This percentage accounted for 85% of the combined total deaths due to TB in HIV-negative and HIV-positive people. The statistics in India showed that 33% of Indians accounted for global TB deaths among HIV-negative people, and for 26% of the combined total of TB deaths in HIV-negative and HIV-positive people.

2.5 *Tuberculosis disease burden in India*

The persistence of high incidence, mortality and drug-resistance of TB in India may be contributed to five reasons:

1. Social and economic determinants- malnutrition, diabetes, smoking of tobacco- are poorly addressed in India.
2. Shortfall in the need of economic investments by the government in order to achieve access to quality diagnosis and treatment of TB.
3. Limited scaling up of rapid, molecular testing
4. Weak implementation of RNTCP in the state level
5. Flourishment of private sector for TB diagnosis and treatment

According to WHO reports, 2017, the TB epidemic is much larger than earlier thought, particularly in the WHO South-East Asia Region (SEAR). This is mainly due to the occurrence of “missing cases” which accounts to nearly 4.3 million. When corrections were included, the number of cases raised by 34% between 2013 and 2015. In SEAR, besides India, Bangladesh, Indonesia and Myanmar are the countries which are included and also substantially contribute to the “missing cases”.

India accounts for nearly quarter of world’s TB burden. In 2016, as many as 28 lakh new TB cases developed and 4.5 lakh people died due to TB disease [WHO, 2017]. The TB epidemic in India is much larger than previously estimated but the incident rate and number of deaths continues to fall as a result of improvised prevention and treatment. Though India ranked to be the major source for the Region’s TB numbers, it actually ranks number six in terms of incidence rate. Between 2010 and 2015, around 49 million lives were saved due to
better diagnosis and treatment regimen. Yet gaps are still persisting which need to be filled in order to meet the “End TB” goals.

2.6 Entry mechanism of bacilli

Upon entry into the alveolar passages of the exposed humans in an aerosol droplet, the bacilli encounter the residential macrophages and dendritic cells. Dendritic cells play a major role in the initial events of antigen presentation and activation of T cells with specific antigens of MTB. Now the macrophages recognize the bacilli through pathogen activated molecular patterns (PAMPs) present on the bacterial surface and thus the host innate immune response is initiated. The recognition is achieved by a number of receptors exposed on the immune cells of the host like toll like receptors (TLR), nucleotide binding oligomerization domain (NOD) like receptors and C type lectins. The interaction of the TLRs in the host cells initiate the signalling cascade events leading to proinflammatory response. Apart from these the fig 2 shows the various receptors involved in the entry of MTB into macrophages. A complex combination of these factors plays an important role in the ability to mycobacteria in establishing a mechanism of entry.

2.7 Tuberculosis pathogenesis

The bacterial load plays an important role in the pathogenesis of TB. An aerosol droplet containing a load of 1-400 bacilli may end up in infection. After the entry of the aerosol droplet into the host, the bacilli may end up with three fates:

a. If the load is less and the host is immunocompetent, the bacteria upon engulfment by the macrophages may get killed by the immune cells present in the lungs.

b. Though there is effective mechanism of killing of bacilli by the host immune cells, some of the bacilli remain alive. The survivors infect the macrophages where effective replication of the bacilli occurs until they burst out of the macrophages leading to further infection.
c. The intracellular bacilli have capacity to exist in dormant stage. The activated T lymphocytes resist multiplication of bacilli and their spread to adjacent compartments. Now the bacilli change their adaptive behaviour by fine tuning the phenotypic expression and persist in resting state or latent state.

In order to define the various microbial factors important for virulence and persistence, molecular and microbiologists decoded the survival strategies of *Mycobacterium tuberculosis*, i.e., physiological and metabolic adaptation to the host environment, dynamics of replication, and synthesis and structures of the cell wall. If the growth of the bacilli inside the host becomes uncontrolled, it causes extensive lung damage ultimately leading to death of the patient by suffocation due to insufficient oxygen. As a result of anoxia, the lung parenchymal cells involved in oxygen uptake get destroyed and due to granulomatous growths, the bronchial passages get obstructed.
2.8 Survival strategies of MTB

MTB upon infection should get adapted to the host environment in order to achieve infection and survive inside the host. MTB adopts three survival strategies: immune modulation, dormancy and phagosomal rupture [Meena and Rajni, 2010; Gengenbacher and Kaufmann, 2012; Conrad et al., 2017] in order to establish a proper niche inside the host.

• Firstly, MTB is a master in modulating the immunity of the host. It interferes with host cell signalling pathways there by carefully balancing the production of cytokines involved in the activation of pro and anti-inflammatory responses [Dietzold et al., 2015; Guirado et al., 2013; ]. MTB balances these responses and delays phagosome maturation, harvests needed nutrients from the host immune cells and stimulates the formation of granulomas [Silva et al., 2012].

• Secondly, during residence inside the granuloms, MTB enters a metabolically inactive and non-replicating state which is resistant to reach of most types of drugs [Gomez and McKinney, 2004]. The bacilli manipulate the macrophages in such a way that they can reside inside for multiple decades by feeding on the required nutrients obtained from accumulation of lipids [Silva et al., 2012; Kapoor et al., 2013; Paige and Bishai, 2010; Shaler et al., 2013; Russell et al., 2009].

• Thirdly, the most severe condition of the bacilli is the rupture of phagosome and release of bacilli in the alveolar environment [Simeone et al., 2012; Sani et al., 2010]. This causes necrosis of the host cells and thus the bacilli will now have access to the nearby macrophages to reside to.

2.9 A delayed T cell response

The T cells which recognize the different peptides and antigens from MTB proliferate to increase their number in order to migrate to the lung. Until this step, the bacilli grow and replicate in an uncontrolled manner and achieve a higher level of infection. The T cell response mentioned above will take weeks to develop and this is one of the disadvantages for the host immune system to fight against MTB and advantage for the organism to establish itself in a proper niche. It is established that when a human is infected with this bacilli, it
takes 6 weeks for the T cells to recognize the bacteria [Wallgren, 1948; Poulsen, 1950]. In other case of infection, the T cell responses can be detected in seven to ten days.

A study conducted on the mice infected with a low dose of aerosolized MTB revealed that there are two phases to the delay [Cooper, 2009]. In the first phase, it is observed that the T cell response do not even initiate until eleven to fourteen days of post infection. In order to establish the same, the bacilli must be first inhaled into the lung where it gets phagocytozed by macrophage. The bacilli happily replicates within the macrophage until the macrophage dies and spills its cargo. This is taken up by another macrophage and the process goes on until the cargo or the bacilli are phagocytozes by dendritic cells. The dendritic cells then transports the bacteria to lymph node approximately nine to eleven days post infection. These cells present antigen to T cell which then initiates response in the lymph node [Wolf et al., 2008].

The second phase of delay occurs after the transport of MTB to the lymph node and T cell responses have been initiated. In order to control the infection, T cells specific for MTB antigens must proliferate, migrate to the lung and produce factors called cytokines that boost the infected macrophages to kill MTB or aid in decrease its replication [Cooper 2009]. This process however, occurs very slowly during infection. In an infected mouse, the T cells do not reach the site of infection in sufficient numbers to support the slowing of bacterial replication until three weeks post infection. They do not peak in number until four weeks of infection. Simultaneously, the bacilli load gets increased in the lung. Thus, if T cell arrives early, it can be proposed that the bacilli are controlled at much lower levels and the mice can survive for longer time. On the contrary, if T cells arrive late, the outcome is worse and it may even be too late to control the infection.

2.10 Granuloma

The alveolar macrophages once infected with MTB, release a number of cytokines in order to recruit various immune cells including more macrophages to the site of infection. Of the various immune cells, dendritic cells play an important role as they present antigens to the T cell of the lymph node where T cell response can subsequently be developed. On the
other hand, the invitation of new non-activated macrophages serves as feeder cells for further growth and multiplication of the bacilli. The recruited cytokines like TNFα and IFNγ accelerate inflammatory cell infiltration. These signalling pave way in the formation of granuloma (fig 3), the hallmark of tuberculosis disease. Granuloma can be defined as an inflammatory mononuclear cell infiltrate that, while capable of limiting growth of MTB, also provides a survival niche from which the bacteria may disseminate. Thus granuloma is composed of blood-derived infected and uninfected macrophages, foamy macrophages, epithelioid cells and multinucleated giant cells (Langerhans cells), B and T lymphocytes and fibroblasts [Russell 2007; Ramakrishnan 2012]. In the presence of activated T cells, the granulomabecomesfullyorganized,withmycobacteria-harboring macrophages at the center surrounded by a rim of lymphocytes. The dynamicity of granuloma causes continues loss of cells by apoptosis and tissue remodelling. The bacilli residing inside the granuloma uptake nutrients from the immune cells and thus cause death of the cells. This leads to formation of cavities inside the granuloma and the core of granuloma shows caesation, necrosis and lack of oxygen. As MTB is a facultative aerobe, the bacilli can persist inside this environment with limited metabolic activities and this state is called the dormant or latent state in which the pathogen may live for decades. In summary, early innate responses of the host to clear the pathogen do little to restrict and much to promote the replication of the same.

2.11 The spectrum of tuberculosis infection

Virulence life cycle of Mycobacterium tuberculosis and progression of TB:

When a person infected with TB sneezes or coughs, the pathogen is carried over as droplets and is capable of spreading to another person through air, a few meters away. This is called primary infection. Upon primary infection, the bacilli enter the alveoli of the lungs which become their ultimate place of residence (fig 4). Now, the entry of foreign materials trigger the immune cells to act immediately causing cell-mediated innate immunity which either results in elimination of the bacilli or emergence of T-cell response and activation of CD4+/CD8+ cells, TNF-α, IFN-γ, ROS, RNS etc. This adaptive response of T-cell activation and its synergy of activations may end up with three fates-
(i) clearance of the pathogen;
(ii) persistence of bacilli as latent infection which is asymptomatic or
(iii) active replication inside the macrophages leading to active TB disease showing symptoms.

The latent infection may persist inside the host for lifetime or may get re-activated and cause disease. Re-activation may occur due to a number of risk factors which include HIV infection, diabetes, genetic factors, immune-suppression, tobacco smoke, alcohol consumption, indoor air pollution etc. Besides, improper intake of medicine and discontinuation of the treatment regimen have also lead to the emergence of drug resistant forms of bacilli which are difficult to treat. Ultimately, the infected people become the carrier of infection and thus the transmission cycle starts over again. Thus the outcome of TB infection is generally distributed between active and latent tuberculosis (fig 4).

2.12 Active TB:

Active tuberculosis disease is contagious and upon left untreated may lead to death of the patient. In active TB, the main organ which is affected by the bacilli is lungs. But the infection can spread to other organs like kidney, brain, stomach and brain. Pulmonary tuberculosis (TB in lungs) accounts for the most cases of TB infection. Extra-pulmonary condition occurs in only minimal population. A person is said to have pulmonary TB (PTB) if he has prior encounter with MTB and the bacterial load is more and the person is immunodeficient. It is more commonly seen by endogenous reactivation of bacteria residing in latent state in the lungs of the infected individuals or re-infection by exogenous bacilli. It is reported that nearly 5-10% of latently infected persons may be converted to active disease if their immunity go down and also due to absence of treatment. In PTB, the bacilli grows and multiplies in the lungs and when the host immune cells try to eliminate the pathogen, cellular immune responses occur which leads to tissue necrosis and formation of cavities.
Fig 3: **Granuloma formation**: The picture represents the stages of a pathophysiological continuum and also MTB life cycle with either retarded growth or metabolic adaptation within the lesion following granuloma disruption. Italics indicate the various cellular and humoral mediators involved in granuloma differentiation. (Courtesy: Ehlers and Schaible, 2013)
Fig 4: Life cycle of *Mycobacterium tuberculosis*: The five stages

Fig 5: Spectrum of tuberculosis infection. (Courtesy: Douglas *et al.*, 2009)
2.13 Primary disease

Primary disease occurs when there is no history of previous exposure to the pathogen previously or not immunized and inhales the aerosol droplets containing MTB. In this type, the source of organism is exogenous and about 5% of the newly infected persons develop significant disease. Upon infection, the bacilli locate themselves in the subplueral mid zone of the lungs or the alveoli (Marais et al., 2004). In primary disease, granulomatous lesions are formed at the site of infection due to delayed hypersensitivity reaction. When the bacterial load is less, infection still persists but does not show up any symptoms. Depending upon the immunity of the individual, the infection is either cleared or progresses to active disease. In immunocompromised and malnourished individuals, the disease may develop without interruption into progressive disease. Cell mediated immune response play an important role in the switch in the state of infection. In PTB, the regional lymph node plays an important role in the protection.

2.14 Primary progressive disease

The infection progresses to disease when the person infected with bacilli becomes immunocompromised. The foci at which infection happens continues to grow and the caseous material disseminate to other parts of the lungs leading to necrosis of the tissues. The infected focus may go on to heal as caseating granulomas are replaced by fibrosis and calcification. These growing lesions will erode the bronchi draining their contents and forming cavities. The bacilli may erode blood vessels and enter circulation, the condition called miliary tuberculosis. Alternatively, erosion of the pleura causes pleural effusion or emphysema. Also, the spread of infection to other parts of the body may lead to extra-pulmonary tuberculosis.

2.15 Extra pulmonary TB

Extra pulmonary TB (EPTB) refers to TB infection in organs other than lungs, for example, pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones or
meninges. The EPTB may occur in isolation or along with a pulmonary focus as in the case of patients with PTB. It constitutes about 15 to 20 percent of all the cases of tuberculosis in immunocompetent patients and accounts for nearly more than 50 percent of the cases in HIV positive individuals. It is majorly present among children of > 4yr and young adults. Lymph nodes are the major victim of EPTB. Diagnosis of EPTB is very difficult and the conventional diagnostic methods have a poor yield and the diagnosis is often delayed. The Ziehl-Nelsen and auramine staining allow detection of bacilli from EPTB samples. The smear positive cases may be localized by examining the biopsy samples rather than the biological fluids. Further, the availability of computerized tomographic scan, magnetic resonance imaging laparoscopy, and endoscopy has helped in the identification of location of EPTB.

The symptoms of EPTB include fever, anorexia, weight loss, malaise and fatigue. Apart from this, people with EPTB manifest symptoms and signs related to the organ system involved. The disease responds to standard antimycobacterial drug therapy.

2.16 Latent infection

Recent WHO (2017) reports that *Mycobacterium tuberculosis* causes nearly two million deaths annually and 10.3 million new cases annually which place the disease among the top three fatal infections. “Dormancy or latency” is an anthropomorphic term derived from the Latin *dormire*, meaning “to sleep,” and has been used to designate multiple conditions in which the bacteria are viable but exhibit reduced metabolic activity. The persons who are infected with the pathogen but do not show any signs and symptoms of the disease are categorized as latently infected people. However, majority of the infected people could combat and control the pathogen. These latently infected (LTBI) individuals may remain with the pathogen residing inside them throughout their lifetime without recurrence. Thus, it can be said that, the LTBI persons act as reservoirs of future source of active TB [Milburn *et al.*, 2007]. Due to immune suppression, the bacilli may get reactivated in the LTBI individuals and nearly 5-10% may get converted to active disease in their life time. The actual number of persons getting converted to active disease depend upon the waning in the host immune response to the bacilli, co-infection with other
diseases such as HIV, diabetes, alcoholic liver disease, malnutrition, use of steroids or other immunosuppressive drugs. It is unknown that reversion of LTBI individuals to active disease is due to reactivation of latent bacilli or re-infection with new load of bacilli.

The detection and treatment of LTBI individuals play an important role in the control of the disease [American Thoracic Society, 2005]. Diagnosis of LTBI remains uncertain because of the lack of clinical signs, symptoms and image findings. Tuberculin skin test and IGRA are the two tests currently under use for diagnosis of LTBI. But these two tests cannot distinguish between active and latent TB infection. Unavailability of appropriate biomarkers in the diagnosis kits which can distinguish between active disease and LTBI from the immunological perspective may also be a reason for its uncertainty. Since treatment regimen for both types of infection differ, addition of surrogate markers to the commercially available kits (Quantiferon) may improvise the situation.

Latent MTB infections are difficult to treat and can persist lifelong. Several in vitro culture studies mimicking the complex host environment such as exposure to hypoxia and nitric oxide resulted in the identification of a phenotypically drug-tolerant, nonreplicating form of MTB. This form of MTB is controlled by the dormancy survival regulon (DosR) (Boon and Dick, 2002; Park et al., 2003; Voskuil et al., 2003). It is shown that DosR is required for survival of the bacilli during dormancy state in vitro and the same can be achieved or induced upon infection of macrophages in animal models and humans (Boon and Dick, 2012). In depth study on DosR regulon is going on owing to its importance for MTB persistence and latency and most studies have focused on transcriptional regulation and only a limited information are available for the respective proteins.

2.17 Factors driving latency

The success of MTB relies in the achievement of latent state which is resistant to host immune response and also antimycobacterial treatment. The major factors which drive the organism to latency are starvation of nutrients and lack of oxygen or hypoxia. Studies have been conducted by growing bacilli in low oxygen condition and it was found that the
organism can well establish itself in hypoxia condition and metabolically regulates the expression of certain genes essential for survival at this stage. Nutrient starvation is the other factor leading to latency. In a granuloma, the bacilli feed on the macrophages and thus deprivation of nutrients from the granuloma due to cell death or necrosis may lead to activation of certain stress related genes in the bacilli for proper survival (Betts et al., 2002).

2.18 Immune response to MTB

The protective and pathological response of the host towards MTB is a complex reaction and involves various components of immune system. It involves an active interaction between the immune cells of the host and the virulence of the pathogen (fig 6). In order to limit the spread of the bacilli and control the infection, generation of T-cell response and the recruitment of inflammatory cells which surround and contain the infected macrophages. The acquired immune response to *M. tuberculosis* infection can be divided into three overlapping phases, namely:

a. The initiation and development of specific cellular immunity.
b. The expression of protective immunity in the lung.
c. The maintenance of protective immunity.

The initiation and development phase is characterized by the activation of T lymphocytes. As soon as the infection is encountered, dendritic cells (DCs) and macrophages constitute the first line of defence and play major role in the clearance of the bacilli [Dorhoi and Kaufmann, 2015; Lowe et al., 2012; Srivastava et al., 2014]. DCs internalise the infective bacteria, process the antigen, migrate to the draining mediastinal lymph nodes and present their antigens to T cells, thereby activating T cells and initiating the onset of the adaptive immune response [Jiao et al., 2002; Marino et al., 2004; Savina et al., 2007]. Many lymphocytes aid in controlling mycobacterial infection in the lung which include CD4, CD8, γδ and some non-classical lymphocytes. The most compelling evidence that CD4 T cells aid in controlling PTB is the fact that TB is one of the diagnostic indicators for AIDS [Garcia et al., 1995 Drobniewski et al., 1995]. On the other hand, macrophages uptake the pathogens by phagocytosis, expose them to acidic, hydrolytically active environment of the
phagosome, thus triggering a signaling cascade that leads to the fusion of the phagosome with the lysosome [Armstrong and Hart, 1971]. Whichever cells are important in controlling bacterial growth, the most important aspect of their development is that they are able to enter the alveolar site where infection is progressing.

In the expression phase of protective immunity, there is upregulation of several selectin, integrin and addressin molecules on both leucocytes and endothelial cells and they mediate further recruitment of other leucocytes extravasation into the lungs [Ebnet et al., 1999]. The ordered structure called the granuloma is then formed as a further step to combat the infection. Macrophages, together with epithelioid cells and multinucleated giant cells (also known as Langhans’ giant cells) and T lymphocytes are also the principal cellular constituent of granulomas [Flynn and Chan, 2001; Flynn and Chan, 2003]. This well established niche serves two purposes- modulates the immune response over a long period of time by maintaining the content of granuloma; provides a proper shelter for the bacilli to survive in the harsh condition.

![Host immune response against Mycobacterium tuberculosis](image)

**Fig 6:** Host immune response against *Mycobacterium tuberculosis*. (Courtesy: Douglas et al., 2008)

The clonal expansion of immune cells towards the site of infection leads to the production of proinflammatory and anti-inflammatory cytokines. While TNF-α, IFN-γ, and
IL-1β play an important role in the shielding function of the granuloma, IL-10 is one of the main negative regulators of the inflammatory response [Kaisho and Akira, 2000; Takeda et al., 2003; Aderem and Ulevitch, 2003].

It is evident from the studies of the murine granuloma that as the time increases, the lymphocyte density lessens as the pathogen residing inside the granuloma feeds on the surrounding cells and this may aid in the burst of the granuloma and release of more bacilli to the alveolar environment exposing themselves to the other nearby immune cells and causing infection. Thus, in order to maintain the phase of protective immunity, maintain the integrity of granuloma and control the bacterial replication, prolonged memory T-cell response is required.

2.19 Various immune cells and cytokines involved in fighting against the infection:

Macrophages

Alveolar macrophages are the first cells infected by the inhaled MTB and they act as first line of defense with highly regulated immune response. The interactions between macrophages and the bacilli are mediated by a number of molecules such as toll like receptors (TLR), FcR, complement receptors (CR1, CR3 and CR4), mannose receptors, surfactant protein receptors, CD14 and scavenger receptors. The activated macrophages stimulate the expression of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-8 and IL-10. The cytokines IFN-γ and TNF-α, show synergistic effect on MTB. They initiate the production of reactive nitrogen species (RNS) by activating nitric oxide synthase-2 (NOS2). The macrophages, after engulfing the bacilli try to fuse with lysosome resulting in decrease in pH of the system there by leading to inhibition or arrest of the growth of MTB [Russell et al., 2007].
**Dendritic cells (DC):**

DCs are antigen presenting cells (APCs) and also seem to play a key role in triggering T cell responses [Tailleux et al., 2003; Tian et al., 2005]. Upon infection with MTB, they become activated through TLR signals. DCs become mature and migrate to peripheral lymph nodes. During this process, DC may express surface markers, adhesion molecules and co-stimulatory molecules (CD80, CD86). DCs induces T cell maturation in the direction of Th1 profile The pathogen impairs DC migration to lymph nodes and their antigen presentation capacity through the down regulation of MHC class II molecules thereby limiting the adaptive immune response.

**Neutrophils:**

Neutrophils are short living but at the same time the most abundant leucocytes and they play crucial roles in the innate immunity of the host. Upon infection with MTB, neutrophils arrive first at the infectious site [Appelberg and Silva, 1989]. They phagocytose the bacteria recognizing them either directly or through Fcγ and complement receptors [Witko-Sarsat et al., 2000]. Neutrophil priming is the initial step of the phagocytosis and the subsequent pathogen killing process. The effective killing of the bacilli needs prior exposure of neutrophils to cytokines (eg. TNF-α, IL-1β), pathogen-associated molecular patterns (PAMPs), chemokines and growth factors or cell interaction with activated endothelial surfaces [Futosi et al., 2013; Summers et al., 2010]. Apart from these functions, neutrophils act as APCs for T lymphocytes [Beauvillain et al., 2007]. They carry antigens from the peripheral sites to the lymph nodes and bone marrow and aid in the generation of Th1, Th17 and CD8+ memory responses [Abi et al., 2011; Duffy et al., 2012; Kalyan et al., 2014].

**Natural killer cells (NK cells):**

NK cells constitute about 10-15% of the total lymphocytes present in the peripheral blood. The best function of NK cells is cytotoxicity, which target cells such as tumor cells, cells infected with viruses or intracellular pathogens like *Mycobacterium tuberculosis*. NK
cells through the production of IFN-γ, activate macrophages and the activated macrophages produce IL-12. IL-15 and IL-18 and recruits more CD8+ and NK cells [Abbas and Lichtman, 2006; Vankayalapati and Barnes, 2009; Cooper and Khader, 2008]. NK cells recognize MTB-infected macrophages through various receptor molecules involved in the lysis of MTB-infected cells [Vankayalapati et al., 2002; Vankayalapati et al., 2005; Esin et al., 2013]. It is evidenced that NKT cell deficiency might be crucial for the development of active TB in MTB infected patients [Rijavec et al., 2011]. Thus the NKT cell levels were found to be less in the peripheral blood pulmonary and extra pulmonary TB patients [Kee et al., 2012].

**CD4:**

The T cells play an important role in the immune response against MTB and are mainly related to the presence of Th1 cells, which produces a number of mediators such as IFN-γ leading to the activation of infected macrophages [Del Prete et al., 1994]. At the first site, the DCs activate T cells present in the lymph nodes which recruits the migration of MTB specific T cells to the blood and then to the area of primary infection in the lung. The activation and migration are also driven by chemokines. Once attaining the infection site, T cells actively participate in controlling the infection [Wolf et al., 2008; Chackerian et al., 2002]. The role of T cells in the protection of infection is demonstrated by the effects of TNF-α blockers. They block the cytokine production by T cells and interfere with the MTB-specific induced proliferation, thus increasing the risk of infection or MTB reactivation [Saliu et al., 2006; Geldmacher et al., 2010; Dixon et al., 2010].

**CD8:**

The role of CD8+ cells in TB control and their mechanism of action is still controversial. Presence of CD8 cells in the airway lumen at the beginning of infection confirmed the role of CD8+ cells. They mainly play effector functions which are represented by the ability

a. To lyse the infected cells such as macrophages and DCs;
b. To produce IFN-γ, but to lesser extent than CD4+ T cells; and

c. To effect the process of killing the bacilli through the production of grazymes and perforins

d. To induce apoptosis of infected target cells through molecules such as Fas or TNF-R family related cell death receptors.

**IFN-γ:**

IFN-γ promotes antigen presentation and the recruitment of CD4+ T lymphocytes and/or cytotoxic T lymphocytes and aid in mycobacterial killing. It is one of the crucial cytokines contributing protective immune response against MTB. It is a proinflammatory cytokine produced by CD4, CD8 and NK cells and activates macrophages to kill intracellular bacilli. IFN-γ, along with other cytokines and chemokines mediates protective immune response. It has been reported that TB-specific IFN-γ production might be useful for identifying patients with progressive infections who may likely to develop the disease [Anderson et al., 2007 B]

**TNF-α:**

It is a proinflammatory cytokine which aids in recruitment of cells to the site of infection, promotes granuloma formation and anti-microbial activity of macrophages [Mohan et al., 2001; Algood, 2005]. It aids in activation of macrophages and induce reactive nitrogen species production. Mice deficient in TNF-α or its receptors are more prone to MTB infections [Bean, 1999]. It plays dual role in the host- protective against MTB [Bean, 1999, Keane, 2005] and development of immunopathology associated with TB [Flynn and Chan, 2005].

**IL-1β:**

IL-1β is a pro-inflammatory cytokine which is mainly produced by monocytes, macrophages, and DCs [Kleinnijenhuis et al., 2018]. IL-1β was shown to mediate signals throught its receptor in response to MTB infection [Sugawara et al., 2001]. It was observed
that IL-1β is targeted by a MTB protein Rv0198c, which negatively modulates IL-1β activity there by resulting in the quenching of the inflammatory response [Master et al., 2008].

**IL 10:**

IL 10 has anti-inflammatory properties in contrast to the other cytokines listed above and it is produced by macrophages and T cells upon infection with MTB. It aids the survival of the pathogen by deactivating macrophage function by downregulating TNFα expression [Fujiwara and Kobayashi, 2005], which in turn reduces the level of IFN-γ by T cells.

All these cytokines are secreted and regulated by macrophages and DCs when the pathogen is sensed by the pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs). These PRRs are responsible for initiating the innate as well as the adaptive immune response to MTB.

### 2.20 Acquired immunity and cellular response

The outcome of infection by MTB and therefore the clinical manifestation of tuberculosis (TB) depend on many combined factors, such as host genetics, bacterial genetics (virulence factors), the health and nutritional status of the host and whether there has been any prior exposure/immunity and vaccination history. The adaptive immune response mediated by T cells is critical for control of MTB infection in humans. This type of immune response executes several effector functions by activating various components of the innate immune system [Maglione et al., 2009]. This response generates antigen-specific effector cells and memory cells. The acquired immune responses can be categorized into

a. Cell mediated immune response which includes T cell activation and its mechanism of action and
b. Humoral response consisting of B cell maturation and antibody production.

The role of T cells and B cells are not mutually exclusive during the course of fighting against the pathogen. Once the infection happens, the dendritic cells sense the foreign
antigens, get activated and then present the antigen to T cells. The T cells then get activated and in turn activate B cells thus eventually cascading the events of adaptive immune response. Thus, both the T cells and B cells go hand in hand working together and try to eliminate the bacilli.

2.21 **Cell mediated immune response:**

A critical factor in containment versus spread of *M. tuberculosis* is the character of the T-cell response or the cell mediated immunity (CMI) that develops in response to infection. CMI primarily target intracellular pathogens that reside in a vacuole within macrophages. As a first step in the events, mycobacterial antigens are presented to MHC class II molecules. Protection against the infection mainly depends upon the expression of α/β T-cells having CD4 or CD8 phenotype. These activated T cells produce several cytokines that attract and activate macrophages and addition lymphocytes to the site of infection. Furthermore, these T cells display cytotoxic activity having ability to control mycobacterial growth through destruction of the infected cells. Bacilli which escape are subsequently ingested and destroyed by the surrounding macrophages activated by T cells. It has been reported that both the subtypes of T cells are involved in CMI againt MTB. Knock out studies (CD4 and CD8 T cells) conducted on mice evidence that the infection is aggrevated upon knocking out of either or both T cell sub-types.

2.22 **Humoral immune response:**

Cell mediated immunity plays an important role in the regulation of bacterial containment in granulomatous lesions in the lungs where the bacteria persist in latent state but do not completely eradicate the bacteria [Kaufmann 2002]. Though it is well established that cell-mediated immunity plays critical role in defense against MTB, By contrast B cells and antibodies generally have been considered unimportant in providing protection [Casadevall and Pirofski et al., 2011; Glatman-Freedman and Casadevall, 1998; Maglione and Chan, 2009]. However, the role of B cell and humoral immune response in defense
against MTB, when completely excluded, becomes problematic. Indeed, it is likely to be possible that many intracellular pathogens exist in the extracellular space at some point in the infection cycle, such that antibodies can act on them [Casadevall, 2003]. MTB has both intracellular and extracellular stages in the course of infection and thus antibodies play a role in containing the infection [Grosset, 2003; Hoff DR et al., 2011; Wayne and Sohaskey, 2001]. Thus during the cascade of events, the MHCII- restricted antigens are presented by antigen-experienced B cells to antigen-specific T cells [Lanzavecchia, 1985; Lanzavecchia, 1990]. The activated T cells then interact with B cells leading to B cell activation. Upon B cell activation, high-affinity antibody responses occur resulting in the development of B cell memory and antibody-producing plasma cells [Vinuesa CG et al., 2005]. Thus it is an established fact that B cells act as professional antigen presenting cells influencing T cell responses [Lund and Randall 2010; Gray et al., 2007].

![Fig 7: Humoral Immune response against *Mycobacterium tuberculosis*. (Courtesy: Lee et al., 2013)](image)

Antibodies control the infection by preventing the entry of pathogen and inhibiting the replication of pathogens and also neutralizing the toxins produced by them. This response
produces antibody dependent cellular cytotoxicity, promoting opsonisation and complement activation. The antibody-mediated protective mechanisms also have the ability to interfere with the adhesion of MTB to cells [Schlesinger et al., 1994]. Many studies have established the fact that antibody mediated responses allow the production of immunoglobulins and also passive immunization as a potential defence in adaptive immune response. Antibody-mediated immunity shapes the host response in reaction to pathogenic attack in a number of ways (fig 7). These include antigen-specific neutralization, regulation of inflammatory responses through complement activation, Fc-γ receptor cross linking, release of microbial products and impact on microbial gene expression upon binding to microbes [Navoa et al., 2003; Yu et al., 2012] therefore, it can be stated that antibody-dependent and independent functions of B cells play an important role in determining disease outcome.

Thus, an efficient cross-talk between innate and adaptive immune responses is crucial to control MTB infection.

2.23 Diagnosis of TB

The process of diagnosis depends upon the purpose of testing (detecting LTBI, active TB disease or drug resistance).

**LTBI:**

Currently two tests are available for the identification of LTBI: the Tuberculin skin test (TST) and the Interferon Gamma Release Assay (IGRA).

**Tuberculin Skin Test (TST):**

The most definitive test to diagnose TB infection is the culture test using the sputum of the person. But this is time consuming as the bacilli has extended growth phase. It takes atleast two weeks to get a result. On the other hand, TST is widely used as test for TB diagnosis which yields results in two days. TST includes the extracted killed TB germs. It is an *in vivo* assay that elicits a delayed hypersensitivity reaction towards tuberculin. The tuberculin or PPD (purified protein derivatives) is given as a shot in the forearm of the person
to be tested for the presence of MTB. At the end of 48 to 72 hrs, the patient is again examined for the presence of swelling around the injected area. The size of swelling reflects the likelihood of infection.

Although TST has several advantages, which include low-resource settings, including low reagent, equipment costs, limited skill and laboratory requirements, it has two major limitations.

1. Repeated BCG vaccination (booster vaccinations) or late vaccination (post-infancy) leads to compromisation in the specificity of TST. [Farhat et al., 2006]
2. The prediction value is limited. Individuals with positive TST results do not progress to active disease [Pai et al., 2014]. Currently, efforts are under way in adding new antigens (RD1 specific) as a replacement to PPD in order to increase the specificity of the test. [Pai and Sotgiu, 2016]

**Interferon Gamma Release Assay (IGRA):**

IGRAs are *in vitro* blood tests which tests the person’s immune reaction to the TB bacilli (cell mediated immune response). When antigens from MTB are mixed with patients’ blood, immune reaction will occur and thus a release of interferon gamma (IFN $\gamma$), a cytokine. Quantification of IFN $\gamma$ interprets the results. RD1 antigens are more specific for MTB than PPD antigens since there the RD1 antigens are absent in BCG vaccine strains or of most species of non-tuberculous mycobacteria [Prabhavathi et al., 2015; Sester et al., 2011]. Limitations of IGRA include poor predictive value like TSTs.

Limitations of IGRA

- IGRA has reduced sensitivity in immune-compromised patients [Farhat et al., 2006]
- The specificity of the IGRA test is poor in patients with suspected active TB in high burden countries and thus this test cannot be used as a rule-in test for active TB in countries with high background prevalence of latent infection [Metcalfe et al., 2011]
• Both TST as well as IGRA tests is neither able to accurately differentiate between LTBI and active TB disease [Farhat et al., 2006; Sester et al., 2011] nor able to distinguish between new infections and re-infection events.

Active TB disease:

Detection of active TB disease includes four main technologies: imaging techniques, microscopy, culture-based methods and molecular tests.

Imaging techniques:

a. Radiography:

Radiographic techniques play an important role in the diagnosis of TB. It is an established triage or screening test and the emergence of digital radiology and softwares eases the process. Since the X-Rays lack specificity, the screening should be followed up with microbiological tests. Currently, advancements in imaging modalities have provided insights into the lung lesions.

Culture-based methods:

a. Sputum smear microscopy:

It is one of the rapid preliminary diagnostic tests especially in case of active, infectious cases. Sputum smear examination is the modest test done to detect the presence of bacilli as the test is very sensitive. The technique employs the respiratory sample which strongly influences the ability to detect PTB. The samples obtained from the patients are simultaneously sent for both sputum and culture test as the culture test is essential to confirm the diagnosis. Since the cost of culturing is more compared to smear test, the tests are often confirmed with the presence of bacilli by acid fast bacilli (AFB) staining smear tests. The sensitivity of sputum AFB has a threshold of detecting 5000-10000 bacilli per ml of specimen [Wu and Wang, 2000].
The sensitivity of microscopy can be increased by using fluorescence microscopy and sputum concentration methods [Steingart et al., 2006a; 2006b]. Although sputum smear microscopy has many limitations like a negative smear does not eliminate the diagnosis of infection, it continues to be the most widely used active TB disease in low-income and middle income countries [Kik et al., 2014].

b. **Culture test:**

This is a confirmatory test for the diagnosis of active disease. This technique require only 10-100 organisms to detect MTB, revealing that the sensitivity of the technique is excellent ranging from 80% to 93% [American Thoracic Society, 2000] with 98% specificity. It is preferred that all specimens to be cultured as the cultures increase the sensitivity of diagnosis and allow speciation and drug susceptibility testing and if needed, genotyping for epidemiological purposes [Bergmann et al., 1999]. There are three types of culture media through which the bacteria can be cultured: solid media, including egg-based Lowenstein-Jensen (LJ), agar based (Middlebrook 7H10 and 7H11) and liquid media (Middlebrook 7H12 and other broths). Solid medium is the standard for culturing mycobacteria. The bacilli grow slower in solid media compared to liquid media. LJ, 7H10 and 7H11 media may detect mycobacteria in < 4 weeks, but need incubation for 2-4 more weeks in order to justify that the specimen is negative.

**Molecular diagnosis of TB:**

**Nucleic acid amplification test (NAAT):**

As an alternative to the current conventional bacteriological diagnosis tests, which have several limitations, NAAT has emerged as a good potential test with rapid turn-around times. This assay detects the presence of mycobacterial insertion element IS6110 for the identification of the MTB complex organisms [Tiwari et al., 2015]. A patient showing positive for AFB smear and positive for NAAT will be indicative of active disease and positive AFB smear and negative NAAT will be indicative of non-tuberculous mycobacterial
(NTM) disease (fig 8). On the other hand, if NAAT result is positive and AFB is negative, it is the decision of the clinician to begin anti-tubercular medication while awaiting results for culture test [CDC, 2009]. The sensitivity of the test was at least 80% in most studies with respiratory specimen and can detect as few as 10 bacilli per sample. Owing to superior accuracy than the conventional bacteriological tests, WHO has endorsed and recommends Xpert/RIF MTB assay as the first line of diagnostic test for adults and children suspected to be infected with MTB [WHO, 2013].

![Fig 8: Diagnosis of TB using NAAT. (Courtesy: Nurwidya et al., 2018)](image)

In HIV-positive individuals, sputum smear test detects only 22-43% of active TB disease [Getahun, 2007]. Thus, Xpert MTB/RIF acts as an initial diagnostic test and is highly recommended by WHO. Apart from Xpert test, detection of lipoarabinomannan (LAM) antigen in urine of the HIV patients has emerged as a potential test to detect the HIV-associated active TB disease [Peter et al., 2016]. The LAM test is now recommended by WHO to assist and expedite the diagnosis of active TB disease in two specific populations: in HIV-positive adult showing signs and symptoms of pulmonary and/or extrapulmonary TB and also who have a CD4+ T cell count of ≤ 100 cells per µl or HIV-positive patients who are seriously ill regardless of their CD4+ counts [WHO, 2015].

**Diagnosis of drug resistance TB:**

The conventional tests for the diagnosis of drug resistant TB include phenotypic, culture-based (testing of ability of bacteria to grow in the presence of anti-TB drugs) and molecular based methods (detection of genetic mutations in MTB that confer drug resistance). Currently, WHO has recommended the implementation of Xpert MTB/RIF as a tool for active TB disease with drug resistance (MDR) [Raizada et al., 2014; Sachdeva et al.,
Apart from Xpert MTB/RIF, two more tests have been endorsed by the WHO: loop-mediated isothermal amplification [WHO, 2016] and molecular line probe assays for rapid drug susceptibility testing of first-line drugs (isoniazid and rifampicin) as well as selected second-line drugs (fluoroquinolones and injectable second-line drugs) [WHO, 2008; 2016].

2.24 Resistance in TB:

The ability of the host to control the infection may directly relate to nutritional status, presence of co-morbidities and genetic predisposition. Anti-Tuberculosis drug resistance has become a major health concern. There are two ways a person may get drug resistance:

- Acquired drug resistance is one way when the treatment for PTB using the DOTS therapy becomes inadequate. The main reasons for this inadequacy can be improper TB treatment regimens, wrong drugs prescribed or sub standard TB drugs used for the treatment.

- The second way of getting drug resistance results from the direct transmission of drug resistance TB bacilli from one person to another.

Researchers have found that primary resistance plays a much greater role in the spread of drug resistance than previously thought. So simply treating drug susceptible TB is no longer sufficient for controlling drug resistance. Thus, we have to specifically diagnose the disease and treat the same in order to control drug resistance TB.

There are two main types of drug resistant TB namely, MDR-TB and XDR-TB. MDR-TB (multi drug resistant TB) is categorized when the bacteria are resistant to at least isoniazid (INH) and rifampicin (RIF), the two most effective TB drugs. It is not clear that when a person is described to have MDR-TB, they are resistant to only these two drugs or other drugs also. So the WHO has now started to refer to “uncomplicated MDR-TB”. This title can be given to a patient with resistance to only INH and RIF but not resistant to any of the second line TB drugs. There is one more category of MDR-TB called as RR-TB in which people will be resistant to rifampicin alone. They may or may not have resistance to other
drugs. MDR/RR-TB means patients with MDR-TB as well as patients with TB resistant to rifampicin.

An XDR-TB (extensive drug resistant) is defined as strains that are resistant to at least INH and RIF and in addition to resistance to any of the fluoroquinolones (such as ofloxacin or moxifloxacin) and at least one of the three injectable second line drugs (amikacin, kanamycin or capreomycin). They require longer duration of treatment regimen (2 years) compared to drug sensitive TB patients (6 months). The treatment requires drugs which are less potent, more toxic and much more expensive.

A third type of resistance, the TDR-TB cases are now been detected. It is sometimes also referred to as extremely drug resistant TB which is very difficult to treat.

Globally, an estimated 4.1% of new cases and 19% of previously treated cases have MDR/RR-TB. WHO surveillance data show that of the 600 000 people diagnosed with MDR-TB in 2016, 240 000 died. Though the number of diagnosing tools have increased, the number of MDR/RR-TB cases detected only reached 153 000. Until 2016, about 8000 patients with XDR-TB were reported worldwide and at least one XDR-TB case was reported in 123 countries. Thus, it can be stated that on average, an estimated 6.2% of people with MDR-TB have XDR-TB.

2.25 Prevention of drug resistant TB:

The most effective and important way to prevent the spread of drug-resistant TB is to follow the drug regimen properly and take all the TB drugs exactly as prescribed by the health care providers. No doses should be missed and treatment should not be stopped early. The health care providers can prevent drug resistant TB by quickly diagnosing cases, following recommended treatment guidelines, monitoring patient’s response to treatment and consistency with the patient record whether the therapy is complete or not.

Another way to prevent resistance is to avoid exposure to known drug-resistant patients in closed or crowded places such as hospitals, prisons or homeless shelters.
2.26 Treatment for tuberculosis disease:

Once the pathogen is encountered, the host immune system tries to eliminate the bacilli but not all the bacilli are destroyed. The remaining bacteria may cause the active disease or may reside inside the host for several years as latent tuberculosis. Thus a proper treatment should be designed to encounter both the dormant stage as well as active stage disease.

The main aim of treating the disease is to cure the patient. The other aims include prevention of spread of TB and prevention of development of drug resistance. The treatment of the disease initiated with the discovery of para-amino salicylic (PAS) in 1940 followed by streptomycin in 1943. Streptomycin showed improvised treatment but had a number of side effects like painful during injection, loss of hearing or sense of balance and emergence of resistance after long term therapy. Soon after, the discovery of isoniazid helped in the reduction of side effects that streptomycin caused. It is a potent drug which is still under use. By the time streptomycin became available, scientists began to think of using combinational therapy in order to avoid the development of resistance. Triple therapy of isoniazid, PAS and streptomycin became standard regimen for more than 15 years until the development of new antibiotic rifampin in 1966. But after the discovery of rifampin, the history of TB drugs became halt with no more discoveries of drugs for more than 50 years.

Fig 9: Treatment outcome of tuberculosis. (Courtesy: Cano-Muniz et al., 2018)
The treatment of TB usually takes 6 to 9 months. There are 10 drugs being approved by U.S. Food and Drug Administration (FDA) to treat TB. Among the approved drugs, the first line anti-tb drugs which are used as the core of treatment include:

- Isoniazid (INH)- cell wall synthesis inhibitor
- Rifampicin (RIF)- transcriptional inhibitor
- Ethambutol (EMB)- inhibition of arabinosyl transferase, cell wall synthesis
- Pyrazinamide (PZA)-targets inactive bacilli residing within an acidic environment

This drug regimen has an intensive phase of 2 months, followed by a continuation phase of either 4 or 7 months. In the intensive phase of treatment, the growing bacilli get killed as an effect of these antibiotics. However, in order to kill the persisting or slow growing strains, the continuation phase of 4 to 7 months with INH and RIF is essential. This therapy is called as DOTS (Direct Observed Treatment Short-course) therapy as recommended by WHO. The amount of drug a patient takes depends upon the weight of the person. These drugs are administered to the patients with active disease and who has not taken any treatment before and they are called new patients. These patients are presumed to have drug susceptible TB unless:

- The isoniazid resistance is high in the new TB patients
- The patient has developed active TB from a person who is documented to have drug resistant TB.

It is recommended that the patients take TB drugs continuously for six months. It is also said that taking drugs three times a week is possible according to DOTS therapy. But it is essential that all the recommended drugs are taken. A patient who does not fulfil the above mentioned bulletins are not given the same drugs as mentioned above. They are probable to take a different and longer course of drug treatment. If they have just the same course of drugs as treatment again, they will not probably be cured.
2.27 **TB treatment failure:**

There are three main causes of TB treatment failure relative to doctors in prescribing drugs, the nature of drugs being delivered, and the patients.

**Doctors – as a cause of TB drug treatment failure:**
- Inappropriate guidelines to patients
- Non compliance
- Absence of guidelines

**Drugs – as a cause of TB drug treatment failure:**
- Poor quality of drugs
- Irregular supply of drugs
- Wrong combination or dosage of drugs
- Due to drug resistance drugs are unsuitable

**Patients – as a cause of TB drug treatment failure:**
- Lack of information about the treatment regimen
- Lack of money for treatment or transport
• Fear of side effects
• Lack of commitment to long course of drugs
• Malabsorption
• Other social barriers.

Normally, it is considered that the patients (90-95%) after three months of drug treatment for PTB will have negative sputum or culture and show clinical improvement. Those who show positive for sputum test even after four months of drug treatment should be classified as treatment failures.

2.28 **TB treatment relapse and recurrence:**

A patient is said to relapse if he becomes and remain culture negative during the treatment regimen but becomes culture positive soon after finishing the treatment. This condition may either be because of reactivation of the person’s latent TB or because they are reinfected with fresh load of bacilli. In any of these situations, it must be considered that the person has drug resistant TB and the treatment regimen should be designed accordingly.

2.29 **Treatment monitoring:**

Every patient should be monitored during their treatment to assess their response to the drug treatment. It ensures the completeness of the treatment and also to identify and manage adverse drug reactions. The PTB patients should be monitored using sputum smear microscopy. WHO recommends that for a smear positive TB patients treated with first line drugs, the patients should be checked for smear at the end of intensive phase of treatment.

2.30 **Treatment of latent TB:**

Treatment of LTBI includes 9 months of ingestion with isoniazid, 4 months of rifampin or 2 months of rifampin and pyrazinamide, although the last combination regimen is no longer recommended due to increased risk of hepatotoxicity among HIV-seronegative
individuals [Jasmer et al., 2002]. A recent study shown that administration of rifapentine and isoniazid for just three months, once a week is as effective as the standard 9 month daily regimen of isoniazid alone and this treatment has a significantly higher completion rate.

2.31 Treatment of drug resistant TB:

A person is said to have drug resistant TB, if he does not respond to at least one of the drugs listed in the first line of anti-TB therapy. The treatment of drug resistant TB is more difficult than for susceptible. It requires the use of second line drugs which are more costly and cause side effects. Also, the drugs should be consumed up to two years for proper cure which may also susceptible to further resistance. Search for treatment with shorter regimen which eases the patients has begun. But not all the patients with resistant TB are eligible for shorter regimen treatment. With the availability of line probe assay, the identification of patients eligible for shorter regimen treatment has become viable. In case, where the drug susceptibility test is not available, treatment decision should be taken based on the patient’s clinical history and recent surveillance data.

Patients with MDR/RR TB are prescribed with a drug regimen containing at least five effective TB medicines during the intensive phase. This includes pyrazinamide and four core second line medicines, one chosen from Group A, one from Group B, and at least 2 from Group C. Using the drugs, an effective TB medicines should be composed, if not, one drug from the Group D2 and others from D3 may be added so that the total becomes five (table 1).

Though there are a number of drugs available to treat the disease, the efficacy and side effects play an important role in extending the life of a patient. Besides, vaccination against TB with live, attenuated BCG originally derived from *Mycobacterium bovis* has helped in reducing the incidence of the disease. But the endurance of the vaccine is less as it becomes ineffective with age and also provides variable protection in case of pulmonary tuberculosis which is supposed to be the greatest burden for global mortality. Thus, the world is in urgent need of diagnostic kits, vaccines and drug targets and drugs which have
capability to cure the symptoms in shorter duration, less side-effects, effective against drug resistant strains and also appropriate to administer along with any other co-infection.

Table 1: Drug regimen for drug resistant TB patients

<table>
<thead>
<tr>
<th>Group A: Fluoroquinolones</th>
<th>Group B: Second line injectable agents</th>
<th>Group C: Other core second line agents</th>
<th>Group D: Add-on agents (not part of the core MDR-TB regimen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levofloxacin (Lfx)</td>
<td>Amikacin (Am)</td>
<td>Ethionamide/Prothionamide (Eto/Pto)</td>
<td>D1 Pyrazinamide</td>
</tr>
<tr>
<td>Moxifloxacin (Mfx)</td>
<td>Capreomycin (Cm)</td>
<td>Cyclosine/Terizidone (Cs/Trd)</td>
<td>D1 Ethambutol (E)</td>
</tr>
<tr>
<td>Gatifloxacin (Gfx)</td>
<td>Kanamycin (Km)</td>
<td>Linezolid (Lzd)</td>
<td>D1 High-dose isoniazid (H&lt;sup&gt;b&lt;/sup&gt;)</td>
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<tr>
<td></td>
<td>Streptomycin</td>
<td>Clofazimine (Cfz)</td>
<td>D2 Bedaquiline (Bdq)</td>
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<td>D2 Deamanid (Dlm)</td>
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<td>D3 p-aminosalicylic acid (PAS)</td>
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<td>D3 Imipenem-cilastatin (lpm)</td>
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<td>D3 Meropenem (Mpm)</td>
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<td>D3 Amoxicillin-clavulanate (Amx-Clv)</td>
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<td>D3 Thioacetazone(T)</td>
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2.32 Solutions for MDR-TB and shorter regimens:

Drug development has lots of phases to be tested before reaching a layman and is very expensive with uncertain results. Thus it is wise to use the existing anti-TB drugs that were never widely prescribed and “re-purposed” drugs (drugs that were originally designed for other diseases that may prove effective against drug-resistant TB) for treating drug
resistant forms of TB. For example, both rifapentine and rifampicin have similar \textit{in vitro} anti-mycobacterial activity but rifapentine has a fivefold longer half-life. Thus this drug can be substituted for rifampicin which is shown to be effective when given once or twice a week [Jindani \textit{et al.}, 2014].

Linezolid is another re-purposed drug and it has achieved successful rate on administration to patients who are resistant to isoniazid, rifampicin or fluoroquinolones [Lee \textit{et al.}, 2012]. But due to its high cost and toxicity, the use of the drug has become limited. Similarly, carbapenems are used for patients with highly resistant strains [Tiberi \textit{et al.}, 2016]. These drugs are expensive and with some exceptions (such as faropenem), they need to given as parenteral administration. Besides all these attempts, it is necessary that in order to improve the treatment of TB, the most promising approach remain the discovery of novel therapeutic compounds and the development of newer regimens.

\subsection{2.33 Proteomics of MTB}

There are many studies which analyse the complex immune response towards MTB in infected individuals, only a few explore the interaction between the host cells and the bacilli [Ernst, 2012; O’Garra \textit{et al.}, 2013; Pieters, 2008; Rohde \textit{et al.}, 2007; Russell, 2007]. Also, exploring the most important components of MTB during infection gives an idea about the genetic makeup of the bacilli. However, the proteome of MTB remains unclear, especially in terms of virulence and pathogenesis [Lew \textit{et al.}, 2011]. Biochemical fractionation and immunological screening based methods were adopted before 1990s in order to characterise many mycobacterial proteins. Many important proteins such as Ag85 complex, MPB64, MPB70, and some cytoplasmic proteins like DnaK, GroEl, and SodA were identified [Daniel and Janicki, 1978; Young \textit{et al.}, 1992] based on these methods.

It was studied that the genome of MTB encodes a total of 4,018 proteins and is elucidated in the Tuberculist database [Lew \textit{et al.}, 2011]. As soon as the whole genome
sequence of MTB became available in 1998, nearly 4000 open reading frames were identified. With the available database comparisons of homologies, function of 61% of proteins could be assigned [Cole et al., 1998]. Researchers have subdivided the genome/proteome of MTB into 10 functional categories as depicted in the fig 11 [Lew et al., 2011]. This genome annotation categorizing each protein has paved way in thorough insight of its function. Random whole-genome high-density mutagenesis was adopted in identifying mycobacterial genes essential for survival in vivo [Sassetti and Rubin, 2004; Talaat, 2004].

Over the last two decades, the field of proteomics has contributed greatly to understand the human pathogen Mycobacterium tuberculosis. Proteomics aims to provide the most detailed insights into cellular processes by analyzing mature proteins, including modifications such as posttranslational processing or cleavage, which cannot be captured by genomics or transcriptomics. In contrast to the genome, the proteome is not static but highly dynamic and it represents a more realistic view of the intricate status of a living cell. In addition, it is studied that the cellular concentration of mRNA and protein encoded by the same locus do not strictly correlate.

Researchers have dedicated their time in determining the cellular architecture and molecular features of the host response in particular the granulomatous response, its formation and the importance of host response in controlling the infection. In vitro studies are conducted by depicting mycobacterial proteome during infection by simulation of the bacilli in hypoxic environments or utilizing the infected cell culture. Further, different models of nutrient starvation and dormancy due to non-replicative persistence have also contributed to the dissection of the bacterium’s intracellular compartments. The results from these simulations and models are pooled together; the common features are extracted with the help of different bioinformatic analysis tools. With the bioinformatic knowledge, new drug targets and vaccine candidates are proposed [Murphy and Brown, 2007; Zvi et al, 2008].
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The analysis of cellular components of *M. tuberculosis* shows important host-pathogen interactions, metabolic pathways, virulence mechanisms, and mechanisms of adaptation to the environment. For example, the analysis of culture filtrates allows the identification of proteins secreted by the bacilli, potential markers of virulence, or immunogenic antigens for the development of new vaccines and diagnostic tools. Cell wall and membrane proteins are involved in the resistance of the pathogen to chemical injury and
play a fundamental role in host pathogen interactions; therefore, the study of the cell wall proteins can be useful in the development of new drugs.

2.34 Comparative proteomics

The goal of comparative proteomics is to analyze proteome changes in response to development, disease, or environment. Proteins present in *M. tuberculosis* but absent in *M. bovis* BCG are valuable antigens for novel diagnostic, therapeutic, and vaccination strategies. Thus, comparative proteomic analysis can be used to differentiate the composition of proteins between the virulent and attenuated mycobacterial strains. Comparision of protein profiles present in two virulent laboratory *M. tuberculosis* strains (H37Rv and Erdman) with those present in two *M. bovis* BCG strains was done by Jungblut *et al.*, 1999, using 2-DE and MALDI/MS analysis. The results revealed that the 56 unique proteins were detected in *M. tuberculosis* and 40 in *M. bovis*.

In order to identify increase or decrease in protein abundance in two or more samples, descriptive and basic expression-profiling proteomics are used where global proteome analysis is performed. In contrast, functional proteomics focuses on the characterization of protein activities, complexes, and signalling pathways [Hinsby *et al.*, 2003; Monti *et al.*, 2005; Pandey *et al.*, 2000]. Thus, functional and activity based proteomic studies are only emerging as tools to define and annotate MTB proteins. The potential of proteomics analysis to fully characterize MTB proteins with respect to physiology and pathogenesis has been confirmed by few studies [Chavadi *et al.*, 2009; Millington *et al.*, 2011; Estorninho *et al.*, 2010; Prisic *et al.*, 2010]

Insight into the proteome of MTB has increased in the past two decades. The first exploration began with the discovery of gel-based proteomics, in which protein fractions from different MTB strains are analyzed by two-dimensional gel electrophoresis (2D-GE) [Betts *et al.*, 2000; Jungblut *et al.*, 1999; Mattow *et al.*, 2003; Rosenkrands *et al.*, 2000; Sonnenberg *et al.*, 1997; Urquhart *et al.*, 1998]. This analysis has provided useful annotation of proteins and thus provided a stepping stone in the field of proteomics of TB.
For a small subset of proteins, affinity based reagents such as antibodies and methods like western blotting and ELISA have been used. These methods are robust and well established but with some disadvantages in terms of cost, time and scaling up. Moreover, generation of these high affinity based protein assays with high efforts has led to a pitfall in the research leaving less-accessible areas mostly unexplored [Edwards et al., 2011].

Identification and quantification of proteins present in the genome of MTB is essential as it gives clue about the role of proteins in a metabolic state. With the discovery of mass spectrometry (MS) - based proteome, high levels of qualitative proteome coverage and improved MTB geneome annotation have been achieved so far [de Souza et al., 2011; Jungblut et al., 2001; Kelkar et al., 2011]. In this technique, proteins are broken down to peptides, and then separated in time by reverse-phase liquid chromatography, ionized, and injected into the mass spectrometer [Aebersold and Mann, 2003]. Shortgun proteomics also known as discovery-driven MS has been used extensively for qualitative and quantitative measurements. Using liquid chromatography coupled to tandem mass spectrometry (LC MS/MS), both labeled and label-free aided in the identification of more proteins in a single experiment. This strategy, in complement with 2D-GE, aid in the most complete analysis of MTB proteins [Malen et al., 2007].

Shotgun proteomics using liquid chromatography coupled to tandem mass spectrometry (LC MS/MS), both labeled and label-free, provided a more ample application, and the number of proteins identified in a single experiment increased considerably. Shotgun strategies when complemented with 2D-GE provided the most complete analysis of MTB proteins [Malen et al., 2007]. In addition, shotgun proteomics, as opposed to gel-based methods, can be more easily applied to characterization of membrane and cell wall proteomes that, due to their intrinsic insoluble characteristics, are difficult to resolve using 2DGE [Wolfe et al., 2010] This application has resulted in extensive descriptions of cytosolic and cell wall proteomes [Rosenkrands et al., 2000; Wolfe et al., 2010; Mawuenyega et al., 2005] whole cell lysates [Rosenkrands et al., 2000; Schmidt et al., 2004] and membranes [Mawuenyega et al., 2005; Gu et al., 2003; Malen et al., 2011; Xiong et al., 2005]
Tandem MS/MS is generally the preferred method used to identify proteins as part of a functional proteomic workflow. In particular, nanoflow liquid chromatography mass spectrometry (LC MS) applications provide higher sensitivity and low solvent and sample consumption when compared with traditional LC MS.

### 2.35 Drug targets

The existing drugs, although have immense importance in controlling the TB infection, have several shortcomings because of the emergence of drug resistance strains of MTB rendering the first line drugs incompatible to use (table 2). Isoniazid and ethionamide are inhibitors of mycolic acid synthesis [Lei et al., 2000; Banerjee et al., 1994], while cycloserine and ethambutol inhibit synthesis of peptidoglycan [Feng and Barletta, 2003] and cell wall arabinogalactan [Deng et al., 1995; Belanger et al., 1996] respectively, weakening the cell wall of the bacterium. Rifampin and Amikacin exert their pharmacological action by inhibiting bacterial RNA or protein synthesis [Telenti et al., 1993; Maus et al., 2005].

![Fig 12: Applications of proteomics: (Courtesy: Carolina et al., 2011)](image-url)
Though they are more effective in controlling the disease in their own respect, they have several short coming including the side effects caused by them which reflect in the damage of the host immune system (for example rifampicin) making them prone for patience non compliance. Moreover, drug resistance renders even the front-line drugs inactive. Another important problem is the inability of anti-mycobacterials in targeting latent form of bacilli. Besides these problems, the co-infection with Human Immuno deficiency Virus has made the situation even worse. For example, protease inhibitors have been shown to be incompatible with rifampicin containing medications. Thus, identification of appropriate drug targets becomes pre-requisite to control the infection.

A drug target is the specific binding site of a drug in vivo through which the drug exerts its action. A specific drug target might have the following characteristics:

1. Drug targets are biomolecules, proteins in precise that could exist in isolated or complex form.
2. These target proteins have special sites which can accommodate small endogenous or extraneous substances such as chemical molecules (drugs).
3. When small molecule binds to the protein of interest, there might be some changes in the overall structure of the protein or in other words, the conformation of the protein may be altered. These changes in structure normally are reversible.
4. The alteration in the structure leads to changes in various physiological responses inducing regulation of cell, organ, and tissue or body status.
5. These physiological responses triggered as a result of conformational change in the protein may play a major role in complex regulation and have therapeutic effect on pathological conditions. [Du, 2004]

Thus, a drug target can be explained as a key molecule involved in a particular metabolic or signal transduction pathway that is specific to a diseased condition.
### Table 2: Targets of current drugs

<table>
<thead>
<tr>
<th><strong>Current drugs</strong></th>
<th><strong>Site of action</strong></th>
<th><strong>Mutations/ causes of resistance</strong></th>
<th><strong>Reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rifampin</strong></td>
<td>β subunit of RNA polymerase</td>
<td>Mutation in (rpoB) locus</td>
<td>Telenti <em>et al.</em>, 1993</td>
</tr>
<tr>
<td><strong>Isoniazid</strong></td>
<td>Activated form binds to InhA enzyme</td>
<td>Inactivation of (katG) gene or mutations in InhA</td>
<td>Winder <em>et al.</em>, 1970; Miesel <em>et al.</em>, 1998</td>
</tr>
<tr>
<td><strong>Pyrazinamide</strong></td>
<td>Active form inhibits fatty acid synthase (FAS) I</td>
<td>Mutations in (pncA) gene coding for nicotinamidase</td>
<td>Scorpio and Zhang, 1996; Boshoff and Mizrahi, 1998</td>
</tr>
<tr>
<td><strong>Ethambutol</strong></td>
<td>Arabinosyl transferase (cell wall biosynthesis)</td>
<td>Mutations in (embB) gene</td>
<td>Takayama and Kilburn, 1989; Sreevatsan <em>et al.</em>, 1997</td>
</tr>
<tr>
<td><strong>Streptomycin</strong></td>
<td>Bind to 16S rRNA and interferes in the binding of formyl-methionyl-tRNA</td>
<td>Missense mutation in (rpsL) or base substitution in 16S rRNA</td>
<td>Honore and Cole, 1994</td>
</tr>
</tbody>
</table>

### 23.6 Approaches to TB drug development

The currently used drugs against TB have been identified over a broad spectrum without knowing the specificity of the drug to the target. Yet these drugs act as starting point for the identification of new agents. On the other hand, a target should be compatible with the existing drugs as well as new sets of molecules binding the target in different way from that of the existing drugs. Interrogating the mode of action of today’s drugs, classical biochemical pathways studies and genetic studies have aided in the identification of a number of specific targets. Besides these techniques, studies on the three dimensional structures of several of the drug targets have made the scenario feasible for the rational design and optimization of
anti-tuberculosis drugs. Targeting the genes which are important for bacterial persistence will provide an alternate approach to chemotherapy and this may also reduce the treatment duration. [Molecular genetics of Mycobacteria, edited by Graham F. Hatefull and William R. Jacobs Jr., 2000]

Complete genome analysis of MTB by high through put sequencing has paved way in exploring the diversity and evolution of the bacilli with respect to humans. By comparing and identifying the potential gene products those are present in the host and absent in the pathogen, the problem of searching for potential drug targets from a large list has been reduced. The antibiotics which are currently under use are essentially inhibitors of certain bacterial enzymes and all enzymes specific to bacteria can be considered as potential drug targets.

Similarly, the study of structure-function properties of proteins may enhance the understanding of the disease or the organism and help in providing inputs for structure based drug discovery. Till date among the 4000 and above gene products as many as 300 of unique mycobacterial proteins have been structurally characterized. The recent release of three dimensional structures in PDB includes RipA peptidoglycan hydrolase (Rv1477) and thiazole synthase (ThiG) complexed with a drug (pubmed id not generated yet). The structure of Rv1885c, chorismate mutase [Agarwal et al., 2007] and Rv3014c, a NAD dependent DNA ligase [Srivastava et al., 2007; 2005] form valuable sources for the design of inhibitors and these have started yielding interesting results. The other structures which form the basis for structure based drug design include enoyl-ACP reductase (InhA) [Rozwarski et al., 1998], quinolinic acid phosphoribosyl transferase [Sharma et al., 1998, IdeR [Pohl et al., 1999], superoxide dismutase [Cooper et al., 1995] and 3-hydroquinase [Gourley et al., 1999].
Neglect of discovery of new drugs during the past four decades came to an end during the early 2000s when the advancements in the scientific era coupled with increased awareness of the problem was enabled. With the prior knowledge of various antibiotics and their targets, phenotypic approaches have been pursued along with target based approaches. However, as in the other areas of antimicrobial drug development [Payne et al., 2007] target-based approaches had limited success. This is due to the fact of lack of knowledge on target vulnerability and issues related to drug penetration, metabolism and efflux confounding the translation of their inhibitory activity in vitro to in vivo assays. Significant advances in mycobacterial genetics, chemical biology, proteomics, imaging and other technologies have helped in increase in the number of front-end of TB drug pipeline with high quality lead molecules which can be effectively used on targets after proper validation.
Target-to-drug

The major criteria used for target selection in a target-based approach of identifying novel antimycobacterials are

- Essentiality for growth and survival of MTB mimicking the conditions encountered during host infection;
- Target selectivity, which implies the lack of human homolog to mitigate the risk of drug toxicity; and
- Target vulnerability

Construction of target gene knockouts by homologous recombination or phage transduction and high-density transposon mutant libraries give an idea about the genes required for the growth of MTB under various growth conditions in vitro [Griffin et al., 2011], during macrophage infection [Rengarajan et al., 2005], or in animal models of infection [Dutta et al., 2010; Lamichhane et al., 2005]. On the other hand, creation of conditional knockdown (cKD) mutants of genes from MTB, by transcriptional silencing followed by regulated protein degradation and also by clustered regularly interspaced short palindromic repeats interface (CRISPRi) [Evans and Mizrahi, 2015; Schnappinger and Ehrt, 2014; Schnappinger et al., 2015; Singh et al., 2016; Carroll et al., 2011] may provide additional information of the genes to be used as drug targets and also for validation. These tools can be used to compare the targets’ vulnerabilities by evaluating the impact of target depletion on MTB in vitro, ex vivo and in vivo [Carroll et al., 2011; Boldrin et al., 2014; Kolly et al., 2014]. They have also been used to assess target selectivity of small-molecule inhibitors in whole M. tuberculosis cells [Singh et al. 2016; Kolly et al., 2014; Djaout et al., 2016; Singh et al. 2015] and to identify molecules that act on a prioritized target or pathway in the bacterium through screening of compound libraries [Abrahams et al., 2012; Park et al., 2015].
Drug-to-target

Phenotypic approaches to TB drug discovery require three key components:

- Chemically diverse compound libraries;
- Cell-based screening assays (preferably under conditions modelled on those encountered during host infection); and
- Methods to deconvolute the mechanism of action of screening hits though this kind is challenging.

Specific compound collections that are designed through computational biology are typically screened for activity against the bacilli in their different stages like replicating stage, non-replicating or latent state and intracellular or in vitro conditions. In case of non-replicating stage, several models (in vitro) have been developed to check the reproducing ability of the organism in that condition and phenotypic drug tolerance [Sala and Hartkoorn, 2011]. These include hypoxia [Cho et al. 2007], nutrient starvation [Grant et al., 2013], various stress conditions which include acidity, nitrogen starvation [Gold et al., 2015] etc.

The advancements in confocal fluorescence microscopy technique has enabled to quantify the growth and survival of intracellular MTB by introducing modification with fluorescent protein in a high through put screening (HTS) format [Christophe et al., 2009]. This type of screening indirectly helps in the identification of non cytotoxic hits that target MTB functions essential for their intracellular growth and survival [VanderVen et al., 2015]. This will save both time and resources. Besides these, HTS can be used to identify molecules that impact bacillary growth by perturbing host cell function and these molecules can be used in host-directed therapy [Stanley et al., 2014].

2.37 Tools for Identification of Drug targets

The development of powerful and sensitive mass spectrometric based methods now allows the accurate identification, quantification and various modifications of almost any
expressed protein [Chen et al., 2014]. These robust techniques have enabled to study the host-pathogen interactions, the proteome of the pathogen, of the infected cells and also subcellular compartments. In case of MTB infection, MS methods have helped in the protein profiling of different mycobacterial strains as well as of clinical and drug resistant isolates from patients. This has tremendously increased our knowledge of their proteome and also the difference in the virulence pattern in case of clinical strains. These methods have also led to the identification of new biomarkers from the infected patients which may be used as surrogates for the betterment of existing diagnostic predictions.

The current trends in drug discovery focus on disease mechanism and their understanding followed by drug targeting and lead compound identification. The bioactive compounds bind to one or more cellular proteins and exert their biological activities. There are two basic different approaches to identify the molecular targets of these bioactive compounds: direct and indirect. The direct approach utilizes the affinity chromatography complemented with compound-immobilized beads. This type is only suitable to identify targets of one drug once and cannot be utilized to apply to many compounds simultaneously. The indirect approach, on the other hand includes systems biology approaches utilizing proteomics, transcriptomics and metabolomics as major tools for target identification. As this indirect technique makes use of comparison of protein expression profiles for a given cell or tissue in the presence or absence of active compounds, the methods have proved successful in target identification of both many compounds and one drug.

A common strategy used in the past decades of drug discovery involves fine tuning structural optimizations of existing drugs which have shown some success in action. This enables to find a newer improved drug which slightly modifies the function of the same target as the lead compound. Thus, it cannot be stated thus these are new targets with new mechanism of action. Traditionally, drug targets have been identified from the knowledge about the function of individual protein molecules, their function and mechanism of action. But not all the proteins whose function and mechanisms are well established can be used as drug targets. Thus potential drug targets identified are taken through a process called validation which involves whole-cell or animal experiments, gene knock-outs or site-directed
mutagenesis that lead to loss-of-function phenotypes. Target validation is one of the critical steps in drug discovery which impounds lots of time and money. Using in silico techniques for identification and validation of drug targets have advantage of speed, low cost and more importantly, provide a systemic view of the microbe at a time addressing to questions which are often difficult to answer experimentally. Thus establishing systems biology concepts to understand the microbe may aid in new opportunities for computational target identification. Moreover, new network-based computational models and systems biology integrate omics databases and optimize combinational regimens of drug development.

**Target identification:**

Chemoinformatic tools aid in the integration of information in several levels to increase the reliability of data and thus present a tremendous potential to advance in silico drug design and discovery. Some of the tools currently under use are similarity searching [Keiser et al., 2009], data mining/ machine learning [Nidhi et al., 2006], panel docking [Li et al., 2006] and bioactivity spectra based algorithms [Cheng et al., 2011]. Network-based drug discovery can also be used for target identification where the drug protein and protein disease networks are integrated. This approach involves correlation between experimental techniques involving genomics, transcriptomics, proteomics, metabolomics, microbiome, pharmacogenomics and databases available in silico is needed for better data interpretation [Barabasi et al., 2011; Billur et al., 2014]. For example, computational frameworks can be developed for drug target identification by relating pharmacological and genomic spaces [Zhao and Li, 2010]. Thus the discovery of omics technologies supports the storing, visualizing and analyzing voluminous biological data.
### Table 3: Web-accessible databases for drug target identification. (Courtesy: Katsila et al., 2016)

<table>
<thead>
<tr>
<th>UTILITY</th>
<th>URL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human metabolome data</td>
<td><a href="http://www.hmdb.ca">http://www.hmdb.ca</a></td>
</tr>
<tr>
<td><em>In silico</em> target identification</td>
<td><a href="http://www.dddc.ac.cn/pdtd/">http://www.dddc.ac.cn/pdtd/</a></td>
</tr>
<tr>
<td></td>
<td><a href="http://www.reactome.org">http://www.reactome.org</a></td>
</tr>
<tr>
<td>Pathway analysis</td>
<td><a href="http://www.pantherdb.org">http://www.pantherdb.org</a></td>
</tr>
<tr>
<td></td>
<td><a href="http://www.biocarta.com">http://www.biocarta.com</a></td>
</tr>
<tr>
<td></td>
<td><a href="http://www.ingenuity.com">http://www.ingenuity.com</a></td>
</tr>
<tr>
<td></td>
<td><a href="http://www.ebi.ac.uk/chembidb">http://www.ebi.ac.uk/chembidb</a></td>
</tr>
<tr>
<td>Drug target database</td>
<td><a href="http://www.drugbank.ca">http://www.drugbank.ca</a></td>
</tr>
<tr>
<td>Protein data bank</td>
<td><a href="http://www.pdb.org">http://www.pdb.org</a></td>
</tr>
<tr>
<td>Disease specific target database</td>
<td><a href="http://thomsonreuters.com/metacore">http://thomsonreuters.com/metacore</a></td>
</tr>
<tr>
<td>Pharmacogenomic data</td>
<td><a href="http://www.pharmgkb.org">http://www.pharmgkb.org</a></td>
</tr>
<tr>
<td>Muti-level drug data</td>
<td><a href="http://r2d2drug.org/DMC.aspx">http://r2d2drug.org/DMC.aspx</a></td>
</tr>
<tr>
<td>Comparative toxicogenomic database</td>
<td><a href="http://ctdbase.org">http://ctdbase.org</a></td>
</tr>
<tr>
<td>Target-toxin database</td>
<td><a href="http://www.t3db.org">http://www.t3db.org</a></td>
</tr>
<tr>
<td>Protein expression information</td>
<td><a href="http://www.proteinatlas.org">http://www.proteinatlas.org</a></td>
</tr>
<tr>
<td>Therapeutics target database</td>
<td><a href="http://bidd.nus.edu.sg/group/cjttl">http://bidd.nus.edu.sg/group/cjttl</a></td>
</tr>
</tbody>
</table>

**Fig 14:** Depiction of drug candidate identification (Courtesy: Katsila et al., 2016)
**Target validation**

Target validation is a time-consuming and money consuming process that identifies and assesses whether a molecule target can be developed as a drug for biopharmaceutical usage. New drug validation supports not only discovery of new drug research but also provide more insight into the pathogenesis of target related diseases. Basically, a drug target validation includes six steps:

1. Discovering a biomolecule of interest.
2. Evaluating its potential as a target.
3. Designing a bioassay to measure biological activity.
5. Performing screening to find hits.
6. Evaluating the hits.

Target validation should be performed in three levels: molecular level, cellular level and the whole animal model level. HTS provides tools for validation of small molecules as chemical drugs. These HTS models are at the molecular level, for example, screening of specific inhibitor involves mixing the enzyme and samples together to detect a decrease in the substrate or to determine an increase in the product in this enzyme catalytic process. This is a cell-free based system. But all the hits obtained during screening are not absolutely reliable as there are many predictable and unpredictable factors. There is a considerable difference between cell and cell-free system. Validation at the cellular level provides the confirmation of cell-free results. As an improvement to this step, animal models are used to further validate the target at the whole level. At this level, the effect of the hit in the animal model is to be considered. If the hit obtained from HTS displays a therapeutic effect in the animal models used, then it may be promising. On the other hand, if there is no effect in the animal model, then results should be interpreted with caution.
Viability of an organism depends upon the correct regulation of gene expression in order that right proteins are produced in response to specific internal and external stimuli. For prokaryotes like *E. coli* and *B. subtilis*, the genetic makeup is known for decades and tools are available to experimentally manipulate the activity of individual genes. But in the case of medically important bacterial like MTB, such tools are rarely available. In order to survive inside the hostile environment of the host, MTB should have a smart play to sense and respond to a wide variety of environments which include naïve and activated macrophages, dendritic cells and evolving conditions within granulomas [Russell *et al.*, 2010]. More recently, genetic switches have been developed which aids in understanding the basis of gene activity in MTB and other mycobacterium. MTB upon infection is subjected to a number of host-derived stresses. In order to respond to these stresses, MTB has evolved a complex network of strategies to modify gene expression and promote survival.

The main stresses faced by the organism are

- Stress due to exposure to oxidizing agents, acquired by the reactive oxygen intermediates and reactive nitrogen intermediates, produced by activated macrophages.

- Stress due to exposure to low pH: MTB has the ability to block phagosome acidification and this block is not complete as there is a slight decrease in pH in mycobacterial phagosome [Deretic and Fratti. 1999]

- Stress due to damage of surface structures: upon infection, MTB resides in alveoli. The alveoli have surfactants which act as mild detergent with antibacterial activity which can damage the structure of its fatty acid-rich cell envelope. Apart from this, granulysin and toxic peptides released by activated macrophages and NK cells act at the bacterial surface. Specifically, these granulysins have been recently shown to be essential for MTB killing after apoptosis of infected macrophages induced by NK cells [Dieli *et al.*, 2001]
• Stress due to hypoxia: In hypoxic condition, the bacilli reside inside the granuloma in an inactive and non-replicative state. This phenomenon is of great importance in MTB pathogenesis but still not completely understood at the molecular level [Wayne and Sohaskey, 2001]. The transcriptional regulators DosR and MprA play essential role in mycobacterial persistence [Boon and Dick, 2002; Park et al., 2003; Zahrt, 2003; Zahrt and Deretic, 2001].

• Stress due to nutrient starvation: inside phagosomes and granulomas, the nutrients and essential elements like iron and Mg$^{2+}$ needed for survival will be reduced [Gold et al., 2001; Buchmeier et al., 2000]. Also during transmission of infection from one person to another, the bacilli encounters other environmental stresses such as nutrient starvation, exposure to UV light, dehydration and low temperature.

In order to respond to these stresses and promote survival, MTB has to build up strategies to modify the expression of genes in transcriptional and translational level. Coordination of these responses is essential and often results in overlapping regulons and stress responders [fig 2]. Besides protecting the bacilli from host immunity, these stress response strategies result in changes in physiology of the bacilli and antibiotic tolerance which precludes eradication of infection [Wayne LG, Hayes LG. 1996; Deb et al., 2009; Baek et al., 2011; Cunningham-Bussel et al., 2013; Franzblau et al., 2012]

Studies show that the MTB genome encodes about 190 transcriptional regulators: 13 factors, 11 two component systems, 5 unpaired response regulators, 11 protein kinases [Av-Gay and Everett. 2000], and more than 140 other putative transcriptional regulators. Several proteins under this classification have been characterized and categorized according to the types of host driven stresses as described above, while other protein regulators respond to still unknown environmental conditions [Ewann et al., 2002;; Zahrt and Deretic, 2001]. The resulting picture is still incomplete, but it suggests very complex regulatory systems with overlapping functions and redundancies.

Also, the emergence of drug-resistant strains of MTB has resulted in its recalcitrance to antibiotic therapy and this shows that we are not equipped to successfully battle the MTB epidemic [WHO, 2015]. Therefore, new therapeutic strategies involving the targets of stress
response could increase the susceptibility of the bacilli to both immune system and antibiotic treatment.

2.39 **Transcription factors and DNA binding proteins**

Transcription factors (TFs) play a major role in the modulation of cellular physiology at the level of transcription. They regulate the expression of genes or operons that are far away and also interact with an upstream region to regulate their own expression. Comparative genomics has gained access to decipher such trans-elements or the transcription factors with cis-regulatory regions of DNA [Bailey *et al.*, 2006]. Only when all the required factors namely the cis and the trans regions cooperate, the transcription reaction occurs [Levine, 2010]. Thus, identifying novel transcription factors required for virulence may lead to the identification of new drug targets.

2.40 **Strategies of DNA binding:**

In order to employ transcriptional machinery, prokaryotes and eukaryotes use different strategies to target transcription factors to specific DNA present in the genome. Prokaryotic transcription factors bind to extended DNA sites and this is enough to ensure specificity in small genomes. On the other hand, eukaryotic transcription factors recognize shorter DNA sequences which may be present multiple times in the genome [Wunderlich and Mirny, 2009] and thus leading to non specific binding. In general, a consensus DNA binding site capable of accommodating a transcription factor can be determined by two processes:

- *in vitro* through a process of selective enrichment, called Selex, starting from any random double stranded DNA oligonucleotide sequence [Tuerk and Gold, 1990; Jolma *et al.*, 2010]
- *in vivo* through the identification and analysis of a series of bonafide cis regulatory regions (CRRs) [Furey, 2012]
The protein structure and the biophysical interactions of the transcription factor plays an important role in proper binding of the response elements [Brent et al., 2008]. A major breakthrough is the identification of TF specificity i.e., how a TF selects the appropriate regulatory targets out of a large number of similar sequences eliciting a more specific cellular response to a signal. For example, genome editing of Zn finger nucleases for genome manipulation has aided in understanding the design principle of this transcription factor [Klug, 2010]. Experiments over the past 40 years suggest that the majority of TF molecules in a cell are in contact with DNA/chromatin at any instant. A best example is the classical studies involving the lacI repressor of Escherichia coli [Lin and Riggs, 1975; Kao-Huang et al., 1977; Phair et al., 2004; Elf et al., 2007]. The results of these studies delineate three types of binding events:

(i) specific functional binding to CRRs with a direct impact on gene regulation;
(ii) specific but nonfunctional binding (to CRRs or elsewhere in the genome);
and
(iii) nonspecific nonfunctional binding, where functionality is defined as transcription regulation.

2.41 Transcription factors present in Mycobacterium tuberculosis:

The Acetamidase system

When the primary carbon source for the growth of Mycobacterium smegmatis short aliphatic amides such as acetamide was used, there is an induction of expression of the acetamidase enzyme encoded by amiE [Draper, 1967; Mahenthiralingam, 1993; Parish, 1997]. Many mycobacterial antigens were produced as a result of this inducible system. Also, the effect of silencing the essential genes (e.g., whmD and dnaA) in M. smegmatis was studied using this system [Gomez and Bishai, 2000; Greendyke et al., 2002]. In the case of MTB, the scenario is not used often or in other words, there is limited use of this system due to genetic instability [Brown and Parish, 2006] and complexity in regulating the operon.
Regulation of this inducible system involves three genes (amiC, amiD and amiA) [Parish et al., 2001; Roberts et al., 2003]. In MTB, this system is being largely replaced by other tools.

**Tetracycline Repressor system**

Tetracycline antibiotic causes antibiotic resistance in many bacteria by effluxing the drug out of the cell using their specific regulation system. This regulation is mediated by a single repressor protein, the tetracycline repressor (TetR). This protein specifically binds to two operator regions namely tetO1 and tetO2 present in the promoter region that drives transcription of the efflux pump [Hillen and Berens, 1994]. When there is unavailability of tetracycline, the tet promoter is bound by TetR and thus RNA polymerase cannot bind the promoter and initiation of transcription is inhibited. On the other hand, when tetracycline is sensed by the cell, it binds to TetR relieving from promoter and thus transcription is initiated leading to the operation of efflux pump to throw the drug out of the cell. The affinity of TetR for tetracyclines is very high (105 fold higher than ribosome’s affinity to tetracyclines) such that the operon is sensitive at even very low drug concentrations [Lederer et al., 1996].

**AraC and LacI**

The main pitfall in many expression systems is the presence of leakiness or expression of protein even in the absence of inducer. One of the examples is the pBAD system. It is one of the most tightly regulated E.coli expression systems [Guzman et al., 1995] which is controlled by two proteins- AraC and CAP. AraC causes repression of the promoter in the absence of arabinose activating the same during the presence of arabinose. CAP, the catabolite activator protein aids in activation of pBAD which is directly proportional to the concentration of cAMP [Schleif, 2010]. Any changes in the activity of pBAD without arabinose can be achieved by adding glucose to the medium as glucose decreases cAMP level in the cell. Protein DNA interactions, protein-protein interactions and cAMP levels play an important role in the tight regulation of pBAD system in E.coli. Unfortunately, pBAD
does not function in M. smegmatis as it does in E.coli [Guzman et al., 1995] and therefore it would be difficult to optimize this system for use in mycobacteria.

IPTG inducible lac system is another frequently used E.coli expression system which depend on promoters that are repressed by LacI [Terpe, 2006]. In mycobacterial, the value of LacI for gene regulation is demonstrated by two studies. The first applied LacI to repress a promoter recognized by the T7 RNA polymerase [Lee et al., 2008] and the second inserted a lac operator (lacO) downstream of a mycobacterial promoter to impose susceptibility to repression by LacI [Kaur et al., 2009].

NitR

NitR is a member of AraC family of transcriptional regulators. This protein controls the expression of nitA which encodes a nitrilase. The nitrilases produced by saprophytic actinomycete Rhodococcus rhodochrous, detoxify nitriles by hydrolizing them to carboxylic acid and ammonia [Kobayashi and Shimizu, 1994]. The molecular mechanism by which NitR acts has not been investigated in detail. The drastic over-expression is achieved via a positive feedback loop controlled by NitR and the activator alone is sufficient to mediate induction of its own promoter NitA [Komeda et al., 1996; Herai et al., 2004]. Studies show that in case of M. smegmatis, NitR strongly activates transcription after addition of either ε-caprolactam or isovaleronitrile, while in MTB, only isovaleronitrile was effective [Pandey et al., 2009]. In mycobacterial, the positive feedback has three main consequences

- induction is strong;
- on a single-cell level the switch is either ON or OFF; and
- intermediate inducer concentrations create two subpopulations, one that has NitR controlled gene expression turned fully ON and one that is still in the OFF state. In contrast, intermediate concentrations of anhydrotetracycline partially activate the TetON system so that the average expression level of most cells increases to levels between the OFF and fully induced states.
**Pip repressor**

Pristinamycin, belonging to the streptogramin group of antibiotics, inhibits protein translation by binding to bacterial ribosomes [Mukhtar and Wright, 2005]. The pristinamycine gene, ptr, encoding a multi drug efflux pump, causes resistance of *Streptomyces pristinaespiralis* to this antibiotic [Blanc et al., 1995]. Pip belongs to TetR family of transcription factors which binds to three sites in ptr promoter (Pptr) and causes repression upon activation with antibiotics. The Pptr is a strong promoter in *M. smegmatis* and *M. tuberculosis* which can be efficiently repressed by Pip on induction with even very low concentration of PI. As a result, this system of repressor has an excellent regulatory range [Forti et al., 2009]

### 2.42 Importance of protein research for Vaccination

It was 100 years ago when the first vaccine for TB was administered to a new born. It is a live attenuated strain of the bovine Mycobacterium species, *Mycobacterium bovis* bacilli Calmette-Guerin (BCG) which is being administered via the skin. In general, BCG provides effective protection against childhood disseminated TB which reduces general mortality during the first years of life of new born by enhancing the responses to other infectious diseases like respiratory viruses. However, its protection against pulmonary disease, the main form of disease manifestation and cause of transmission is unsatisfactory [Da Costa et al., 2015; Xing et al. 2014; Kaufmann et al. 2017; Davenne and McShane, 2016]. Thus, the development of new vaccines for TB with improved effectives is essential in order to control the disease and also to reach the set goal of End-TB strategy by 2035 (WHO). It was investigated that the variations in the efficacy of BCG is due to difference in the strain, the age during vaccination, or methodological differences [Clemens et al., 1983]. Moreover, the inability of BCG to provide immunity at the time of primary infection may increase the number of latently infected individuals worldwide. These LTBI persons have a risk of developing the active disease when they become immunocompromised due to many reasons like HIV infection, malnutrition, diabetes etc [Corbett et al., 2003]. Thus it can be stated that BCG fails to elicit an immune response against these latent antigens. Also the protective immune response decreases with age and its efficacy in eliciting response in adults and older
individuals is less than satisfactory. So, there is an urgent requirement for new vaccines with improved performance and efficacy for the control of the disease.

Due to the fact that live vaccines can cause life-threatening disease in immune-compromised patients, attempt to develop a safe vaccine by improving or replacing the current BCG is necessary. Subunit vaccines may be used as an alternative because of their relative safety profile and standardized production. Development of subunit vaccine for TB depends on the identification of antigens that induce appropriate T cell responses. In general, protective immunity to TB is conferred by Th1 CD4 and effector CD8 T cells [Flynn, 2001] and thus an effective vaccine requires the generation of T cell-mediated immune response. In addition, antigens associated with latency may also be included in the vaccine as they may protect individuals from developing into active disease.